Aspects of ovarian function in a line of sheep with a novel X-linked maternally-imprinted gene that is associated with an increased ovulation rate

By

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A thesis submitted to the Victoria University of Wellington
In fulfilment of the requirements for the degree of
Doctor of Philosophy
In Cell and Molecular Bioscience

Victoria University of Wellington
2006

TE WHARE WĀNANGA O TE ŪPOKO O TE IKA A MĀUI
VICTORIA
UNIVERSITY OF WELLINGTON

20 November 2006

Supervisor's Statement for Liz Feary

I can affirm that, to the best of my knowledge, Liz Feary has carried out the research for her PhD thesis according to the requirements of the Victoria University of Wellington statutes as set out in the Calendar.

Liz has been jointly supervised by Dr Bill Jordan and me. We have provided advice about methodology, research, resources and analysis. Liz carried out her research project independently and therefore the thesis represents her own work.

Dr Ken McNatty

Presentations and Posters (See also Appendix 1)

June 2004	Presentation at Victoria University on PhD project background, methods and interim results.
June 2004	Poster presentation for the MacDiarmid young scientist awards and attended the awards and science communication workshops.
November 2004	Poster presentation at the Medical Science Congress 2004 Queenstown.
August 2005	Presentation at Wellington Health and Biomedical Research Society 58th Scientific meeting
October 2005	Presentation at the combined Endocrine Society of Australia and Society for Reproductive Biology Annual Scientific Meeting, Perth, Australia
April 2006	Abstract accepted for the International Ruminant Reproduction symposium in Wellington August 2006

Acknowledgements

This project has been supported financially by the Foundation for Research, Science and Technology, and Ovita with an Enterprise Scholarship.

The following people have made a large contribution in supporting me in my endeavour to complete this PhD.

Dr Ken McNatty for his expertise, wisdom and willingness to take on a 'mature' student. His incredible fortitude and ability to hold and lead and exceptional team through a very difficult time, with integrity.

Dr Bill Jordan for being a willing partner in a supervisory role with Dr Ken McNatty. His expertise and wisdom and ability to impart his vast knowledge of proteomics.

Dr Judy Bond, Jonathan Dunne, Fiona Kwan for their assistance, and patience in getting me through the proteomics part of my thesis.

All the support staff at Wallaceville AgResearch for always being willing to help and giving me advice on so many things. Especially Margaret (IT) and Susan (library) Lillian (Statistics).

Karen Frogley for her incredible ability and compassion in enabling me to work through my past and have a future.

Selwyn Feary for his love and support, it has never diminished through some extremely testing times and financially challenging months.

Sheinach for always believing I had the ability.

The reproduction team at Wallaceville Animal Research.
The day I received notification that I had my scholarship for this PhD, AgResearch announced the closure of Wallaceville animal research centre. The reproduction team through a very turbulent and emotionally difficult time have been able to produce world

class science and provide support and technical instruction for me to learn the skills to complete a PhD.

Dr Jenny Juengel who was not supposed to be my supervisor but who became a mentor and teacher on many experimental procedures and her guidance in writing and reviewing papers.

Dr Janet Crawford for her encouragement and support and initial editing and suggestions in my writing up of this thesis.

Dr George Davis for his knowledge and willingness to share all his information on the discovery of the Woodlands sheep mutation.

Dr Jilly Evans for inspiring me to return to Science.

Abstract

Fecundity is a term that refers to the number of offspring produced per female. It combines fertility (i.e. ability to produce offspring) and prolificacy (i.e. number of offspring). Ovulation rate i.e. the number of mature eggs released from the ovaries during one reproductive cycle in sheep, as with other mammals, is controlled by an exchange of hormonal signals between the pituitary gland and the ovary.

Genetic mutations affecting ovulation are commonly referred to as the fecundity genes (Fec). The most obvious outcome is the number of offspring produced. There is already evidence of a number of major genes affecting the ovulation rate in sheep, specifically the Booroola, Inverdale, Hanna and more recently the Woodlands gene. The sheep carrying the Woodlands gene arose because the mutation was first recognised on a farm in Woodlands, Southland, New Zealand. Woodlands have a novel, X-linked maternally-imprinted, fecundity trait referred to as $FecX2^{W}$, where Fec = fecundity, X = X chromosome, 2= 2^{nd} mutation identified on X and W= Woodlands.

The studies in this thesis investigated ovarian follicular development in both 4-week old Woodland carrier (W+) and non-carrier (++) lambs and adult ewes and evaluated some aspects of the endocrine interactions between the ovary and pituitary gland. The purpose was to identify potential physiological effects of the $FecX2^{W}$ gene on ovarian function. A confounding issue during these studies was the discovery that a large ovary phenotype (LOP) which was present in many of the W+ but not ++ lambs at 4 weeks of age was in fact a coincidence and not linked to the $FecX2^{W}$ mutation.

The key findings from the studies of lambs and/or ewes that were carriers (W+) or non-carriers (++) of the $FecX2^{W}$ gene were:

1. No genotype differences were present either in the numbers of primordial (i.e. Type 1/1a follicles) or developing preantral (i.e. Types 2-4 follicles);
2. Significant genotype differences were present in the numbers of small antral (Type 5) follicles (W+>++; $p<0.05$);
3. An earlier onset of antral follicular development in W+ vs. ++ ewes with irregularities in morphology between the basement membrane and stroma in the former;

4. No genotype differences in the onset of gene expression during follicular development or in the cell-types expressing GDF9, BMP15, α inhibin, β_A inhibin and β_B inhibin, FSHR, ERα, or ERβ;
5. No genotype differences in the levels of GDF9 or BMP15 gene expression in oocytes throughout follicular growth;
6. No genotype difference in the diameters that follicles reached in W+ vs. ++ ewes;
7. Some lambs at 4-weeks of age had unusually large ovaries with an exceptional level of antral follicular development that is reminiscent of a polycystic ovarian condition. The underlying cause of this condition is unknown.

In conclusion, the physiological characteristics of ovarian follicular development in ewes with the FecX2^W gene is different from that in ewes with the Booroola, Inverdale, Hanna or other recently identified mutations.

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Chapter 1 - Introduction

1.1 Fertility

Despite an increase in the world's population, there remains a large percentage (15%) of couples that cannot have children (Matzuk *et al.*, 2002). Almost a quarter of these clinical infertility cases appear idiopathic in nature and this is probably due to our incomplete understanding of the mechanisms regulating fertility. Failure to ovulate can be the consequence of hypothalamic-pituitary and/or ovarian malfunctions and it is thought that genetic defects underlie many unrecognised pathologies. Ovulation is a critical rate-limiting step influencing fecundity (Scaramuzzi *et al.*, 1993). Research that leads to a greater understanding of folliculogenesis (the processes by which an egg develops through to ovulation) will aid in developing new diagnostics, methodologies and therapeutics for improving the potential to ovulate and thereby the fecundity of humans and other mammals. This thesis investigated a highly novel line of sheep that have an X-linked, maternally imprinted, gene that is associated with an increased ovulation rate. The aim was to explore aspects of ovarian function associated with or causing the increased ovulation rate. This is the first known X-linked, maternally-imprinted, gene affecting ovulation rate and it is anticipated that the underlying physiological mechanisms will offer new insights into fertility regulation.

1.2 Hypothalamic-pituitary-ovarian endocrine system

The endocrine system acts by hormones that are synthesized and released by one organ and delivered to the target organ via the blood circulation (Guyton & Hall, 2000). The three endocrine tissues in the ovary are the stroma/interstitium, the follicle and the corpus luteum. The stroma or interstitium refers to tissue around the follicles, corpora lutea and vascular system (Peters & McNatty, 1980). The follicle consists of an oocyte (egg) surrounded by a basement membrane and a squamous layer of pregranulosa cells. Once the follicle has begun to grow, the oocyte enlarges and the granulosa cells proliferate in number. As the follicle begins to grow a vascular system develops adjacent to the basement membrane of the follicle together with a concentric alignment of differentiating cells outside the follicle; these are referred to as theca cells (Peters &

McNatty, 1980; Knobil, 1988). As the granulosa cell numbers increase, they are distinguished by where they establish around the follicular wall (mural granulosa) or as a raised nest where the oocyte is situated (cumulus oophorus). A characteristic feature of the developing follicle is its ability to secrete hormones such as oestradiol (Figure 1). After ovulation, the follicle develops into the corpus luteum which is a transient endocrine gland whose primary function is to secrete progesterone. The process by which this transformation occurs is termed luteinisation because the trigger for ovulation is a pre-ovulatory increase in the plasma levels of LH (Knobil, 1988; Pangas *et al.*, 2002). This dramatic increase initiates differentiative changes in the granulosa cells so that they secrete progesterone which in turn is essential to support the early stages of a pregnancy.

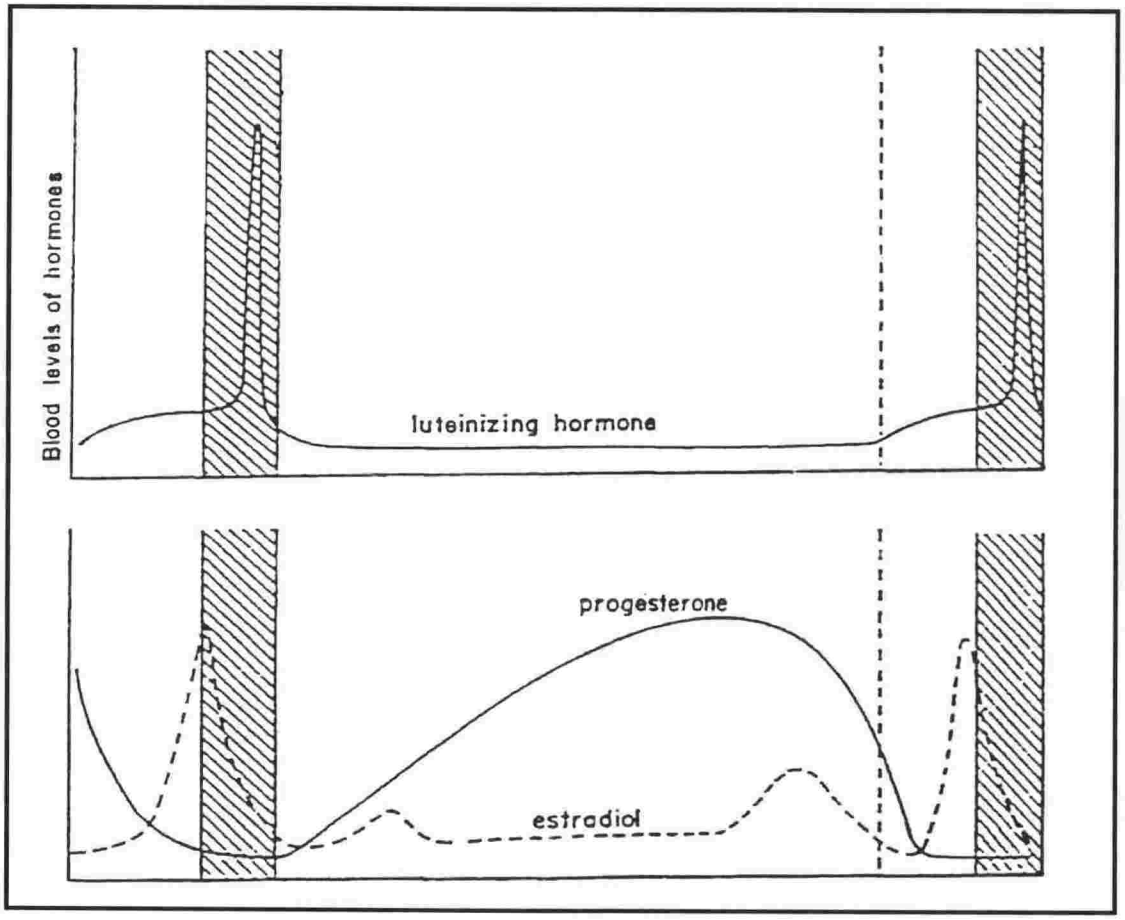


Figure 1: Relative level of luteinising hormone and steroid hormones during the oestrous cycle of the ewe.

Shaded bars refer to the preovulatory period when oestrus and mating occur.

In humans (and pigs), the theca cells secrete both androgens and oestradiol during follicular growth and they continue to secrete these steroids after ovulation (Evans *et*

al., 1983). However, in many other species, theca cells secrete mainly androgens before ovulation but not afterwards as part of the CL when the steroid-secreting cells are predominantly secreting progesterone. The intricate exchange of endocrine signals between the hypothalamus, pituitary gland and ovary plays an important role in determining number of follicles that ovulate during each oestrous cycle (ovulation rate). The normal oestrous (in rodents, livestock) or menstrual cycle (in primates) is regulated by inter-relationships among the hypothalamic-gonadotrophin–releasing hormone (GnRH); pituitary, ovarian and uterine-derived hormones. The hormones communicating between the pituitary gland and the ovary, namely follicle stimulating hormone (FSH), and luteinising hormone (LH) (collectively named gonadotrophins), and ovarian hormones such as oestradiol (E_2), progesterone (P_4) and inhibin are well documented (Figure 2; Scaramuzzi et al.,1993, for a review).

Ovarian follicular growth is a continuous process with no resting phases so that follicles either grow to ovulation or the majority to undergo atresia (Peters *et al.*, 1975; Scaramuzzi *et al.*, 1993; Webb *et al.*, 2004). The initial phases of follicular growth are gonadotrophin-independent and are characterized by stage and cell specific expression of locally produced growth factors and growth receptors (McNatty *et al.*, 2001). However the cyclical pattern of follicular growth, ovulation and CL function is controlled primarily by the hypothalamic–pituitary axis via GnRH and fluctuating gonadotrophin secretions from the anterior pituitary gland.

1.3 Physiology of the ovary

Ovarian function in all mammals has many common mechanisms but some general characteristics of female reproductive activity can vary widely. For example, the characteristic of seasonality where some female mammals have a defined breeding season (e.g. horses and sheep) whereas others can breed throughout the whole year (e.g. cattle and humans).

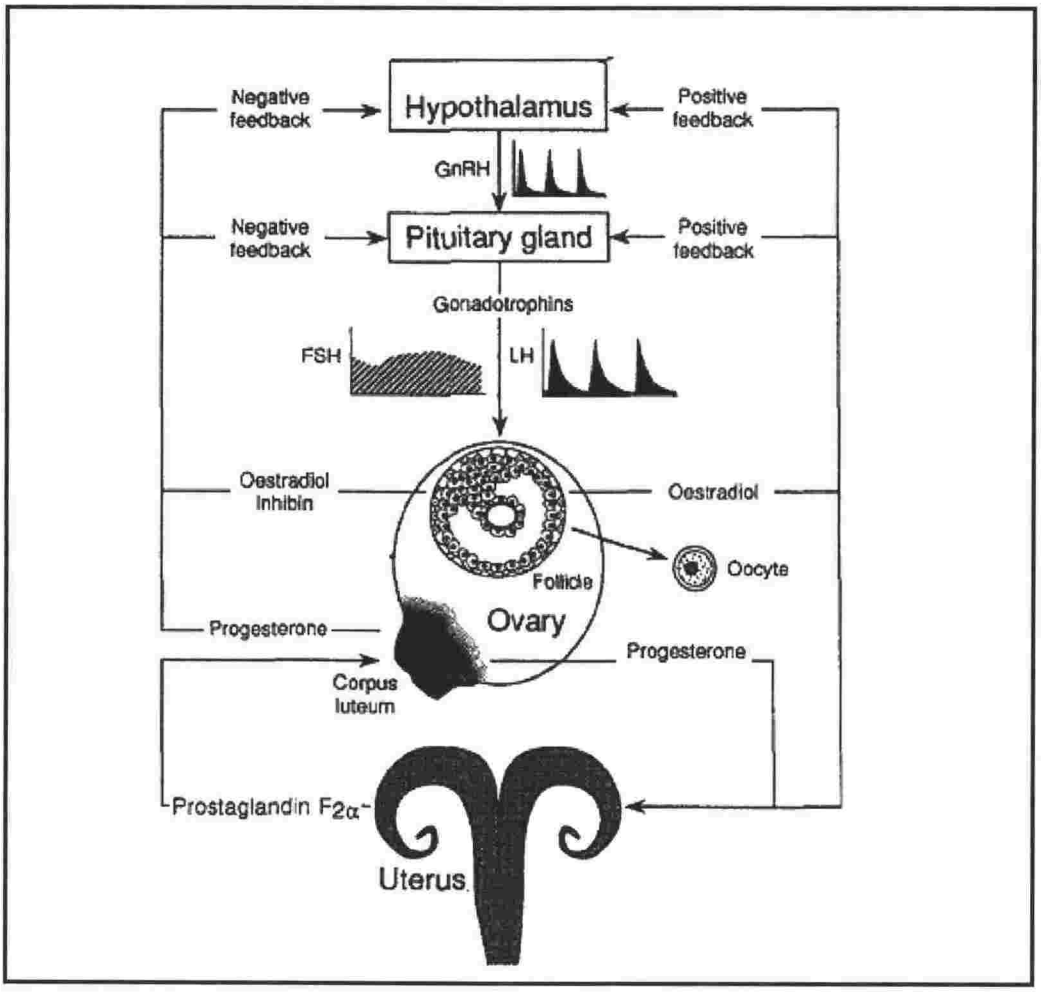


Figure 2: The hypothalamic-pituitary-ovarian-uterine system.

Shows the negative and positive feed back loops between the hypothalamic (GnRH), the pituitary gland releasing LH and FSH, oestradiol and inhibin from developing follicles and progesterone from the corpus lutea after ovulation. The progesterone negative feedback to the hypothalamus and pituitary prevents further development of follicles to ovulation and is required for maintenance of pregnancy. If pregnancy does not occur, the corpus luteum regresses under the influence of prostaglandin F2α from the uterus and a new oestrous cycle begins (Figure modified from Scaramuzzi *et al.*, 1993).

Photoperiod plays an important role in seasonal breeders, with the shortening of daylight hours providing an enhanced melatonin signal for the onset of the breeding season (Tamarkin *et al.*, 1985). Mammals can also be separated based on the type of ovulation. Spontaneous ovulators such as humans and sheep have definite cycles with sequential intervals of follicular and the luteal phases. The duration of oestrous cycle intervals can also be quite variable from 4-5 days for a rodent to 17 days for sheep, 21 days for horses and cows, and 28 days for primates. In the absence of spontaneous ovulatory cycles some animals must be mated to induce ovulation e.g. camelids and cats (Monniaux *et al.*, 1997). Ovulation rate differs widely among species and among

different breeds within a species (Scaramuzzi *et al.*, 1993). Ovulation rate determines the number of mature oocytes available for fertilisation that determines the number of offspring born per pregnancy (Scaramuzzi *et al.*, 1993). In sheep, the ovulation rate normally varies between one and three and this is a determinant of reproductive efficiency.

1.4 Ovary

The ovaries (ovarium = egg receptacle) or female gonads are paired glands that have the same embryonic origin as that of testes (Tortora, & Grabowski 96).

During fetal life and/or shortly after birth females acquire a finite number of oocytes for their reproductive life (Rodgers *et al.*,2003) and although recent research has questioned this dogma (Johnson *et al.*, 2004), the former remains the accepted view.

1.5 Follicular formation and classification

A schematic outline of how follicles develop in the sheep ovary is shown in Figures 3 and 4. In sheep, clusters of oogonia and pregranulosa cells produce membrane materials to isolate themselves from the surrounding tissues. The result is the formation of ovigerous cords that contain the oocyte – pregranulosa cell clusters. The formation of these cords was observed to occur at Day 38 of fetal life. By Day 45, ovigerous cords were isolated from the ovarium interstitium and vascular system by an enveloping basal lamina (Figure 3; Sawyer *et al.*, 2002). The occurrence of apoptosis from day 75 appeared only to affect germ cells and not pregranulosa cells. Therefore it was hypothesised that the excess pregranulosa cells then migrated to surround the remaining oocytes within the ovigerous cords to become part of the newly-formed population of follicles (Juengel *et al.*, 2002; Sawyer *et al.*, 2002).

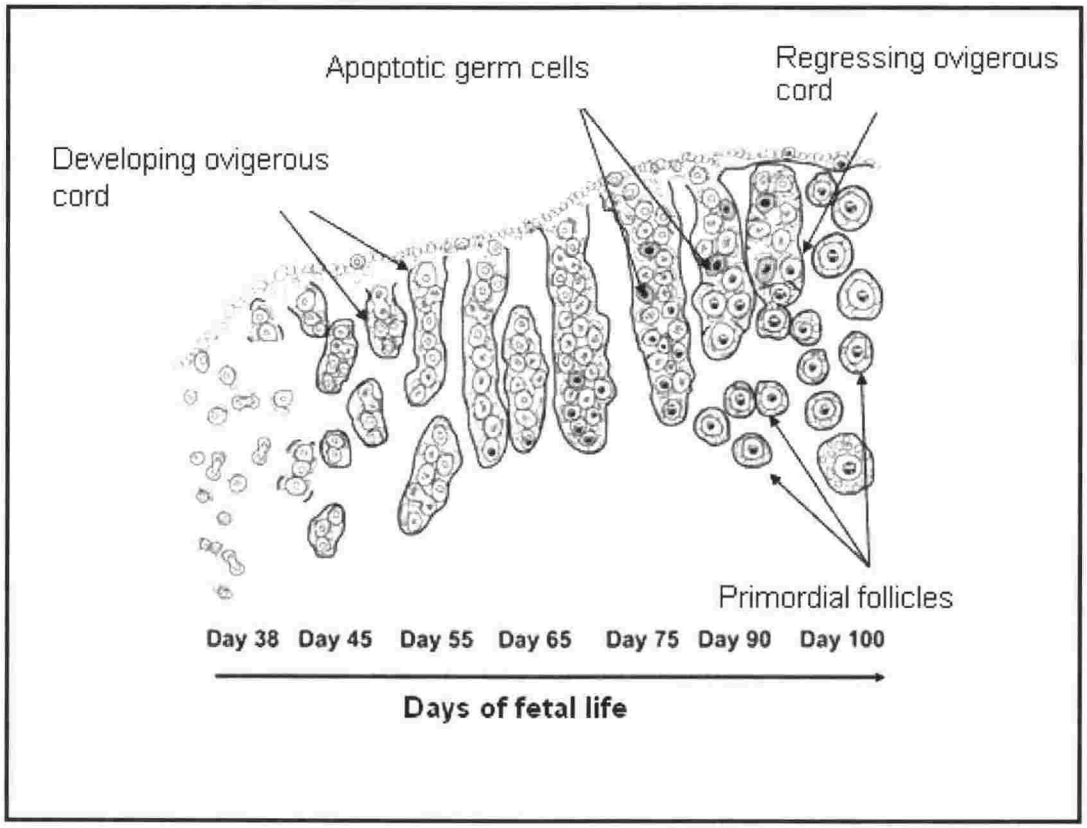


Figure 3: Ovigerous cord development during fetal life in sheep.

This schematic shows the ovigerous cords developing from day 38 during fetal life and then regressing as primary follicles are formed at days 90-100 in sheep. (Sawyer *et al.*, 2002)

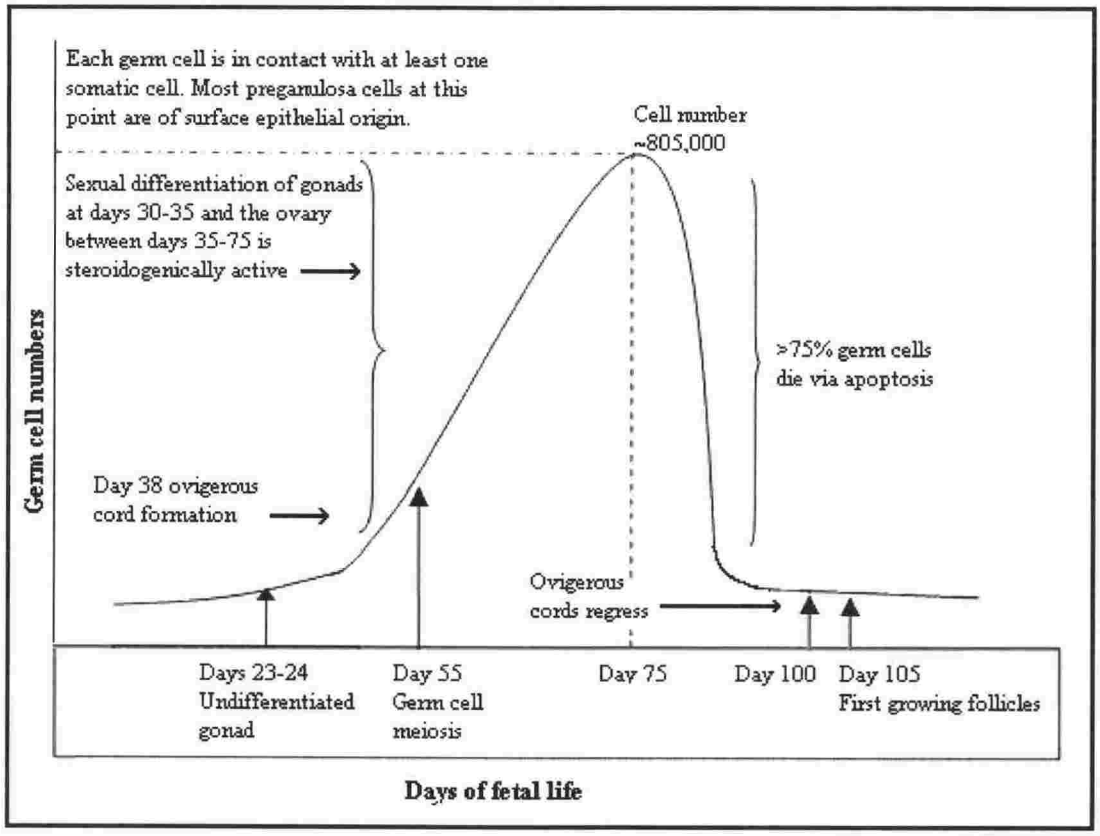


Figure 4: Schematic of time line of germ cell and follicular development during fetal life in sheep.

This diagram shows the increasing number of germ cells to a peak of ~805,000 at day 75. Germ cell numbers then plummet and the excess granulosa cells are thought to attach to the remaining germ cells. The x-axis is number of days of gestation and the y-axis indicates number of germ cells (data from Sawyer *et al.*, 2002)

1.6 Folliculogenesis

Folliculogenesis is a highly selective process and begins when a follicle leaves the pool of primordial follicles and enters the growth phase. The pool of primordial follicles tends to be found in the outer regions of the ovarian cortex. Once these follicles leave this pool, they are committed to growth but the mechanism(s) for this initiation are unknown (Shimasaki *et al.*, 2004). Recruitment of excess pregranulosa cells during fetal life may lead to the variability observed in numbers of granulosa cells present in the newly formed Type 1 and 1a follicles using the follicular classification method established by Lundy et al. (1999). This classification method describes an oocyte surrounded by either: a single layer of flattened granulosa cells (Type 1 follicle); a single layer of both flattened and cuboidal granulosa cells (Type 1a follicle) ; one, but

less than two complete layers of cuboidal granulosa cells (Type 2); two but less than four complete layers of cuboidal granulosa cells (Type 3); four to six layers of cuboidal granulosa cells and no antrum (Type 4); or a variable number of layers of cuboidal granulosa cells and the presence of an antrum (Type 5 follicle or antral follicle). Antral follicles appear at approximately Day 135 of fetal life in sheep.

After the transition from the primary (Type 2) to the secondary (Type 3) stage of folliculogenesis, the basal lamina of the follicle becomes surrounded by mesenchymal cells which will differentiate into the theca interna and theca externa layers (Shimasaki et al., 2004).

Receptors for both LH and FSH can be identified on theca and granulosa cells respectively. In the ewe, many gonadotrophin-responsive follicles develop between 1 and 2.5 mm (Scaramuzzi *et al.*, 1993). Thereafter, as follicles develop beyond 2.5-3.0 mm in diameter, they become increasingly dependent on FSH making them vulnerable to atresia when plasma FSH concentrations are low. The larger follicles also require LH for the thecal cell production of androgen as a substrate for aromatization to oestradiol by granulosa cells (Scaramuzzi et al., 1993). The conversion from gonadotrophin-dependent to an ovulatory follicle requires a low but critical concentration of FSH (Scaramuzzi et al., 1993). There appears to be a window during which gonadotrophin-dependent follicles remain viable. There is a shift in the steroidogenic activity of granulosa cells from a predominantly oestradiol-secreting to a progesterone secreting cell. This occurs close to ovulation and is referred to as luteinisation. It is well established that the process of luteinisation of granulosa and/or thecal cells is initiated by the pre-ovulatory surge of gonadotrophins (Yamamoto *et al.*, 2002).

In addition, to the importance of gonadotrophins for follicular development it is evident that factors produced within the follicle are also required (Yamamoto *et al.*, 2002). Bi-directional communication between the oocyte and surrounding somatic (e.g. granulosa and theca) cells are now known to be essential for normal folliculogenesis (Dong *et al.*, 1996; Galloway *et al.*, 2000). The results from studies on oocyte secreted factors and their interactions with granulosa cells have shown that the ovary itself contributes to the outcome of increased fecundity. These localised signals between oocytes and somatic cells within individual follicles, referred to as paracrine signals (Figure 5), are known to

play an important role in establishing the number of follicles that ovulate (Moore *et al.*, 2003).

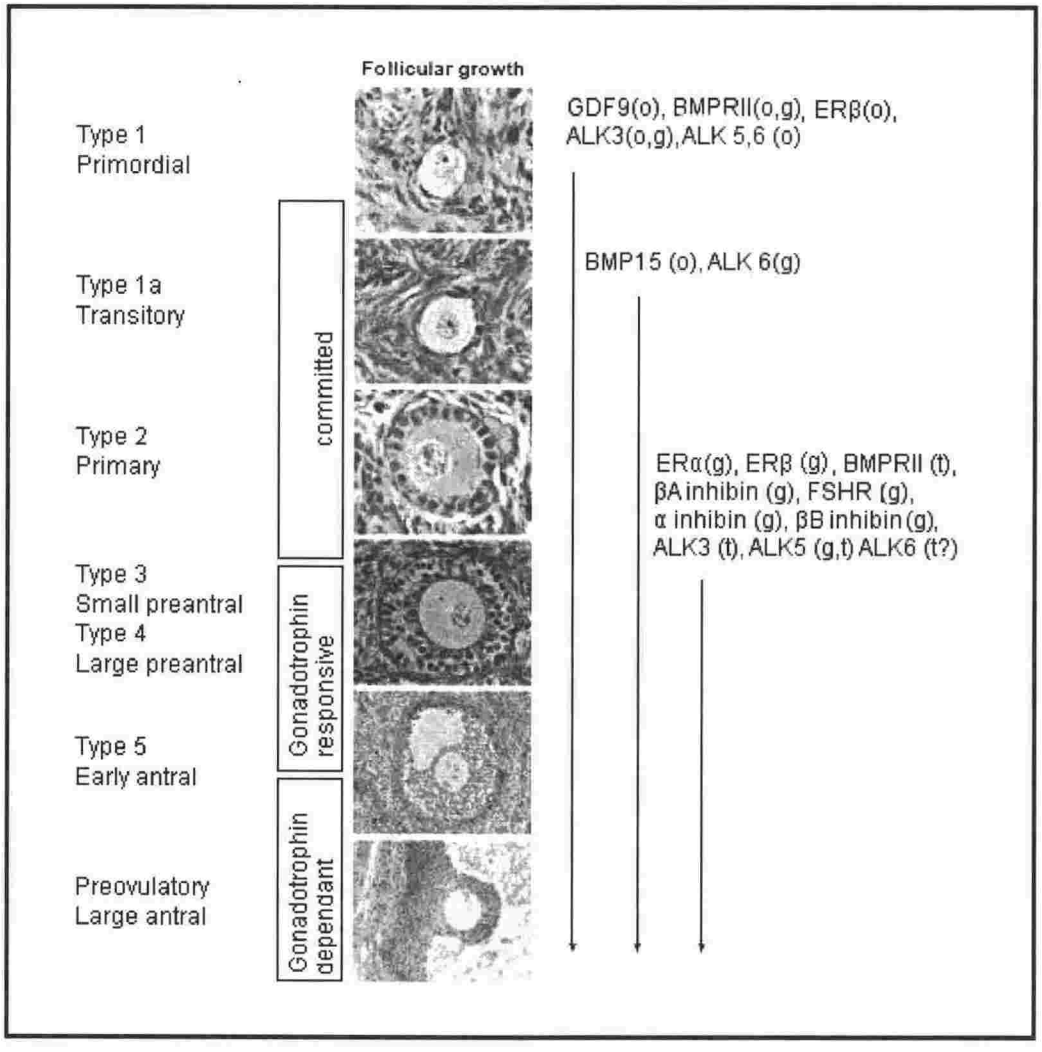


Figure 5: Follicular growth and gene expression.

A summary of the various stages of follicular growth in ewes together with the onset of expression of certain genes in (o) oocytes, (g) granulosa cells and (t) theca interna–externa (t?) theca interna but not certain. The solid vertical arrows indicate observations that all genes once expressed continue to be expressed at least until the time of the pre-ovulatory LH surge but only a very small percentage of follicles survive apoptosis. Abbreviations: Growth differentiation factor-9 (GDF9), Bone Morphogenetic Protein 15 (BMP15), Oestrogen receptor α and β (ERα, β), Follicle stimulating hormone receptor (FSHR), Activin receptor-like kinase 6, 5, 3 (Alk6, 5, 3), Bone Morphogenetic Protein Receptor Type II (BMPRII). (Data from Montgomery et al, 2001; Juengel & McNatty, 2005).

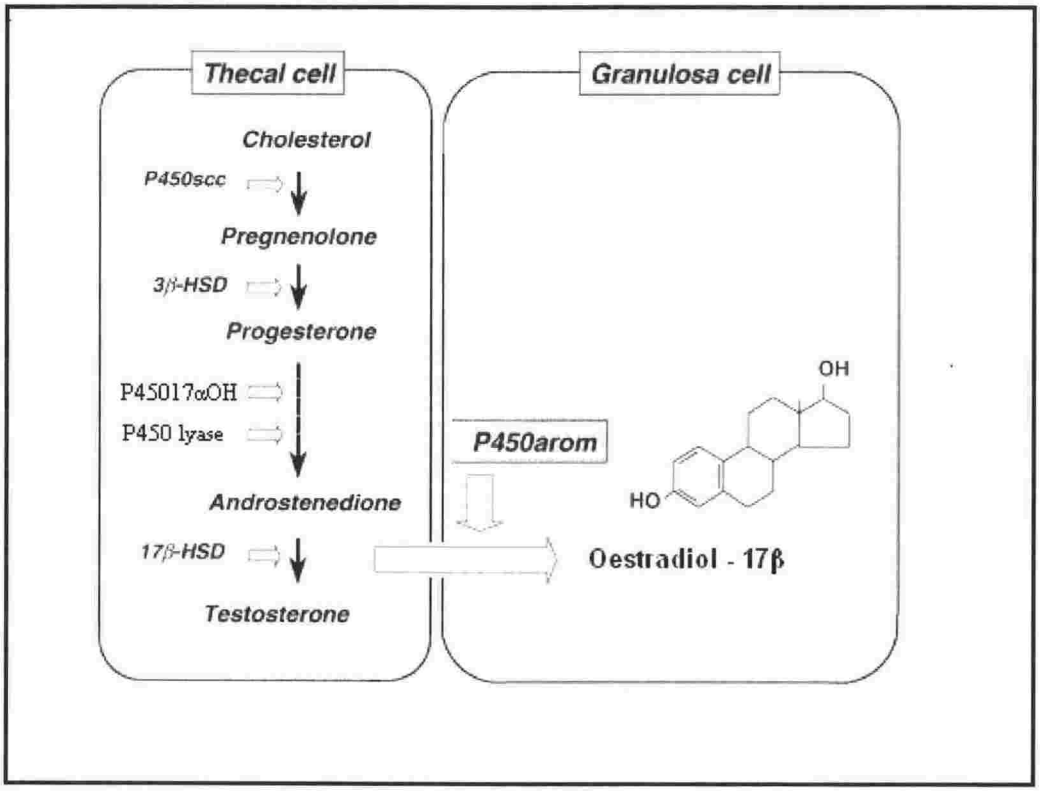


Figure 6: Steroid pathways in the ovary

Major steroid pathways in the ovary including the two-cell theory for oestradiol production. Cytochrome P450 side chain cleavage (p450scc), 3 β hydroxy-steroid dehydrogenase (3 β-HSD) cytochrome, P45017α hydroxylase and P450 lyase (P450lyase) (17αOH), and cytochrome P450 aromatase ($P450_{arom}$).

1.7 Steroidogenesis

A major role of the developing follicle is to synthesise steroid hormones. Steroidogenesis and especially the production of oestradiol-17β is facilitated through the complementary actions of the granulosa and thecal cells known as the two-cell theory (Figure 6). Under the stimulating effects of LH, thecal cells produce androgens but in many species, due to the absence of the P450 aromatase enzyme these cells are incapable of converting androgens to oestradiol. However, granulosa cells have the ability to convert androgens to oestradiol via the aromatase enzyme but lack the ability to synthesise significant levels of androgens de novo. Therefore, thecal cells may provide the substrate to the granulosa cells for the conversion of androgens to oestradiol.

At many levels, steroids contribute to the success of the reproductive process; not only as paracrine agents acting on adjacent cells but as autocrine agents acting within the cell they are produced (Knobil, 1988). They also act on a wide variety of target tissues and organs that contribute to the success of the reproductive process (Knobil, 1988; Logan *et al.*, 2002). In sheep, the onset of steroidogenic capability occurs in theca cells just before the antrum forms. The production of steroid hormones by the developing follicle is dependent upon the presence and activities of several key proteins, namely, steroidogenic acute regulatory protein (StAR), cytochrome P450 17α hydroxylase (17αOH), 3β-hydroxysteroid dehydrogenase (3βHSD) and cytochrome P450 aromatase ($P450_{arom}$) (McNatty *et al.*, 2000; Logan *et al.*, 2002). Logan et al (2002) showed that ontogeny of expression of these enzymes occurred at discrete stages of follicular development in both neonatal and adult sheep.

As FSH concentrations increase during the early follicular phase of the oestrous cycle, there is a concomitant increase in aromatase enzyme activity and a rise in the plasma concentrations of oestradiol. During the gonadotrophin-dependent stage, follicles have a requirement for threshold levels of FSH which decreases the closer the follicle is to the pre-ovulatory stage of growth. Thus the normal variation in FSH throughout the oestrus cycle is likely to influence the viability of many follicles as the levels fall below their threshold (Scaramuzzi *et al.*, 1993).

1.8 TGF-β family

Although it is well established that the hypothalamic-pituitary-ovarian endocrine system is critical for normal ovarian follicular development, it is also known that intra-ovarian factors are essential (Shimasaki *et al.*, 1999).

Some of the key intra-ovarian factors are members of the TGF-β superfamily. Members of the TGF-β super family share several characteristics including a pre-pro region and a mature region. Often the smaller mature region is the biologically active moiety. Typically, the mature regions of these proteins are arranged as disulphide–linked homodimers or heterodimers. Examples of the latter include the inhibins A and B, and activin AB (Figure 7). There are over 30 structurally related but functionally diverse

proteins in the TGF-ß superfamily consisting of anti-mullerian hormone (AMH), the three TGF-β isoforms TGF-ß1, TGF-ß2 and TGF-ß3, two inhibins (A and B), three activins (A, B and AB), twenty bone morphogenic proteins (BMP1-20) and 9 growth differentiation factors (GDF1-9). The description Bone Morphogenetic Protein was first given by Urist et al (1965) to the active components in demineralised bone and bone extracts that were capable of inducing bone formation at ectopic sites (Shimasaki *et al.*, 2004). BMPs are considered to be multifunctional proteins that regulate growth and differentiation in many cell types. They play important roles during embryogenesis in mammals, amphibians and insects in addition to bone development, immune and inflammatory responses, wound healing and hematopoiesis (Wilson *et al.*, 2001). The GDF proteins are closely related to the BMP group (Knight & Glister, 2003).

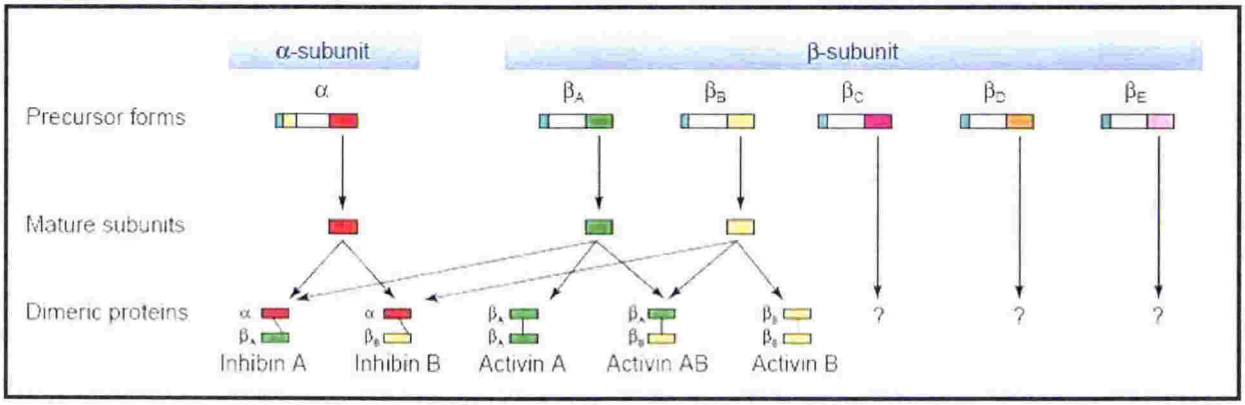


Figure 7: Representation of the interaction between activin and inhibin subunits

They are synthesized as precursor proteins before maturing to expose a C-terminal region that binds to either mature c-terminal regions thereby culminating in either homo and heterodimers of inhibin and activin (Ethier & Findlay, 2001).

In general the TGF-β family is found in a wide variety of tissues with several being expressed in ovarian follicles. In some lines of sheep mutations in some of these TGF- β superfamily genes or a related receptor have been identified as the causal factors with respect to changes in ovulation rate (i.e. BMP15 and BMPR1B, otherwise known as ALK6) (Galloway *et al.*, 2000;Wilson *et al.*, 2001).

The TGF-β family signal through a receptor complex involving two types of related trans-membrane serine/threonine kinases namely, a type-I (alternatively known as activin receptor-like protein kinases; ALKs) and type-II receptor (Ethier & Findlay, 2001; Juengel & McNatty, 2005). It is believed that upon ligand binding, the type-I

receptor recruits the type-II receptor or directly binds to a pre-existing complex of type-I and type-II receptors which is then phosphorylated. The activated receptors subsequently phosphorylate specific intracellular second messengers called Smads (either Smad 2 and 3 or 1, 5 and 8) which in turn interact with Smad 4, a common member of the TGF-β signalling pathways (Shimasaki *et al.*, 2004). Smads 1/5/8 or 2/3 are intracellular signalling proteins which interact with Smad 4 and the complexes translocate to the nucleus where they interact with transcription factors to subsequently regulate the expression of target genes. Smad 1/5/8 are specific for BMP ligands whereas Smad 2/3 are specific for TGF-β ligands (Figure 8). Seven members of Type-I receptor and five members of Type-II receptors have been identified in mammals (See Table 1). There is likely to be considerable promiscuity between the various BMP and TGF-β ligands. It is known that GDF9 binds to BMPRII and activates downstream responses mediated by Alk5 and Smad 2/3. TGF-β and activin interact with their particular TGF-β or activin Type II receptors, followed by activation of Alk5 and Alk4 respectively (Mazerbourg *et al.*, 2004) whereas BMP2 binds to BMPRII and Alk3. Combinations of the receptors complexes allow for differential ligand binding or differential signalling in response to the same ligand but many of these interactions remain to be resolved (Derynck & Zhang, 2003). In the ovine follicle, GDF9 is expressed in oocytes from the Type 1 through to the preovulatory stages of growth as is BMPRII in the oocyte (Juengel & McNatty, 2005; McNatty *et al.*, 2005a) Alk6 and BMPRII are expressed in both the oocyte and granulosa cells (see Figure 5). As follicles grow beyond the Type 1a stage of growth oocytes synthesise and secrete BMP15 and BMP15 plays an essential role in the transition between primary and secondary stages (Shimasaki *et al.*, 2004).

Homodimeric activins of β_A and β_B are produced in wide variety of tissue and stimulate the synthesis of FSH by direct action on gonadotropes (Findlay *et al.*, 2001). Maintenance of normal reproductive function is dependent on precise regulation of gonadotrophin biosynthesis and secretion. Control of gene expression for the unique β subunits of both gonadotrophins is regulated by circulating gonadal steroids as well as by hypothalamic-derived GnRH. In the ewe, activins are produced by granulosa cells (Figure 5) and the pattern of expression changes during folliculogenesis. In rats, the activins act to promote granulosa cell proliferation and potentiate FSH actions by increasing FSH receptor expression on granulosa cells (Shimasaki *et al.*, 2004). In sheep

and rats, inhibins produced by granulosa cells (Figure 5) are released into the systemic circulation to suppress FSH secretion from anterior pituitary (Findlay *et al.*, 2002). They may also have a local role in the ovary as a potent antagonist to the many actions of activin (Findlay *et al.*, 2002).

Table 1: Receptors of the TGF-β family.

Type I subtypes (alternative nomenclature)	Type II subtypes				
	TGFβR-II	ActRIIA	ActRIIB	BMPRII	AMHRII
Alk1	TGFß				
Alk2 (Act RIA)		BMPs activin	BMPs activin	BMPs	AMH
Alk3 (BMPRIA)		BMPs	BMPs	BMPs	
Alk4 (Act RIB)		activin	activin nodal		
Alk5 (TGFβRI)	TGFß				
Alk6 (BMPRIB)		BMPs			
Alk7			BMPs nodal	BMPs	AMH

The seven type-1 and five type-II receptors associated with TGF-β superfamily and in blue are some of the ligand-receptor combinations that appear to form active signalling complexes on the surface of responsive target cells (Knight & Glister, 2003).

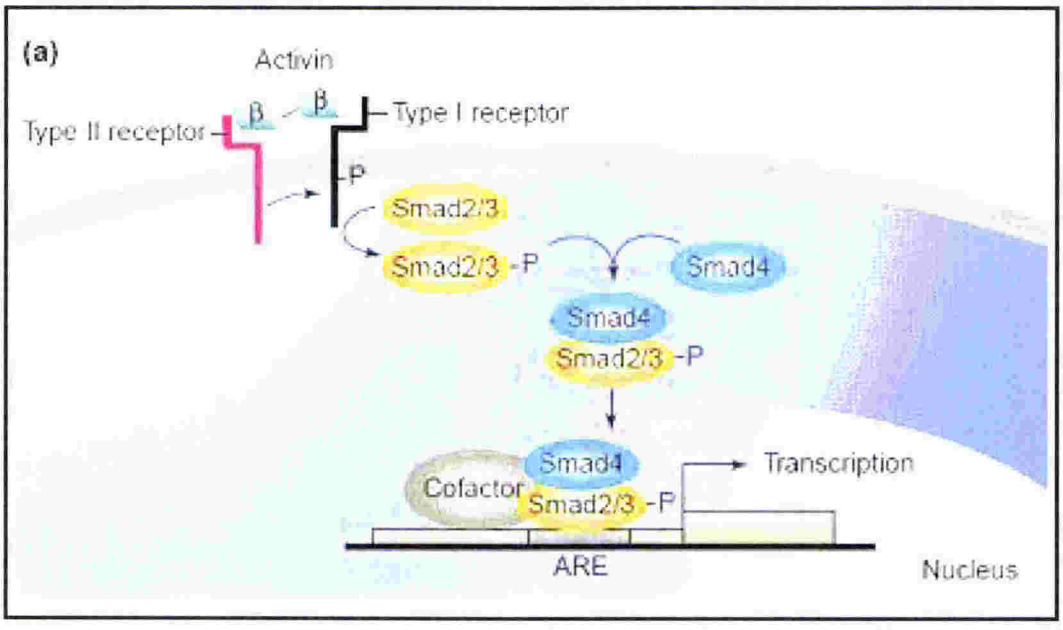


Figure 8: An example of the signalling mechanism initiated following activin binding to the type II receptor.

The type II receptor recruits and phosphorylates the type I receptor, which leads to phosphorylation of Smad 2 and 3 which in turn dimerize with the Smad 4. Once translocated into the nucleus, this complex links with cell-specific transcriptional co-factors and bind directly to the specific response element (e.g. activin response element; ARE) (Ethier & Findlay, 2001).

Anti-mullerian hormone (AMH) is best known for its effects during sex differentiation (Donahoe, 1992). Under control of the *sry* (*sex* determining *region* on the *Y* chromosome) gene on the Y chromosome, gonads in the chromosomal male differentiate into testes and AMH in the male signals regression of the anlagen of the female duct. (Visser & Themmen, 2005). However, AMH is also known to have a functional role in females during folliculogenesis. It is the only known negative regulatory factor for the primordial to primary transition phase of follicular growth (Skinner, 2005). Ovaries of AMH-knockout mice are depleted of their primordial follicles earlier than that of control mice (Jonard & Dewailly, 2004) AMH is inhibitory to early follicular growth but as it is not produced by resting follicles. It is likely to act via a paracrine mechanism as a localized growth-rate regulator. It continues to be expressed in growing follicles until they become steroidogenically active and thus at a differentiation state when they are to be selected for dominance. In sheep ovaries, AMH mRNA is present in granulosa cells of preantral and early antral follicles but not in primordial or medium to large antral follicles (Baarends *et al.*, 1995; Schmidt *et al.*, 2005).

1.9 GDF9 and BMP15 (alternatively named GDF9b)

The receptors identified thus far for BMP15 are BMPRII and ALK6 and for GDF9 are BMRII and Alk5 (Juengel & McNatty, 2005). Depending on the species BMP15 and GDF9 have either the same or different ontogeny of expression in the oocyte. These two genes have mutations identified as affecting ovulation rate in sheep, with BMP15 having five different, and GDF9 having one point mutation (McNatty *et al.*, 2005). From studies in mice it was reported that GDF9 was produced by oocytes and that targeted deletion of the GDF9 gene led to abnormal oocyte development and granulosa cells that did not proliferate (Dong et al, 1996). Consequently, folliculogenesis was found to be blocked at the primary stage of follicular growth. Similarly in sheep devoid of functional GDF9, folliculogenesis was impaired during preantral development and the animals were sterile (Hanrahan *et al.*, 2004). Mice deficient in BMP15 are fertile but have reduced litter size (Matzuk, 2000) whereas in sheep, BMP15 is essential for follicular development (Galloway *et al.*, 2000). Ewes which are homozygous for the Inverdale and Hanna mutations have a loss of functional BMP15, are sterile and fail to produce follicles beyond the primary stage of growth (Galloway *et al.*, 2000). Recently a BMP15 mutation associated with ovarian dysgenesis in women has been identified, which poses the question of whether there is a difference in the action of BMP15 between species depending on whether they are mono-ovulatory (sheep and humans) or poly-ovulatory, (mice). A difference in homozygous mutations between mice and sheep is that in sheep, GDF9 is incapable of rescuing the defects in early follicular development caused by inactive BMP15, whereas in mice GDF9 appears to compensate for the lack of BMP15 and is able to promote folliculogenesis (Liao *et al.*, 2002).

1.10 Cyclic AMP

Binding of FSH and LH to their respective heterotrimeric G protein-coupled receptors on ovarian granulosa cells activates the effector adenylyl cyclase. Adenylyl cyclase then converts intracellular adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) (Saxena *et al.*, 2004). Once cAMP is synthesised, it either binds and activates specific protein kinases such as protein kinase A (PKA) or is degraded by cyclic nucleotide phosphodiesterases (Heldin & Richards, 1988; Torres, 2004). Activation of

PKA requires an intracellular threshold concentration of cAMP resulting in phosphorylation of either cytoplasmic substrates or nuclear transacting factors (e.g. cAMP regulating element binding protein; CREB) thus controlling gene expression (Sassone-Corsi, 1998).

By measuring the amounts of cAMP produced by granulosa cells in vitro it is possible to determine the responsiveness (and sensitivity) of the cells to FSH and / or LH. For example, Booroola ewes with the mutation that enhances ovulation rate, were found to have granulosa cells that were more sensitive to FSH and LH earlier in follicular development than granulosa cells from wild-type animals (Henderson *et al.*, 1987). This suggested that the Booroola follicles were maturing earlier in follicular development than the wild-type controls (Henderson *et al.*, 1987).

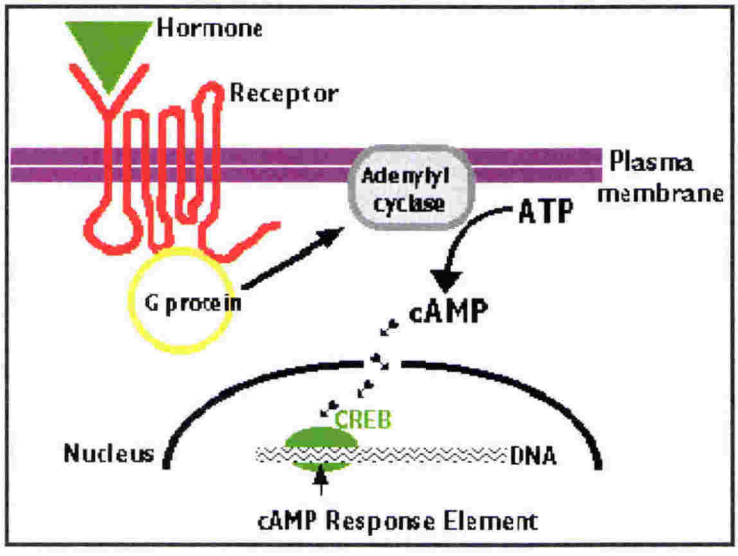


Figure 9: Example of how cAMP acts as a second messenger.

The hormone binds to a site on the extracellular portion of the receptor. The receptors are trans-membrane proteins that pass through the plasma membrane 7 times, with their **N-terminal** exposed at the exterior of the cell and their **C-terminal** projecting into the cytoplasm (from www.users.rcn.com on cell signalling).

1.11 Sheep model

In general, advances in our understanding of mechanisms involved in human fertility have relied on information from several different animal models (e.g. primates, rodents). Animal models have defined several key signalling pathways and proteins involved in reproductive physiology and many knock-out mouse models have been produced improving our knowledge of the genetic basis of mammalian infertility. An alternative approach to identifying factors regulating ovulation rate has also been to identify mutations that influence the target phenotype. In this respect sheep are an excellent model for human fertility being generally monoovular (ovulating only one egg during one reproductive cycle) and follow a similar pattern of gonadal and follicular formation during fetal development. Also, sheep have been a valuable model as a number of natural mutations responsible for increasing offspring or causing infertility have been identified. The naturally occurring mutations in sheep affecting ovulation rate have all been shown to be part of the Transforming Growth Factor-β (TGF-β) superfamily and a related cell membrane receptor. These studies have also indicated that the BMP sub-family of the TGF-ß family are key regulators of follicular growth (Souza *et al.*, 2004).

1.12 Natural mutations in sheep

Models of follicular selection indicate that multiple ovulations are influenced by concentrations of FSH near the time of follicular selection and by intraovarian factors (Scaramuzzi *et al.*, 1993; Galloway *et al.*, 2000; Matzuk, 2000). By studying natural and induced mutations that influence a phenotype (e.g. increased ovulation rate or the number of offspring), a number of genes affecting ovulation rate have been identified. Piper and Bindon (1982) presented the first evidence of segregation of a putative major gene with effect on litter size in Booroola merino sheep. Davis et al. (1982) showed the same effect on ovulation rate in New Zealand Booroola flocks. The mutation was subsequently mapped to sheep chromosome 6 and identified as a point mutation in BMP1B (Alk6) (Mulsant *et al.*, 2001; Souza *et al.*, 2001; Wilson *et al.*, 2001). Sheep with a Fec BB (Fec for fecundity and B for Booroola) mutation have a single A to G substitution at the nucleotide position 746. The resultant replacement of glutamine with

arginine at position 249 of the protein (Souza *et al.*, 2004) is in a highly conserved area of the receptor. A consequence is that females with a single copy (B+) of the mutation have an ovulation rate of 3 or 4 whereas to those with two copies (BB) have an ovulation rate of between 5 and 14. One physiological consequence of the Alk 6 mutation is that oocyte diameter in BB ewes is larger in smaller sized follicles relative to that in equivalent size follicles in ++ animals. The near maximum oocyte diameter is reached in follicles 236 and 383 um in diameter in BB and ++ ewes respectively (Figure 10). It was suggested that when the oocyte reached this near maximum diameter it signals a larger proportion of granulosa cells to enter a differentiative pathway in preference to a proliferative one (Wilson *et al.*, 2001).

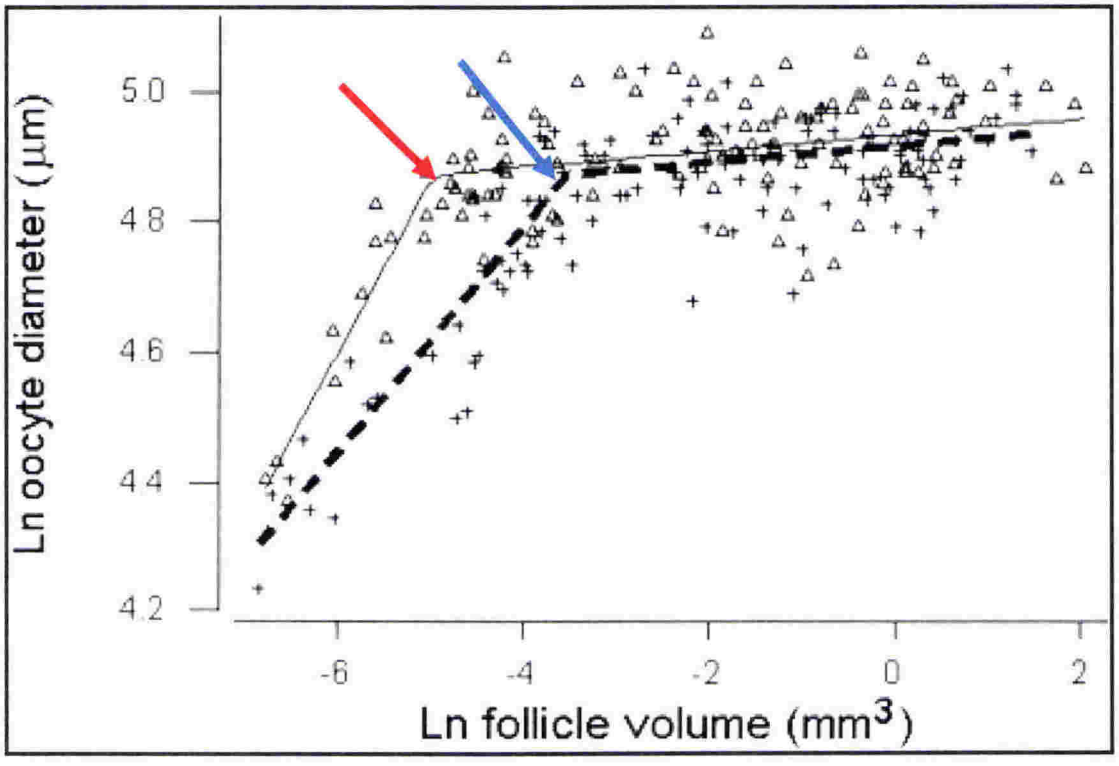


Figure 10: Relationship between logarithm (Ln) oocyte diameter (µm), and logarithm (Ln) of follicle volume (mm^3) with respect to Booroola genotype (BB=△ ;++=+).

The solid and dotted lines represent the best-fit lines for the BB and ++ genotypes, respectively (Wilson *et al.*, 2001). The red arrow shows the earlier maturation of the Booroola (BB) oocyte compared to the blue arrow of the control (++) oocyte.

Two strains of highly prolific sheep, the Inverdale and Hanna carry causal point mutations in the BMP15 gene (see Table 2). Sheep that are heterozygous carriers of the gene display increased fertility because of an increase in ovulation rate whereas sheep homozygous for the mutation are infertile (Galloway *et al.*, 2002). The Inverdale gene

(FecX^{I}) was first identified in descendants of a Romney ewe with consistently high lambing rates. Segregation studies showed the gene to be carried on the X-chromosome (Davis *et a.l.*,2001). The Hanna sheep (FecX^{H}), whilst unrelated to the Inverdale, displayed the same X-linked phenotype.

Sheep with the FecX^{I} mutation have a single nucleotide substitution (T-A) at nucleotide position 92. The resulting substitution of a valine to aspartic acid is again in a highly conserved region of the mature protein (Galloway *et al.*, 2002). Sheep with the FecX ^{H} mutation have a single nucleotide (C-T) substitution at nucleotide position 67: this translates to a stop codon at amino acid residue 23 of the mature protein.

Table 2 Polymorphic sequence variations in BMP15 and GDF9.

Gene	Allele	Base change	Coding residue (aa)	Mature peptide residue	Amino-acid change
BMP15	FecX^{G}	C → T	239	–	Gln → STOP
	FecX^{B}	G → T	367	99	Ser → Leu
	FecX^{I}	T → A	299	31	Val → Asp
	FecX^{H}	C → T	291	23	Glu →STOP
	FecX^{L}	G → A	321	53	Cys →Tyr
GDF9	FecG^{H}	C → T	395	77	Ser → Phe

The variations that affect ovarian follicular development and ovulation rate in sheep (Hanrahan *et al.*, 2004).

In ewes that are homozygous for FecX^{I} or FecX^{H} or have a copy of both mutations FecX^{I} FecX^{H}, (i.e. II, HH or HI), the ovaries are abnormal and they fail to ovulate. In Irish sheep breeds other point mutations have been observed in BMP15 that affect ovulation rate in a similar manner to that described for the Inverdale and Hanna phenotypes (e.g. FecX^{G} and FecX^{B}, see Table 2). In addition, a point mutation has been identified in the GDF9 gene of some Irish sheep (FecG^{H}, Table 2). As with the aforementioned BMP15 mutant animals, those heterozygous for FecG^{H} have higher ovulation rates than the corresponding wild-types whereas those homozygous for FecG^{H} are sterile with abnormal follicular growth during the preantral phase of growth

(Hanrahan *et al.*, 2004). Ovine BMP15 maps to the X chromosome and ovine GDF9 maps to chromosome 5 with both genes encoding a pre-proprotein region and a mature protein region (McNatty *et al.*, 2004). At present, five different point mutations in BMP15 in sheep and one in GDF9 have been identified and each one has a major effect on ovulation rate. GDF9 and BMP15 differ from other TGF-β superfamily members studied thus far in that changing concentrations of these two factors in vivo leads to incremental changes in ovulation rate in sheep, (Juengel *et al.*, 2002; McNatty *et al.*, 2003). In sheep, GDF9 mRNA expression is observed at the earliest stage of follicular formation in fetal life as well as in primordial or type 1 follicles right through to the large antral follicles (Bodensteiner *et al.*, 2000; Mandon-Pepin *et al.*, 2003). However, BMP15 mRNA expression is first observed by in situ hybridisation and immuno-histochemistry in Type 2 follicles through to the antral follicles (Juengel & McNatty, 2005). A point mutation in the proregion in BMP15 has been found in humans (Pasquale *et al.*, 2004), and the carrier women who are heterozygous for this mutation are sterile with follicular growth blocked during early follicular development apparently because the resulting BMP15 protein product is acting as a competitive antagonist.

Recently a line of sheep known as Woodlands have been identified with a highly unusual pattern of inheritance of ovulation rate. Whilst there is no direct evidence to suggest this is a single genetic mutation, the inheritance pattern is consistent with a major genetic effect. However, the increase in ovulation rate is relatively small (i.e. 0.4, or an extra egg every 2nd or 3rd ovulation). The Woodlands genotype is unusual in that it is the first gene to be identified as *imprinted* on the X chromosome and results in increased ovulation rate (Davis *et al.*, 2001). The following section discusses the inheritance patterns of major traits and genomic imprinting before expanding on the Woodlands.

1.13 Inheritance

Mendelian inheritance is where each parent donates a set of chromosomes to offspring so maternally and paternally encoded information is expressed equally. An understanding from Gregor Mendel's principles of inheritance is that the genetic makeup is expressed in the body (i.e. its phenotype) and is not associated with the sex of the parent. Most genes give rise to the same phenotype whether they are inherited

from the mother or father; however, in genomic imprinting the phenotype is dramatically different depending on the parent of origin. This phenomenon was discovered by two types of mouse experiments. Nuclear transplantation was performed to produce embryos that had only one of the two sets of parental chromosomes (uniparental embryo) or embryos that inherited specific chromosomes from one parent (uniparental disomy). In both cases, the finding was that the mammalian genes could function differently depending on whether they came from the maternal or paternal chromosomes (Reik & Walter, 2001).

1.14 Genomic imprinting

Genomic imprinting is an epigenetic mechanism of transcriptional regulation through which expression of a subset of mammalian genes is restricted to one parental allele (Verona *et al.*, 2003). That is, while the gene of one parent's copy is expressed, the other is silent. Over 70 imprinted genes have been identified in mammals with nine of those being in sheep (Verona *et al.*, 2003). It appears that genomic imprints change during the life cycle of the organism. Imprints are first established during the developmental progression of germ cells into sperm or eggs. Once fertilisation has occurred the imprints are maintained as chromosomal duplicates and thereafter undergo segregation in the developing organism. It appears that in germ cells of the new organism, the imprints(s) are erased at an early stage (Figure 11). The establishment of the imprint follows at a later stage of development (Reik & Walter, 2001 Figure 11). In somatic cells the imprints are maintained and modified during development. Loss of imprinting is involved in a variety of disease syndromes and cancers (Reik & Constancia, 1997). Embryos containing only maternal or paternal genomes develop abnormally and are characterised at birth as having diseases such as Prader-Willi (PWS), Angelman (AS), and Beckwith-Wiedemann due to loss of imprinting (Verona *et al.*, 2003). These diseases are often caused by large mega-base deletions: in the case of PWS the deletion occurs on the paternal chromosome whereas in AS the affected chromosome is always of maternal origin (Tilghman, 1999). A feature of imprinted genes is that they often cluster on a chromosome forming large imprinted domains e.g. ~ 80% are physically linked with other imprinted genes (Verona *et al.*, 2003). There are

also effects of position and copy number which suggests that regional control may also be required to achieve perfect imprinting (Reik & Constancia, 1997).

Imprinting is a particularly important genetic modification in mammals and is thought to influence the transfer of nutrients to the foetus and the newborn from the mother.
Haig and Westoby in (1989) suggested that imprinting arises in polyandrous mammals as a result of a conflict between males and females over the allocation of maternal resource to offspring. Fathers will favour strategies for extracting maximum resource from the mother for their offspring, whilst mother will protect the offspring by selecting strategies favourable for long term survival. This hypothesis can be extrapolated to the case of the Woodlands gene whereby the sire passes on genes to produce more offspring from daughters' i.e. granddaughters, whereas the mother turns the gene off when passing to her daughters so they in turn have less offspring and minimise their maternal investment or burden.

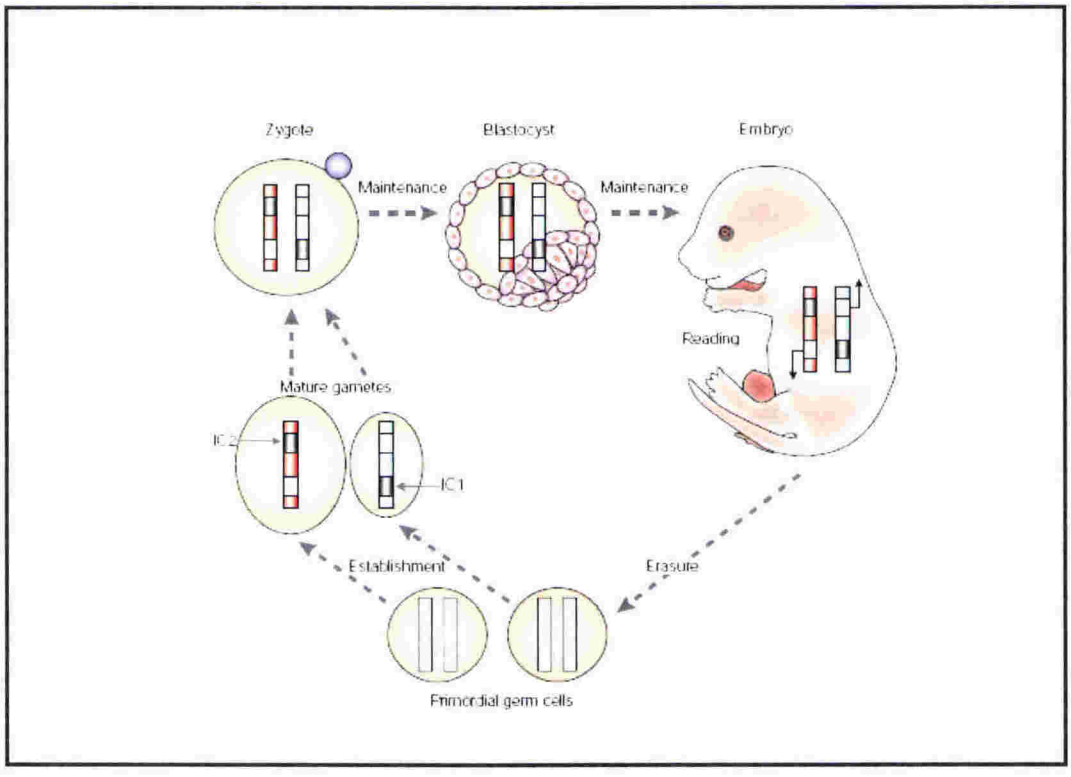


Figure 11: Key stages of genomic imprinting.

Erasure of the imprint on both parental chromosomes during germ cell development, followed by a new imprint established according to the sex of the germ line for the next generation, to maintenance for that generation and then reading of the stably propagated imprint. Successive cycles of erasure, establishment nd maintenance are a key feature of the imprinting process (Reik & Walter, 2001; Constancia et al., 2006).

1.15 Woodlands mutation (FecX2^{W})

The unusual pattern of inheritance in FecX2^{W} animals is due to the fact that females have an increased ovulation rate only if they receive the gene from their fathers (i.e. maternally imprinted; Figure 12; Figure 13). Furthermore, males only express the gene (i.e. have daughters with high ovulation rates and referred to as expressor carriers) if they inherit the gene from a mother that carried, but did not express the gene (i.e. normal ovulation rate and referred to as silent carriers) (Davis *et al.*, 2001). The FecX2^{W} which is present in both silenced (normal ovulation rate) and expresser (higher ovulation rate) females, was hypothesized to affect some aspect of ovarian development during fetal life thereby resulting in altered follicular growth during fetal and neonate life

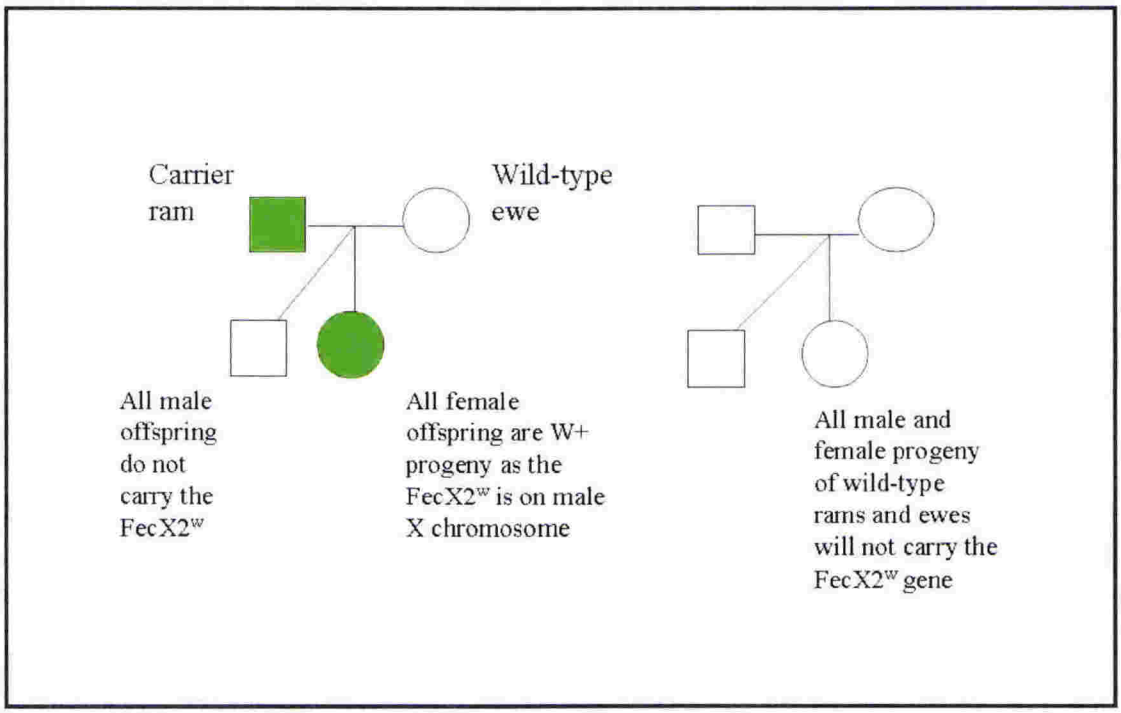


Figure 12: A breeding profile in FecX2^{W} carriers (W+) and non-carriers (++)

On the left, progeny of a wild-type ewe crossed with a Woodlands carrier ram giving rise to all the female offspring as carriers. On the right, a wild-type ewe crossed with a wild-type ram giving rise to off-spring none of whom inherit the FecX2^{W} gene.

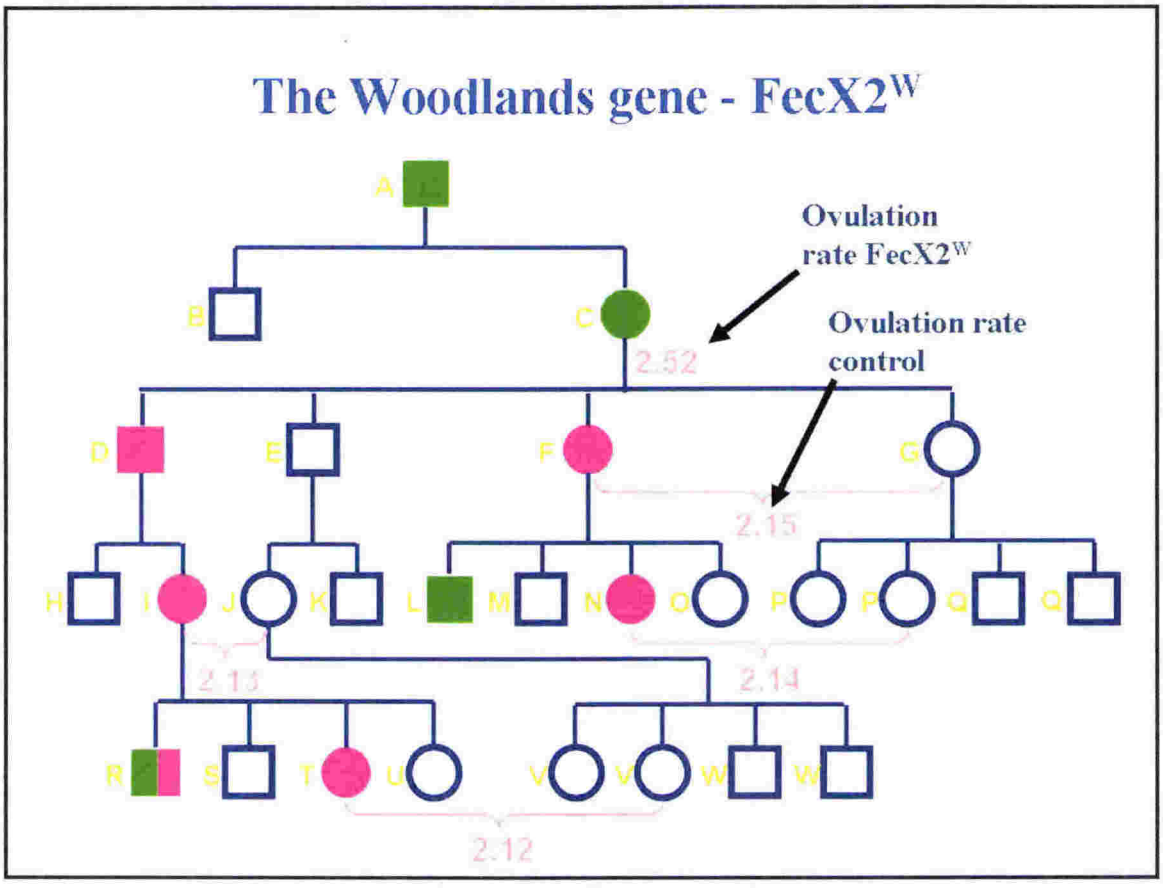


Figure 13: Woodlands inheritance pattern

A multi-generation family showing descendants of an expressor male (A) green, which sires an expressor female (C) green circle. Open squares are (male) and circles (female) non-carriers. Pink circles are silent females; i.e. they have the gene but do not express it. Numbers represent mean ovulation rates (Adapted from Davis et al 2001).

1.16 Dr George Davis and the discovery of Woodlands

In 1984, the ovaries of 15 two-tooth daughters of a Coopworth ram, (79-754) were observed using a laparoscopic technique. From examination of the ovaries by using this method it is possible to identify the number of ovarian follicles that have ovulated by counting the number of corpora lutea (CL). The number of CL can be considered to represent the ovulation rate. From examining the daughters of the aforementioned Coopworth ram, the mean ovulation rate at the end of the first cycle of mating was 2.60 compared with an ovulation rate of 1.78-2.20 from daughters of five other Coopworth rams used in the same year. Such a large difference suggested a possible major gene effect. Subsequent follow–up progeny tests, using the laparoscopic technique, were

carried out at AgResearch Invermay (Mosgiel, NZ) on Coopworth rams in 1987 and this included two grandsons and two great grandsons of ram # 79-754. When daughters of these rams were laparoscoped in 1989 there was no difference in ovulation rate between these ewes and controls.

In 1992, the ovulation rate records of all daughters, granddaughters and great granddaughters of ram # 79-754 were studied. All had been laparoscoped twice as two-tooth (18 month old ewes) and the mean ovulation rate was found to be 2.53 in the daughters, 2.18 in the granddaughters and 2.19 in the great granddaughters. This observation, coupled with the unimpressive performance of the four rams progeny tested in 1987 seemed to indicate that the outstanding performance of daughters of ram # 79-754 had been some 'flash in the pan' event with no evidence of it carrying on in subsequent generations.

From 1991 to 1999, another 108 Coopworth rams were progeny tested (i.e. lambing rates of female offspring examined). Most of these tests involved mating a single ram to ~ 70 ewes (and retaining 30 daughters) were carried out on research farms (69) and the remaining tests (39) were on seven commercial farms in Otago and Southland. Initially, the tests involved rams descended from ewes where there had been some signs of genetic segregation as shown by close relatives having markedly different prolificacy. This extensive progeny testing failed to produce clear evidence of any major gene. Throughout this time the occasional Coopworth ram showed up in the Woodlands flock with daughters that had higher ovulation rates than their contemporaries.

Sire 79-754 Revisited

By 1997, sire 79-754 and his descendants were back in contention because Dr Davis had found that a number of high ovulating ewes all traced back to him through their sires. A progeny test of 15 rams showed only two of the fifteen rams had daughters with high ovulation rates.

There had been seven Coopworth rams over the years in the screened prolific flock with high breeding values for ovulation rate i.e. they had daughters with high ovulation rates. All seven descended from a Lincoln University ram, but so did many of the sires used in

the flock. Daughters of high breeding value rams (by definition high ovulation rates) were followed through to their daughters (half should be carriers) and granddaughters (1/4 should be carriers). This revealed that the latter two groups of females had the same ovulation rates and were no different from controls (other ewes in the screened prolific flock unrelated to this family). Data showing that ewes inheriting the gene from their sire had high ovulation rates whereas those inheriting from their dam did not. Evidence that the gene was on the X-chromosome came from three sons of a high breeding value ram (all three were low) and that the coefficient of variation for ovulation rate is low in daughters of a high breeding value ram which like Inverdale, suggests that all carry the gene. Had only half been carriers this segregation would have given a high coefficient of variation. The mean ovulation rate of this small sample of ewes was the same as single copy expressers. This supported the hypothesis that they have one expresser copy (from their sire) and one silenced copy (from their dam).

1.17 The large ovary phenotype; a confounding factor

Originally animals carrying at least one copy of the FecX2^{W} gene were thought to have the large ovary phenotype at 4-weeks of age whereas non-carriers of the FecX2^{W} ovulation were thought to have normal size ovaries at 4-weeks of age. This was the situation as we understood it when the work in this thesis was started. Subsequently, with more extensive studies of carriers and non-carriers of the FecX2^{W} mutation, it was revealed that the large ovary phenotype was not co-inherited with the FecX2^{W} gene but randomly distributed amongst carriers and non-carriers. Before we were aware of this, we had made the decision to undertake quantitative morphometric studies and both qualitative and semi-quantitative in-situ hybridisation studies of ovaries of known FecX2^{W} carriers and non-carriers (i.e. 4-week old lambs and sexually mature i.e. adult ewes). In those studies, the 4-week old lambs also had both the large ovary phenotype and were carriers of the FecX2^{W} gene whereas ewes which were known non-carriers had ovaries of a normal phenotype. However other 4-week old lambs in these studies were classified as carriers and non-carriers based on the large ovary phenotype alone and not by pedigree of the rams. This was because Dr Davis and his colleagues thought initially that the large ovary phenotype at 4-weeks of age could be used as a marker for the FecX2^{W} mutation. Some if not all of these rams subsequently turned out not to be

carriers of the FecX2^{W} gene. For these studies we decided to determine some morphometric characteristics of the ovaries (i.e. size of cortex, size of ovary, how many large follicles and number of germ cells), the cAMP responsiveness of granulosa cells to FSH and hCG/LH and to establish functional as well as morphological differences to distinguish the large ovary phenotype from 4-week old lambs with the normal ovarian phenotype. Studies by Dr George Davis which were taking place at the same time as those described herein also showed that the large ovary phenotype was not evident at 2 weeks of age or in most ewes at 6 weeks of age. The cause and long-term consequences of the large ovary phenotype are currently not known. However, the large ovary phenotype is now known not to be associated with the FecX2^{W} mutation.

Ovaries from 4-week old lambs with the FecX2^{W} mutation and the large ovary phenotype were approximately six fold heavier than normal ovaries and contained ten times more large fluid filled follicles. This suggested the Woodlands ovary may be polycystic by nature and be a model for polycystic ovarian disease. Stein and Leventhal first described the PolyCystic Ovarian Syndrome (PCOS) in 1935 (Norman, 2002). They reported the association of polycystic ovaries in women with amenorrhea, hirsutism and obesity. Morphological and histological descriptions of ovaries from these patients showed a thickened tunica albuginea (the external lining of the ovary), hyperplasia of the theca interna and cortical stroma and multiple subcapsular follicles in various stages of atresia. PCOS is a heterogeneous disorder that may result from alterations in LH: FSH ratios, high intra-ovarian concentrations of androgen or follicular atresia. PCOS is the most common endocrine disorder in women of reproductive age and the leading cause of anovulatory (no menstrual cycle) infertility (Jonard & Dewailly, 2004). Often, these women do not show the extreme ovarian phenotype described by Stein and Leventhal. A characteristic feature of ovaries in these women are their larger size due to an excessive number of fluid-filled ovarian follicles (Franks *et al.*, 2000). Although the population of antral follicles is increased, there is some evidence to suggest that the disorder in folliculogenesis involves abnormalities during preantral follicular growth (Hughesdon, 1982). Women with the Stein-Leventhal condition had higher than normal number of small preantral follicles. In women classified with the PCOS condition were also found to have more small atretic follicles (Qyandt, 1993). A major marker of the polycystic ovarian morphology is excess androgen production (hyperandrogenaemia), and the theca and interstitial cells of

polycystic ovaries have shown to be the main source of the excess androgen (Webber *et al.*, 2003). Regardless of symptoms in women with polycystic ovaries, the thecal cells produce approximately 20 times more androstenedione in primary culture compared to thecal cells taken from normal ovaries (Franks & McCarthy, 2004).

Increased steroidogenic activity was not only confined to androgen production as other steroids are produced in excess in women with the PCOS condition. Steroidogenesis in the ovarian theca cell is primarily regulated by luteinising hormone (LH), which upon binding to the receptor promotes the cAMP cascade. Women with PCOS have elevated LH levels and evidence suggests that theca cells in these women may be hypersensitive to LH action. Additionally, increases in expression of aldehyde dehydrogenase 6 and retinol dehydrogenase 2 which play a role in all-trans-retinoic acid biosynthesis, and transcription factor GATA 6 resulted in the subsequent increased expression of 17α hydroxylase providing a functional link between altered gene expression in PCOS thecal cells (Wood *et al.*, 2003).

Chapter 2 - Aims

The aims of these studies were to examine selected parameters of ovarian function in Woodlands female sheep thought to carry the $FecX2^{W}$ mutation. The initial hypothesis being tested was that $FecX2^{W}$ in both silenced and expressor females affect some aspect of ovarian development during early fetal life resulting in altered follicular growth during late fetal and neonatal life. The overall aim of this PhD study was to test this hypothesis by investigating ovarian follicular development from 4-week old lambs and adults identified as carriers of the Woodlands gene and to evaluate some endocrine interactions between the ovary and pituitary gland. A secondary aim was to examine the unusual ovary phenotype in 4-week old lambs and to assess the gonadotrophin-responsiveness of the granulosa cells harvested from these ovaries. In the long-term, understanding the relationship and interactions of $FecX2^{W}$ with ovarian development, follicular growth, ovulation rate and fecundity is likely to assist in discovering the identity of the mutation(s) affecting ovulation rate in these animals. Given the novelty of this mutation, it is likely to involve a mechanism not currently known to be associated with reproduction. In turn this should lead to new and improved methods to regulate fertility in a wide range of mammals.

Chapter 3 - Methods

3.1 Animals and tissue preparation

In these and for all animal experiments where blood samples and ovaries were collected, ethics approval was granted by the AgResearch Invermay Agriculture Research Centre and the Wallaceville Animal Research Centre (Ethics numbers INV 595/03 and WA 803). A Coopworth Woodlands ram which was a progeny tested carrier ($FecX2^{W/Y}$) and another that was a progeny tested non-carrier ($FecX2^{+/Y}$) were mated with non-carrier Romney ewes. All female progeny from the mating between a carrier ram and non-carrier ewes were heterozygous carriers of the $FecX2^{W}$ gene, whereas those from the mating of the non carrier ram and ewes are non-carriers of the $FecX2^{W}$ gene. Birth dates and pedigree of the progeny were recorded. Progeny from each mating (n=6 per genotype) were euthenased with intravenous pentobarbital as 4-week-old lambs and the ovaries recovered. From each lamb a peripheral venous blood sample was obtained for further study. The blood samples were centrifuged at 500 g and the plasma collected and stored at -20 °C until assayed for hormones. After weighing the ovaries, one ovary was fixed in paraformaldehyde for in situ hybridisation and the other in Bouin's fixative for histological studies. Adult Coopworth ewes aged 6-10 years (n=8) that were also sired by one of three progeny tested pedigree $FecX2^{W/Y}$ rams or one of five progeny tested $FecX2^{+/Y}$ rams, were euthenased by exsanguination. Their ovaries were recovered and one was fixed in paraformaldehyde for in situ hybridisation and the other in Bouin's fixative for histological studies. The tissues were serially sectioned at 5 μm in paraffin wax.

For the cAMP studies, animals were selected on the assumption that lambs with the Large Ovary Phenotype (LOP) at 4-weeks of age carried the $FecX2^{W}$ gene whereas those with the Normal Ovarian Phenotype (NOP) were non-carriers. These animals were euthenased using cervical severing dislocation and exsanguination as anaesthesia affects pituitary hormone release. The animals were selected using a laparoscopy procedure. The reason using this selection criterion was that we had insufficient numbers of $FecX2^{W/Y}$ rams and it was thought that producing female offspring

associated with LOP at 4-weeks of age were carriers of the FecX2^{W} mutation. As mentioned, this subsequently was found to be incorrect.

3.2 Histological analysis

Ovarian volume $\mathbf{V}_{(o)}$ and ovarian cortex volume were estimated by the Cavalieri principle (Gundersen, 1986; Gundersen & Jensen, 1987). The ovarian cortex was defined as the outer region of the ovary containing all the germ cells. The total area and area of cortex area (**a**) in μm^2 of every 100th section was determined by point counting using a 10 mm grid for the cortex and a 20 mm grid for the ovarian volume. The volume **(V (o) = ∑a.h)** was calculated from the sum of the area x the distance between the sections **(h),** where **h** = every 100th 5 μm section thus 500 μm or 0.5 mm used to determine the area.

The area (**a**) in μm^2 of every 100th section (or every 10th slide each 5 μm thick, with 2 sections on each slide) was determined by point counting using a 10 mm grid for the cortex and a 20 mm grid for the ovary volume. The volume **(V (o) = ∑a.h)** was calculated from the sum of the area x the distance between the sections (**h**) used to determine the area,

Germ cell number was estimated by the dissector method first described by Sterio (1984) and since by (Smith *et al.*, 1993 & Smith *et al.*, 1997). Adjacent section pairs were used and these were 5μm apart. (Smith *et al.*, 1997).The tissues were sampled systematically with a minimum of 10 pairs of sections per ovary (Figure 14). The area chosen to count was selected at random using a clock as reference. An area of cortex was picked at random and measured (Figures 15, 16). This was then used to calculate the dissector volume, which is the proportion of the cortex used for counting follicles. Primordial follicles (using the oocyte nucleus as a reference) were identified in the first section of a pair and those follicles appearing in the second section of the pair, which were not present in the first were then counted (N). The dissector volume was calculated to give an area where primordial follicles could be counted.

Primordial follicles were identified and crosschecked with the adjacent tissue. The dissector volume was calculated to give an area where primordial follicles could be counted.

Cortex volume = $\sum$ pnts X (10/12.5) 2 X 0.5

In this formula, 10 = grid size, 12.5 = magnification and 0.5 mm is the distance between sections examined (every 10th at 5 μm = 0.5 mm). This gives an estimate of the volume of the cortex.

Dissector volume = $\sum$ pnts X (10/125) 2 x .005

The total number of Type 1 and 1a follicles was estimated from this number using the total cortex volume and the dissector volume (Cortex volume/dissector Volume x $\sum$ N). This method reduces the probability of counting follicles twice and giving an over exaggerated number of germ cells. All other types of follicles (Type 2-5) were identified and counted. The Type 5 follicles for the Woodlands lambs had previously been counted and measured by Peter Smith, (AgResearch, Wallaceville).

The cortex volume was measured using a projection microscope. The slide with ovary sections is projected onto a piece of paper and outline of the ovary and cortex drawn (Figures 14-16). A 10 mm grid was used to count how many points lay inside the outlined area, this was done on every 10th slide.

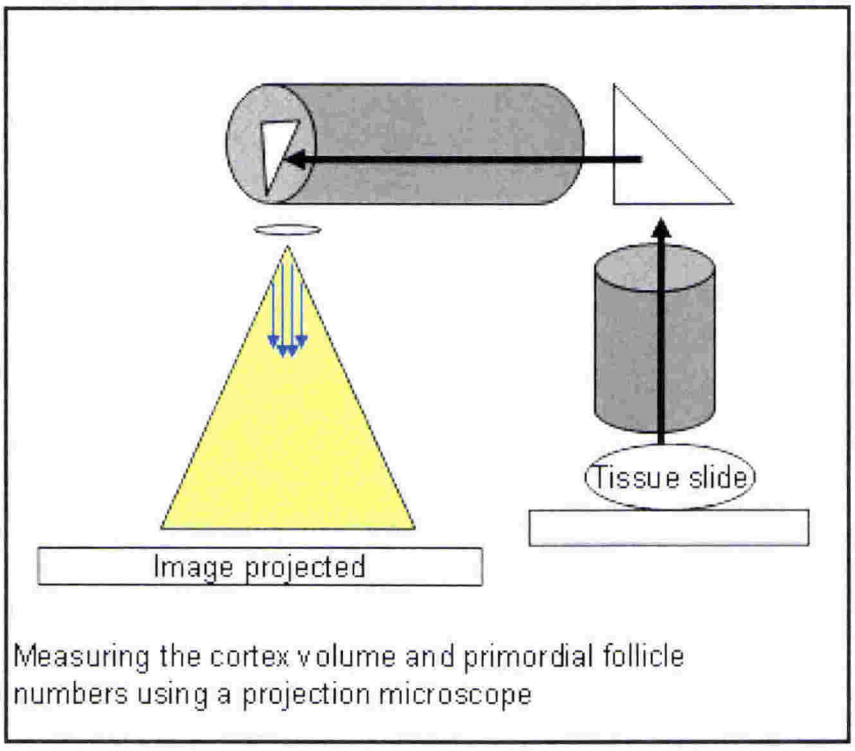


Figure 14: A schematic of how the tissue section on the slide is projected onto paper where it is measured.

The slide is placed under the microscope objective on the right hand side and the image is projected on to paper on the left.

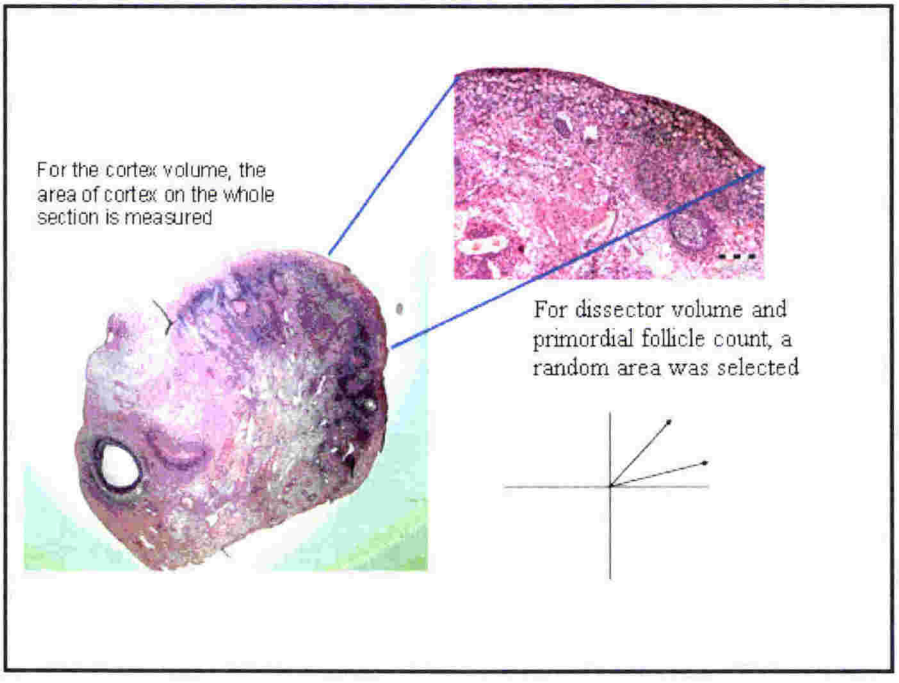


Figure 15: A photograph of ovary tissue

Ovary tissue in the left hand image and the randomly selected area for measurement and counting of primordial follicles shown on the right hand side at an increased magnification.

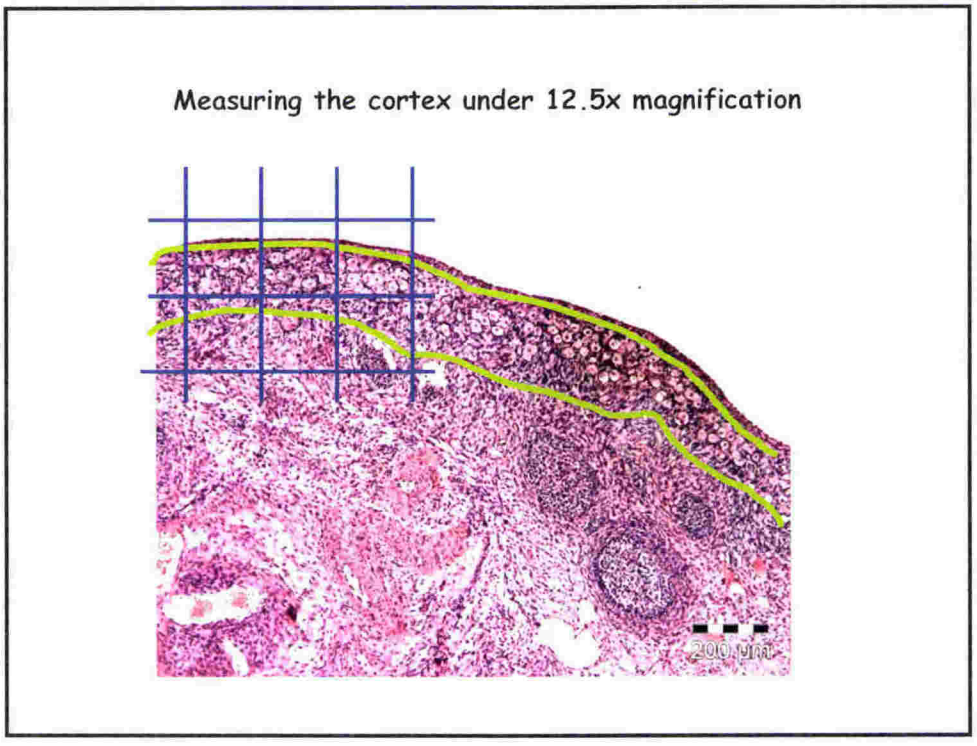


Figure 16: Photo of outlined cortex and 10cm grid over the top

Corner points are counted only if they fall in the cortex volume area.

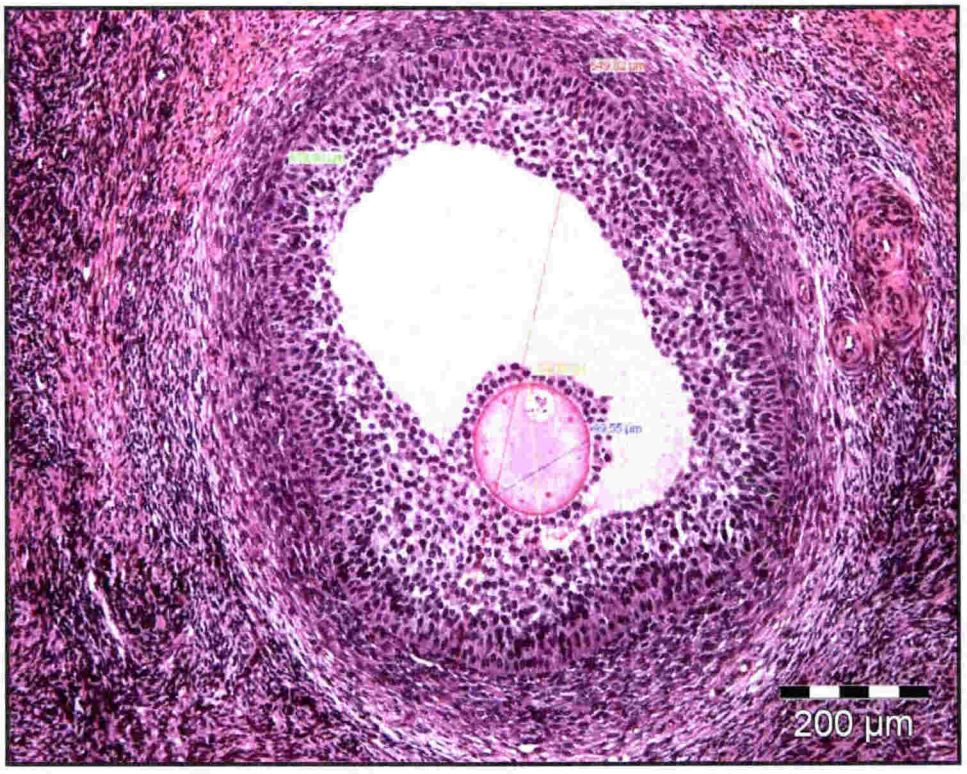


Figure 17: A Type 5 follicle with two measurements made of the oocyte and two of the follicle

The measurements are made from the basement membrane for the follicle.

The diameters of the follicles and oocytes for adult follicles were measured using a Leica DMR microscope linked to Analysis software (version 3.2). For each animal, the diameters of the oocyte and follicle were measured; two measurements of each diameter were made at right angle to each other and then averaged. For counting the number of granulosa cells, oocytes were visualised in the optical plane containing the nucleolus and their diameters recorded. The total number of granulosa cells around the circumference in the same optical plane was then counted. A minimum of 20 granulosa cell-oocyte complexes were counted for Type 1 and 1a follicles and all Type 2 follicles were counted. All other follicular types in each slide were identified, counted and both oocyte and the follicular diameters were measured.

This method has been used extensively by the Reproduction Group at the Wallaceville Animal Research Centre and refined by Peter Smith.

3.3 Follicular classification

Developmental stage

The classification system used in this study was that described in the introduction(Chapter 1,section 1.5), namely a Type 1 follicle being a single layer of flattened granulosa cells; Type 1a, a single layer of both flattened and cuboidal granulosa cells; Type 2 follicle one, but less than two complete layers of cuboidal granulosa cells; Type 3, two but less than four complete layers of cuboidal granulosa cells; Type 4 follicle, four to six layers of cuboidal granulosa cells and no antrum and Type 5 or antral follicle, a variable number of layers of cuboidal granulosa cells and the presence of an antrum (Lundy *et al.*, 1999). Lambs at 4-weeks old were found to have ovaries with a large range of follicle types up to type 5 (<1mm) so it was appropriate to look for possible differences in follicular development between controls and Woodlands lambs.

Functional status (Atretic vs. non-Atretic)

For the Type 2, 3, 4 and 5 follicles:

Each slide was studied and those containing an oocyte were counted. All atretic follicles were also counted.

Atresia was determined when one or more of the following parameters were noted:

- >10 pyknotic thecal cells
- Disorganised thecal layer
- Disorganised granulosa layer
- Loss of basement membrane integrity (this is subjective but will usually be associated with other markers of atresia)
- Oocyte nucleus deformation
- >5 pyknotic granulosa cells

These definitions of atresia were made based on a paper by Jolly et al. (1997) and following discussions with Peter Smith (Wallaceville AgResearch). If a follicle did not show any of the atretic characteristics then these are considered to be non-atretic

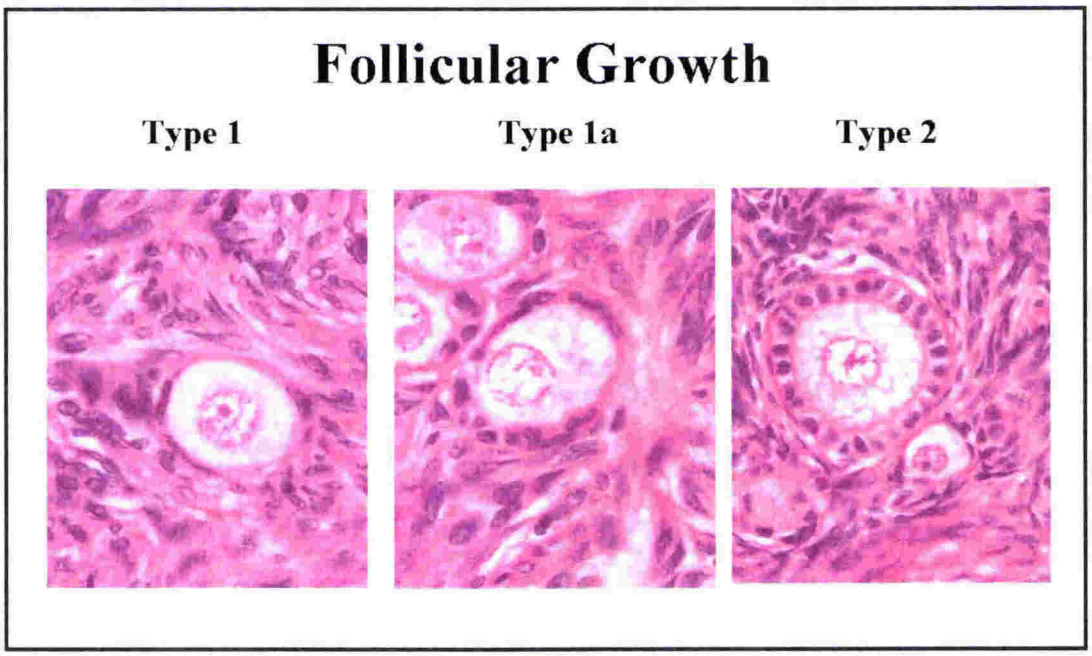


Figure 18: Follicular growth

Photomicrograph showing a Type 1, (all flattened cells) and Type 1a with a few cuboidal cells. At the primary (Type 2) stage, follicles are considered to be committed to growth and either grow through to ovulation or undergo atresia.

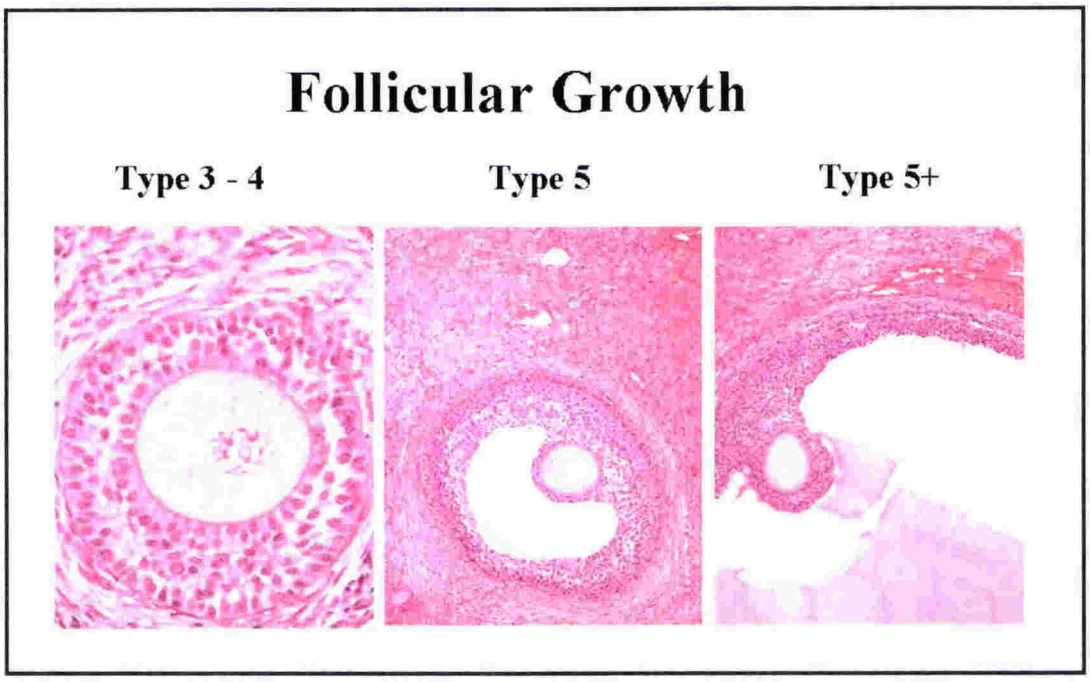


Figure 19: Follicular growth

The picture on the left shows a follicle growing through the pre-antral stage by increasing numbers of granulosa cell layers and becoming increasingly gonadotrophin-responsive. A Type 5 follicle is one with an antrum (fluid filled space) which continues to enlarge throughout development until ovulation or atresia intervenes; the oocyte by now is close to the maximum diameter. In sheep there can be up to 8 layers of granulosa cells in an antral follicle. Type 5+ follicles were those considered to be > 1mm diameter in the present study: these follicles eventually become gonadotrophin-dependent.

3.4 In-situ hybridization

Using established in-situ hybridisation techniques (e.g. Galloway *et al.*, 2000; Juengel *et al.*, 2002; Quirke *et al.*, 2001; Figure 20) the patterns of gene expression for hormones, growth factors and hormone and/or growth factor receptors including g Growth Differentiating Factor (GDF) 9, Bone Morphogenetic Protein (BMP) 15, α inhibin subunit, follicle stimulating hormone receptor (FSHR), oestrogen α and β receptors (ERα, ERβ) and β_A inhibin and β_B inhibin were examined. These studies were performed on the paraformaldehyde-fixed ovaries collected previously from 4-week old FecX2^W carrier (n=4) and non-carrier (n=4) 4-week-old lambs.

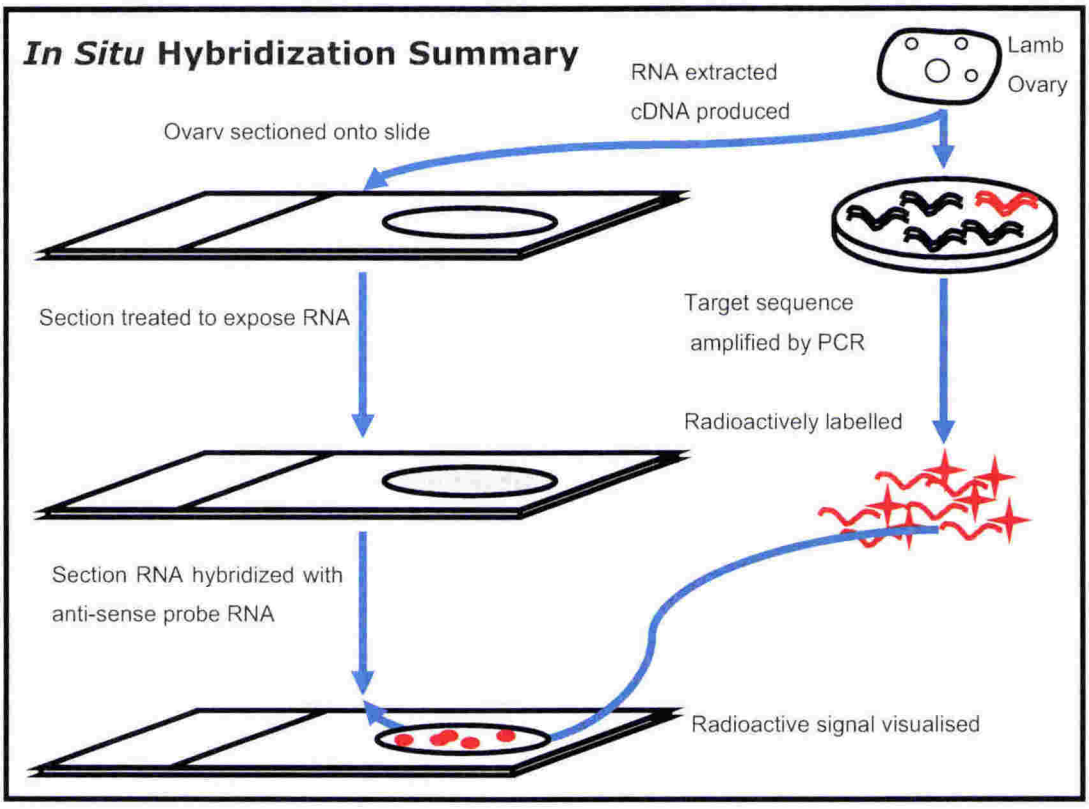


Figure 20: An outline of the method for in situ hybridisation

(Prepared by A.Bibby, AgResearch, Wallaceville).

Cells stocks of E.coli containing the gene insert of interest were grown overnight on streaked plates (see Appendix 2 for plasmids). The plasmid stocks for all genes were available before these studies began, Samples of colonies were then removed and placed into liquid growth medium and again grown overnight. These mini preps were then processed using a Quiagen® mini prep kit, to extract the DNA. The concentration of DNA was measured on a photometer to calculate the amount of enzyme to linearise the DNA ready for making a radioactive probe.

All the in-situ hybridisations were incubated overnight at 55 °C for homologous probes or 50 °C for non-homologous probes. The hybridisation solution that contained 45,000 cpm/ul of ^{33}P-lablelled antisense RNA was generated with T7 or SP6 RNA polymerase using the Riboprobe Gemini system (Promega). Non-specific hybridisation of RNA was removed by RNAse A digestion followed by stringent washes. This was followed by an overnight incubation on a Kodak film to observe any probe activation and then dipped in emulsion (LM-1; Amersham, Auckland, New Zealand). The slides were then stored for 2-4 weeks at 4° C, developed in Kodak developer D-19 (Radiographic supplies,

Christchurch New Zealand) and then counterstained with hematoxylin. The in-situ hybridisation technique is used extensively by the reproduction team at Wallaceville AgResearch and has been refined for use in sheep (Figure 20); (Tisdall *et al.*, 1999, Quirke *et al*, 2001, Logan, 2002).

All follicles with signs of atresia, i.e. lack of distinct basement membrane, degenerate oocytes or pyknotic granulosa cells, were excluded from the study. Hybridisation was considered to be specific when the intensity of the signal within a tissue was different, as measured by visual assessment, to a tissue or background that is not expressing the gene of interest (Juengel *et al.*, 2000; Quirke *et al.*, 2001).

3.5 Comparisons of concentrations of GDF9 and BMP15

Comparisons of the strength of expression were also studied, looking specifically at the two growth factor genes expressed exclusively by the oocyte, namely GDF9 and BMP15. Silver grains were counted in the follicles by using a Leica DMR microscope linked to Analysis Software (version 5). Using 40X magnification of a follicle, an area was drawn around the oocyte where GDF9 and BMP15 were expressed. Because GDF9 was such a strongly expressed gene, the time for developing the emulsion during the in-situ process was decreased to two weeks so that silver grains could be counted. The BMP15 gene was left for the normal three-week period.

A measurement of the area of the oocyte was made and then the silver grains were tagged and the computer software calculated how many had been tagged. These numbers were then saved in a windows XL format for further analyses. The number of silver grains per unit area was calculated and any background number of grains was removed to give a reasonably accurate measure of silver grain intensity for each oocyte (Figure 21). After the oocyte was traced, the software then calculated the area within the tracing. Three random areas were also measured outside the stained tissue and averaged to give a background count (40 x magnifications).

This method was used to measure the intensity in all Type 1 to Type 5 follicles.

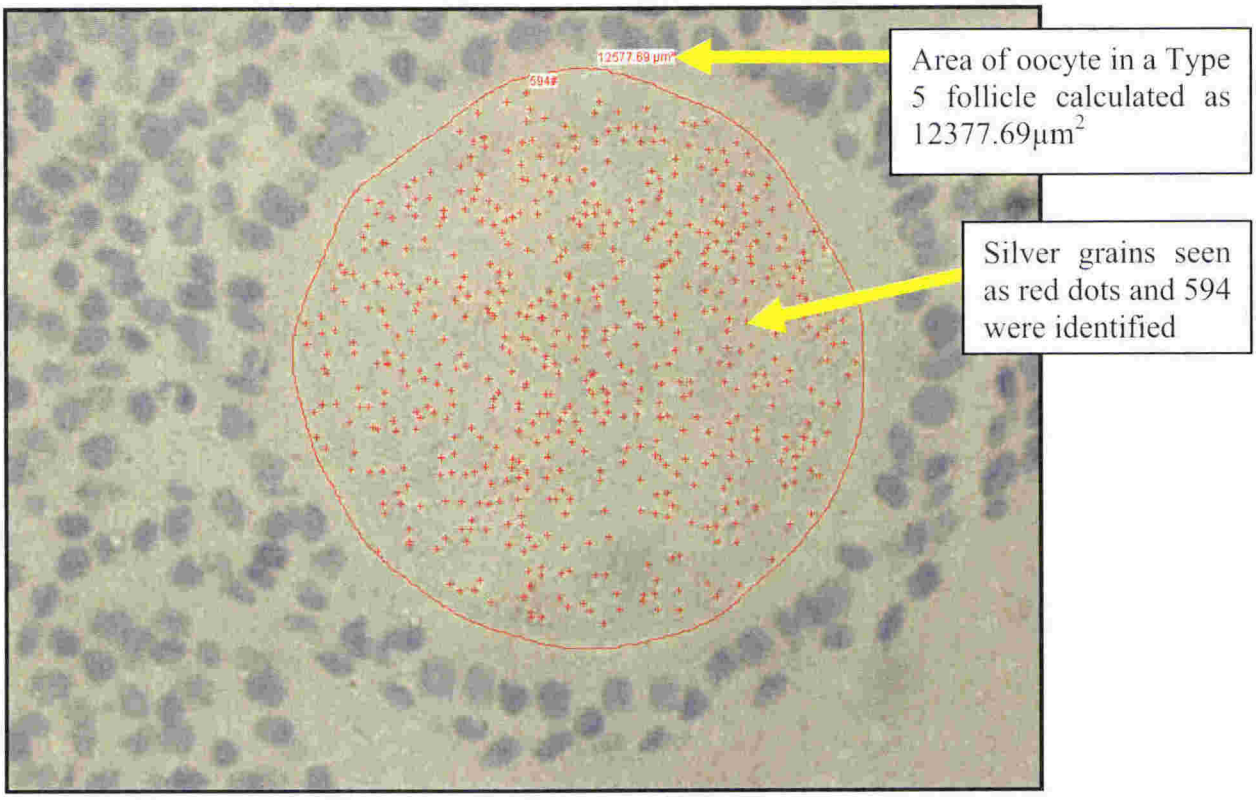


Figure 21: An example of a photograph of a type 5 follicle.

This figure shows the oocyte circled and the silver grains counted (as red dots) to determine the levels of expression of BMP15.

3.6 Cyclic AMP assay

The cAMP assay was used to measure the responsiveness of granulosa cells to follicle stimulating hormone (FSH) and luteinising hormone (LH). For these studies, ovaries were weighed and follicles dissected from the stroma and the follicular diameters were measured. Thereafter the granulosa cells were scraped out of the follicle into a petri dish with 1 ml of media (Dulbecos Modified Eagle media + 0.1% BSA w/v + 0.2 mM Hepes+ 3 isobutyl-1-methyl xanthine).

The numbers of granulosa cells in each petri dish were counted using a haemocytometer and thus the number of granulosa cells in each follicle could be calculated. Follicles were classified as non-atretic or atretic. Non-atretic follicles had an oocyte enclosed in cumulus oophorus cells and looked intact and with no debris evident in the follicular fluid when the follicle wall was disrupted. Furthermore non-atretic follicles had a visible vasculature in the theca at 40X magnification under the dissecting microscope.

In contrast, atretic follicles were characterised by the presence of debris in the follicular fluid, <25% of granulosa cells of the maximum number expected for a follicle for every mm diameter, of the follicle and no obvious thecal vasculature or a degenerative-looking oocyte (McNatty *et al.*, 1985). After calculating how many granulosa cells were present, approximately 300 µl of cells were added to 300 µl of media to achieve a cell concentration of about 200,000 per ml.

The tubes containing the cells were then incubated either alone or after addition of FSH to a final concentration 10 or 100 ng/ml, or hCG as a surrogate for LH at a final concentration of 1000 ng/ml. The FSH added was ovine (o) FSH–(2002) prepared by Dr Lloyd Moore at Wallaceville. This preparation had 1.4x receptor activity of United States Department of Agriculture–ovine FSH 19-selective immunoaffinity purified reference preparation (USDA-oFSH-19-SIAFF-RP2). The hCG (CR-121) was prepared and supplied by Dr Robert Canfield, Columbia University, New York (CR-121 = 13450 iu/mg). The tubes were then incubated in a 37 °C bath for 45 min to activate the cAMP followed by 15 min at 80 °C bath to stop any catabolism of cAMP and then stored at -20 °C until assayed for cAMP.

The RIA assay has been described in detail by (Frandsen & Krishna, 1976; Henderson *et al.*, 1987). The method adopted was that described by Henderson et al., (1989).

Briefly, 100 ul of standard or unknown sample was prepared in the same buffer/media. Acetylation was used to increase the sensitivity of the test. This was performed by mixing one volume of acetic anhydride with two volumes of triethylamine. Five microlitres of acetylation reagent was added to all samples and standard tubes (including controls) and then mixed immediately by vortexing the tubes.

100 ul of antibody (that had been diluted containing 0.1% BSA so that it bound 30-40% of the Succinyl cyclic AMP Tyrosine Methyl Ester-Iodine125 (ScAMP-TME-I125) tracer was added. The iodinated cAMP tracer was added in 0.05 M Na Acetate buffer, ph 6.5 containing 2% (w/v) BSA. The tubes were incubated at 4 °C overnight and then 50 ul of a secondary antibody diluted 1/20 in sodium acetate buffer was added and the tubes incubated at room temperature for 60 min. The secondary antibody was sheep anti rabbit IgG. Thereafter, 1 ml of 14% w/v PEG-8000 phosphate buffered saline was

added to the tubes which were then centrifuged at 4000 rpm for 25 min at 10 °C. Subsequently the liquid was decanted and the precipitate counted on gamma counter (Wallaceville, AgResearch; 1470). The detection limit of the cAMP RIA was 2 femptomol/tube and the granulosa bioassay had a limit of detection of 0.2 picomoles /million cells.

3.7 Follicular fluid recovery and preparation for protein identification

Follicular fluid was collected from the same animals as the granulosa cells for cAMP studies. These were collected and assessed retrospectively to have been taken from animals with either unusually large ovaries (large ovary phenotype; LOP) or the normal ovarian phenotype (NOP). Each ovary was weighed and all visible follicles dissected under a dissecting microscope.

The diameters of the antral follicles were determined and these were divided into groups of those ≤1mm and anything larger was put into separate container. All antral follicles were punctured and the follicular fluid aspirated into a pre-weighed Eppendorf tube. The tubes were spun at 300g for 5 min to remove any sediment and then put into 0.1% (w/v) BSA and 0.01M phosphate buffered saline (PBS) and snap frozen on dry ice. Because the samples had been preserved in 0.1% BSA and PBS, it was subsequently found to be necessary to remove these before analyses. To achieve this, an albumin and IgG removal kit supplied by Amersham Biosciences (Sweden) was used.

Fifteen microlitres (µl) of follicular fluid (usually pooled) was added to 750 µl of the albumin and IgG slurry, and shaken for 30min at room temperature. This was then transferred to a microspin column and spun for 5 min at 6500 g. This gave a filtrate of 500 µl to be used for running the gel. To precipitate the protein, the Calbiochem protein precipitation kits (KP 31480 and KP31484) were used with 200 µl of filtrate. This was added to 0.8 ml of precipitation agent at -20 °C and incubated for 20-60 min. This preparation was then centrifuged for 10 min at 10 000g and aspirated from the pellet. The pellet was subsequently washed and centrifuged again for 2 min at 10 000 g. After aspiration, the pellet was left to dry for between 5 min and 1 hr. This is considered to be a standard method for precipitating proteins when preparing for isolelectric focusing

and 2D gels. A 2D quant kit (Amersham Biosciences) was used to quantify the protein before adding to the isolelectric focusing strips. This requires the use of a BSA standard curve from 0, 5,10,15,20 & 25 ug/ml BSA with the absorbance read at 450 nm.

3.8 1D and 2D gels

Initially, a one-dimensional SDS polyacrylamide gel electrophoresis (1DE) was carried out using Tricine gels (Table 3; Figure 22). The method used a three layer system with 4% stacking gels cast above the 10% and 15% gel layers to enhance separation of smaller proteins in the bottom where the higher percentage gel was located.

Table 3: Preparation of Tricine gel layers.

Gel percentage	4%	10%	15%
Acrylamide(ml) 30%T 2.95%C	0.67	3.33	5
Gel buffer (ml) Composition	1.24	3.33	3.33
Glycerol (ml)	-	1.3	1.3
H2O (ml)	3.05	2	0.33
TEMED (µl)	10	10	10
10% APS (µl)	30	30	30
Total (ml)	5	10	10

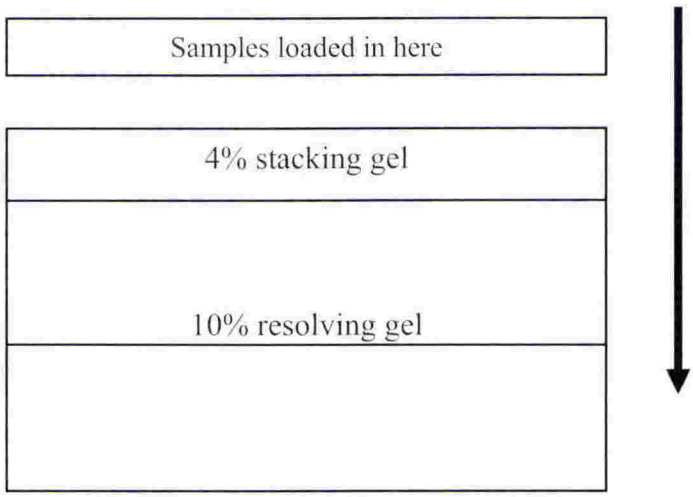


Figure 22: An outline of the 1D gel

The gel was electrophoresed, in an Invitrogen (Carlsbad, USA) X-cell sure lock™ gel system, for 1 hr at 200 V using a Bio-Rad Power Pack 200. To minimise overheating, the gel box was put on ice to maintain a constant temperature. Gels were then fixed overnight in 40% methanol and 10% acetic acid and stained in Biorad (Hercules, CA). Bio-Safe Coomassie Blue G-250 for 2-3 days. Molecular weight markers were SeeBlue ® Pre-stained Standard from Invitrogen.

3.9 Isoelectric focusing and two-dimensional gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2DE) was carried out using first-dimension isoelectric focusing on Amersham Biosciences immobilized pH gradient (IPG) strips followed by a second-dimension SDS PAGE. Follicular fluid samples (20 µl) samples were passively loaded onto the Immobiline ™ IPG Dry Strips pH 3-10, 7 cm long, by inclusion in the rehydration buffer that was applied to the strips overnight. Isoelectric focussing was carried out in an Ettan IGPhor II (Amersham, Biosciences) system using the following steps 1-3 after the initial start up voltage of 300 V:

Step 1 1000 V for 1 hr

Step 2 5000 V for 1 hr

Step 3 5000 V for 3 hr

After isoelectric focusing the strips were equilibrated first in 1% [w/v] dithiothreitol for 10 min followed by 2.5% [w/v] iodoacetamide for 10 min. The second-dimension electrophoresis of the equilibrated strips was on an Invitrogen™ 10-20% Tricine or 4-12% Bis Tris gels. The gels were fixed overnight in 40% methanol and 10% acetic acid in the case of the Bis Tris gel or 50% ethanol and 2% phosphoric acid for the Tricine gel. The gels were then washed for 3 x 10 min in MilliQ water and stained in Coomassie Blue G-250 (34% methanol v/v, 17% ammonium sulphate w/v, 2% phosphoric acid v/v, and Coomassie Blue G-250, 0.075 g per gel) for 2-3 days or in Sypro® Ruby (Invitrogen) for at least 3 hr. When using Sypro® Ruby the gels were covered in tinfoil to protect them from light. Sypro® Ruby stained gels were washed in a solution of 10% methanol and 7% acetic acid for 30 min and then twice in MilliQ water for 5 min and scanned using a Fuji FLA 5100 fluorescence scanner (FujiFilm , Fuji Photo Corporation LTD., Minato-ku, Tokyo, Japan). The Coomassie Blue G-250 stained gels were scanned using a Molecular Dynamics laser densitometer (Molecular Dynamics, Sunnyvale, CA). The scanned images were imported into Amersham Biosciences ImageMaster 2D Platinum™ software for analyses. ImageMaster software was used was used to compare gels to detect differences in protein spot intensity (abundance) between samples.

For protein identification, spots were manually excised from Coomassie stained gels and processed to produce tryptic peptides for matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF) analyses. The excised gel pieces were put into 0.5 ml Eppendorf tubes and washed twice with MilliQ water. The spots were then destained with 50 mM ammonium bicarbonate/acetonitrile (ACN) 1:1 [v/v] while mixing on a shaker. The solution was changed every 45 min and the process was continued until all obvious stain was removed from the gels. Approximately 100 µl of 100% ACN was added and allowed to stand for 3-5 min so that the gel pieces were fully dehydrated. The gel pieces were put into a SpeedVac (Labconco, Kansas City, Missouri, USA.) vacuum centrifuge for 5 min to evaporate the ACN. Two µl of trypsin (0.25 mg/ml in 50 mM ammonium bicarbonate) was then added to the gel pieces and left at room temperature for approximately 10 min; this was to make sure the pieces had absorbed the trypsin. Twenty µl of 50 mM ammonium bicarbonate was then added and the tubes incubated in a 37 °C water bath for 3 hr.

The solution digest was then removed from the tube and transferred to a clean Eppendorf tube. Three washes of 30 min each in 0.5% trifluoroacetic acid (TFA) /ACN 50:50 were done and the wash solutions were added to the original digest. ACN was then added to the gel pieces and shaken for 30 min before adding the solution to the accumulated extract. The final sample was concentrated to dryness in a SpeedVac and stored at -20 ^{o}C until analysis. For MALDI-TOF, the samples were rehydrated in 10 µl of ACN: 0.5% TFA (1:1 v/v). Two µl of this sample was then mixed with 2 µl of the matrix solution (alpha-cyano-4-hydroxycinnamic acid [CHCA] 10 mg/ml in acetonitrile: 0.1% TFA 1:1 v/v). Thereafter 1 µl of this mixture was spotted onto a MALDI plate and left to dry.

3.10 MALDI-TOF

(Matrix-assisted laser desorption/ionisation time-of-flight) mass spectrometry

Mass spectrometry is an analytical method that determines the mass-to-charge ratio (m/z) of ions (Figure 23). In MALDI-TOF, ions are created by mixing the analyte with a small organic molecule that absorbs light at the wavelength of the laser, the matrix. The analyte becomes incorporated into the crystal lattice of the matrix and is then irradiated with a laser. The laser causes desorption and ionisation and the ions are then accelerated into the MS analyser. In TOF mass spectrometry, the analyser is a chamber under vacuum that contains no electric fields. The ions drift through the analyzer with the kinetic energy obtained from the potential energy of the electric field (Westermeier & Naven, 2002).

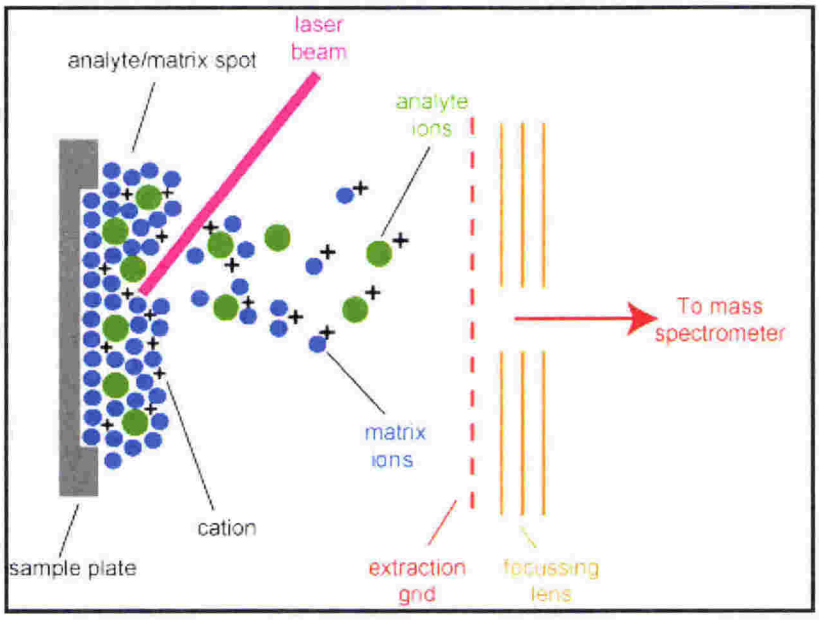


Figure 23: A schematic diagram of the mechanism of MALDI

From http://www.chm.bris.ac.uk/ms/theory/maldi-ionisation.html

3.10.1 Use of the MALDI, voyager-DE™ PRO, BioSpectrometry Workstation

Maldi spectra were acquired using the following instrument settings

Instrument mode

- Positive ion mode

Spectrum acquisition

- Mass range, e.g. 700-3500
- Low mass gate (Da) e.g. 600

Calibration

- Matrix α-cyano-4-hydroxycinnamic acid.

Measured masses were calibrated using the following common ion trypsin masses that were present in all spectra 805.4167 and 2273.1599 Da (these are m/z values).

3.10.2 Matching tryptic masses to sequence databases

Masses of tryptic peptides measured by MALDI-TOF mass spectrometry were matched to sequence databases to obtain probability based identifications of the parent proteins. Peptide mass fingerprints were searched using ProFound;

(http://prowl.rockefeller.edu/profound_bin/WebProFound.exe). Searches were made against the NCBInr database (using either "all taxa", "mammalia", or "other mammalia" taxonomy), using protonated monoisotopic masses, with one missed trypsin cleavage, complete modification of cysteine by iodoacetamide, partial modification by methionine oxidation, and between 0.1 Da or 5-50 ppm mass tolerance in the search settings. Criteria for a match included number of peptides matched; sequence coverage and use of Z scores to determine significance. Theoretical spot positions on 2D gels for unmodified polypeptides were predicted from known sequences using the Compute pI/Mw algorithm at www.expasy.ch.

3.11 Statistical analysis

4-week old lambs and adult ewes

For the germ cell numbers, oocyte diameter and follicle diameter, the data were normalised by a loge transformation and analysed using a 2-Way ANOVA with ewes as blocks. The oocytes and follicles to be measured were chosen randomly with a minimum of 20 counted from each animal for Type 1, and 1a but all Types 2, 3 4 and Type 5 follicles were counted and measured.

When the variance between the data sets were unequal, genotype differences were examined using the non-parametric *Kruskal-Wallis* test. This was the case for the type 5 follicles in the adults.

When measuring diameters, two measurements were made at right angles to each other and using the *Analysis version 3 digital imaging solutions (Soft Imaging System GmbH, Cyberjaya Malaysia)* these measurements were captured and saved to excel format. The duplicate measurements were than averaged and standard errors of the mean calculated.

The in-situ hybridisations were distinguished visually by observation of the signal, and whether it appeared stronger or lighter than the signal from the controls. For closer examinations of the silver grain intensity for GDF9 and BMP15, the data sets were analysed using REML to estimate the animal effect as they were too unbalanced for an ANOVA.

Chapter 4 - Results

Histological and morphometric analyses of ovaries from Woodland lambs and ewes

4.1 Introduction and Aim; Lamb ovaries

The observation had previously been made that the 4-week old Woodlands lambs had a larger ovary when compared to its wild type counterpart (Figure 24). Tissue sections at 5μm thickness had been prepared to determine why the ovarian mass was larger, and every 10^{th} section of 5 μm was examined using a Leica microscope. Type 5 follicles were identified and counted and it was found that there were ten times more of these follicles in the Woodlands lambs than in the control animals. It was hypothesised that the increase in the number of antral follicles may have been due to an increase in the number of Type 1/1a follicles, or, alternatively in and increase in the overall number of follicles growing to maturation. If true, this would account for the Woodlands ewes having an increase in ovulation rate. Further analyses were undertaken to test this hypothesis. Using a projection microscope, the total number of small follicles (Types 1 and 1a), and respective volume of the ovary and cortex, was calculated for each animal. Type 2, 3, and 4 follicles were also identified and counted using the method described by Lundy et al (1999).

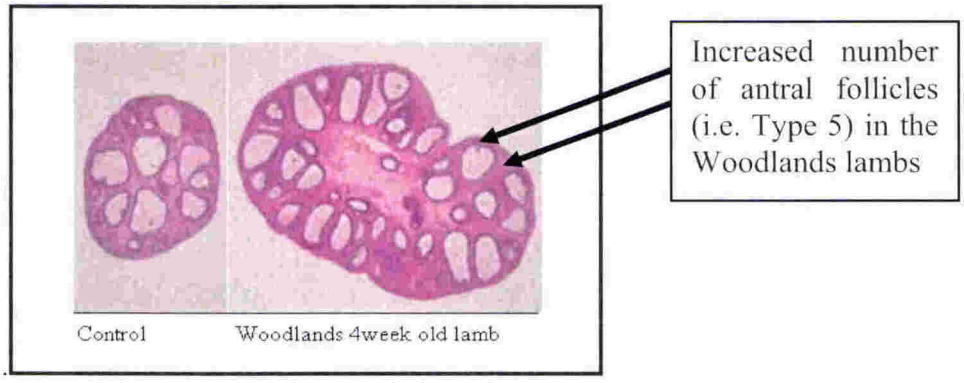


Figure 24: Ovarian sections from 4-week old lambs.

Non-carrier lamb Left panel and Right panel from a $FecX2^{W}$ (Woodlands) lamb (photo courtesy of P.Smith).

4.1.2 Results

In 4-week-old lambs, the mean volume of the ovarian cortex of the Woodlands (W+) phenotype was 30mm^3 compared to 9mm^3 controls (++). There was no difference in the number of granulosa cells in the largest cross-section of the Type 1, 1a or Type 2 follicles (Table 4). Moreover, there was no difference in the numbers of primordial follicles (i.e. Type 1/1a follicles, Figure 25) or in the pre-antral follicles Types 2, 3 and 4 (Figure 26). However there were 10 times more Type 5 follicles and the ovary was 6-fold larger than the control ovary (Figure 24).

No differences were observed between the genotypes in the follicular or oocyte diameters for any follicular type (Figure 27). However based on studies being undertaken by Dr Davis at the time of these studies it became evident that the large ovary phenotype was not strongly associated with the Woodlands fecundity allele. Therefore, it was uncertain whether animals carrying the Woodlands gene had different follicular populations compared to the controls. The major difference between the Woodlands and control lambs was in the Type 5 follicle population with a larger difference in the number of non-atretic small and larger antral follicles, (e.g.1453 vs. 186 $\leq$ 1mm diameter, $p < 0.01$, 776 vs. 46 > 1mm diameter, $p < 0.01$). There were also a higher percentage of atretic follicles for the Woodlands animals compared to controls compare 49% vs. 18%. Another difference seen in the 4-week old lambs was the size of the cortex volume which was ~ 49% larger in the Woodlands animals although very similar numbers of germ cells were counted, suggesting that the size effect was due to the greater number of Type 5 follicles in the W+ animals. While not statistically different, the mean oocyte diameter tended to be consistently larger in the W+ genotype (Figure 27b) and this finding needs to be investigated further with larger number of animals.

Table 4:

Granulosa cells in largest cross section of Type 1,1a and 2 follicles (Mean ± sem)				
	W+		++	
Follicle Type	Mean	sem	Mean	sem
1	5.25	0.49	6.05	0.26
1a	11.73	0.74	13.33	0.95
2	24.75	1.89	26.82	2.27

There were no significant differences between the Woodlands (W+) and the control (++) lambs in the numbers of granulosa cells in Type 1,1a and 2 follicles.

In Booroola sheep, there was a genotype difference in the pattern of accumulation of granulosa cells during follicular development which may have been due to the oocyte reaching a larger diameter earlier than in the wild-type (Wilson *et al.*, 2001). It was hypothesized that the larger oocytes in the Booroola ewes signalled a greater proportion of granulosa cells to enter a differentiative pathway as opposed to a proliferative one (Wilson *et al.*, 2001). This may be one mechanism whereby the Booroola follicles reached maturity at a smaller size compared to wild-type controls. It remains to be determined for Booroola ewes whether there are genotype differences in the granulosa cell composition or ultra-structural characteristics in the Type 1 and 1a follicles. However it has been established that there are no genotype differences in the populations of small follicles present (Smith *et al.*, 1997). To determine if any genotype differences in the numbers of granulosa cells was present in Woodlands animals a minimum of 20 Type 1 and 1a follicles as well as all Type 2 follicles were examined. No significant differences were found in the numbers of granulosa cells surrounding Type 1, 1a or Type 2 follicles (Table 4).

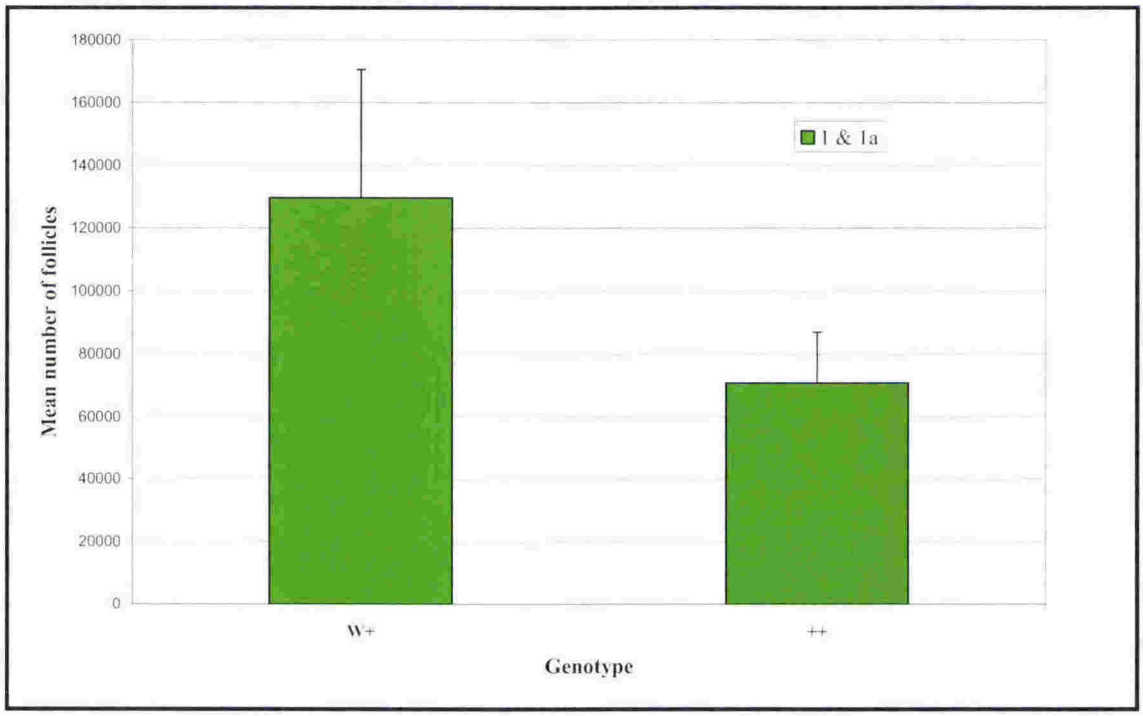


Figure 25: Number of Type 1 and 1a follicles (+ sem) in 4-week old lambs carrying the FecX2^{W} gene compared to control lambs (++) (n=6).

No significant differences between W+ lambs compared to the controls ++. (n=6)

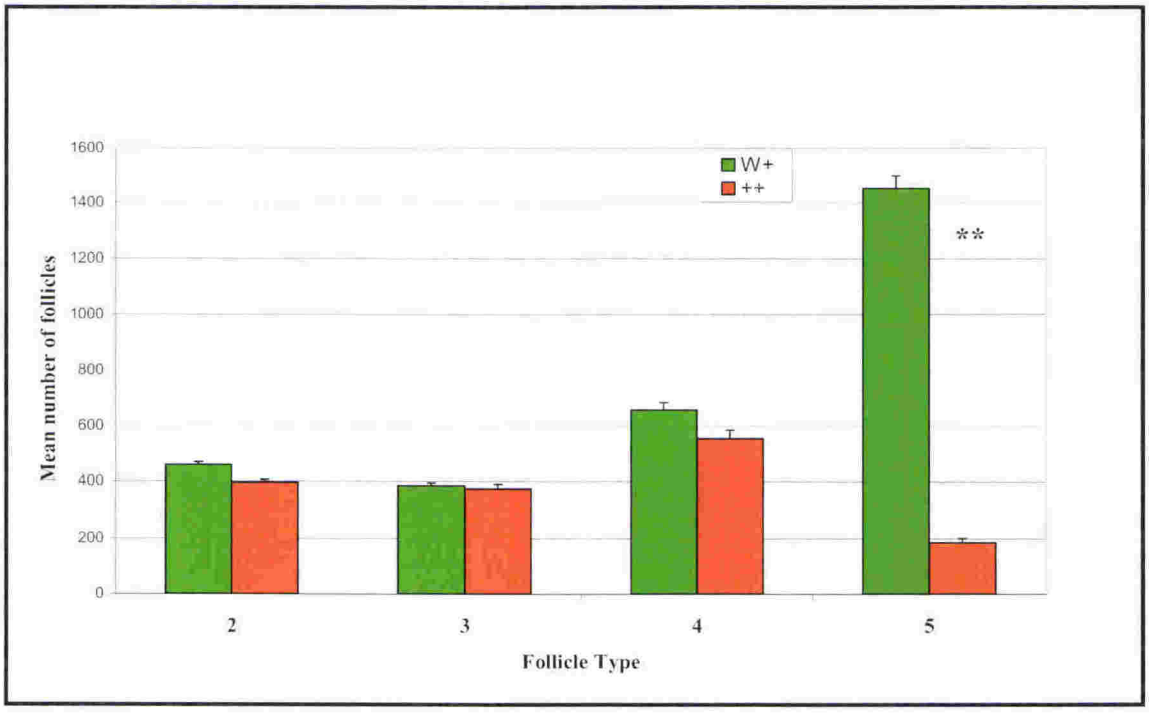
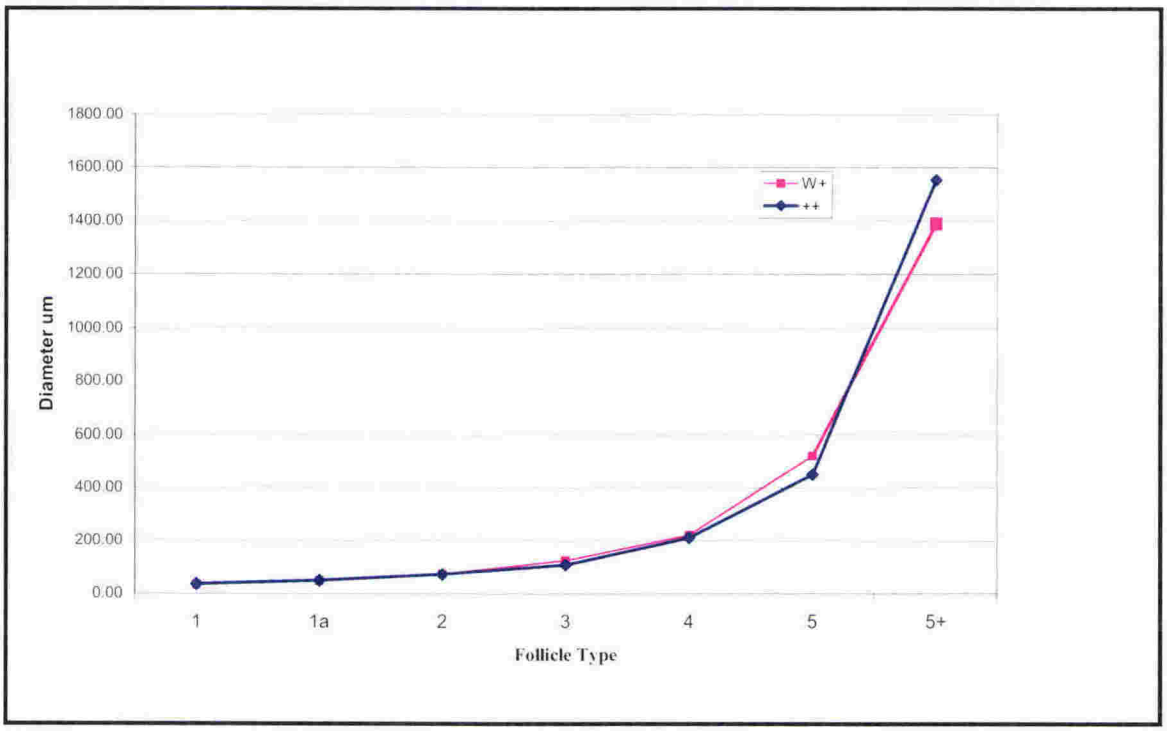


Figure 26: Mean number (+ sem) of follicles in 4-week old lambs carrying the FecX2^{W} gene (W+) compared to control lambs (n=6).

There were significantly more Type 5 antral follicles in the W+ lambs compared to the controls ++ (** $p<0.01$).

(a)



(b)

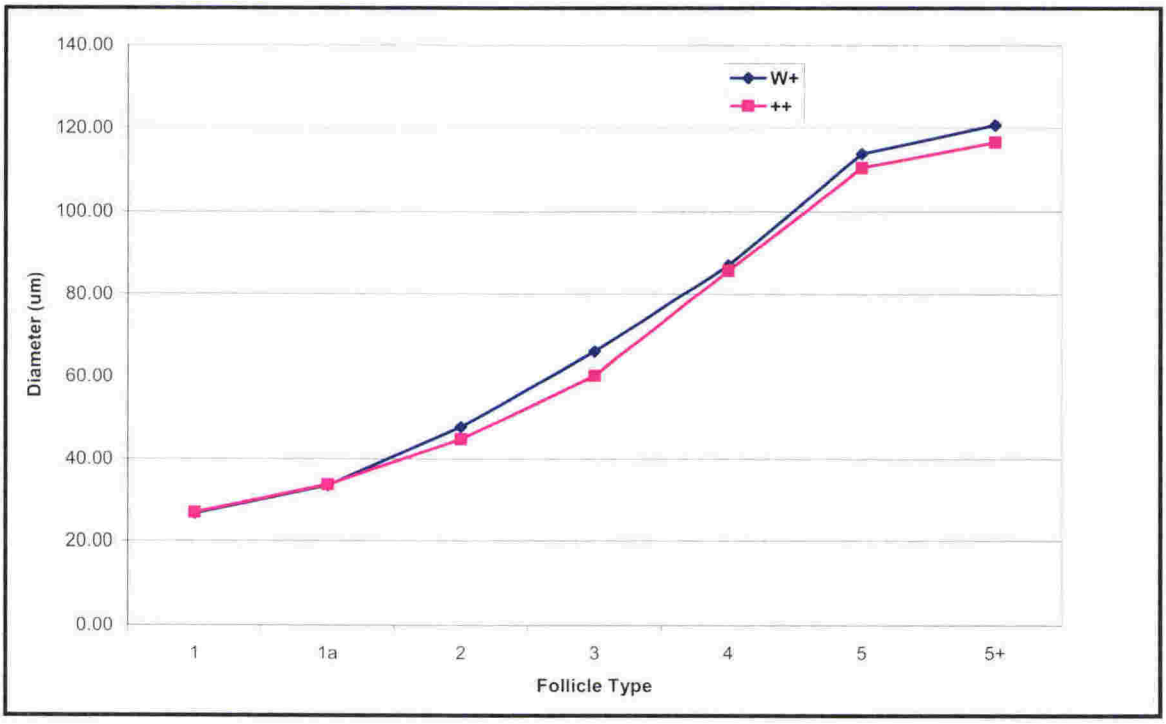


Figure 27: The mean diameters of the ovarian follicles and ooctyes.

(a) Diameter of follicles and oocytes (b); in 4-week old Woodlands (W+) lambs and controls (++).For both follicular and oocyte diameter there were no statistical differences found between the Woodlands (W+) and control lambs (++).

The changes in oocyte diameter in Woodlands animals were more like the Inverdale mutation than the Booroola mutation, as in the Inverdale sheep no genotype differences in oocyte diameter were noted (Smith *et al.*, 1997) whereas in the Booroola ewes the oocyte diameters were larger during preantral follicular growth relative to wild-type (Wilson *et al.*, 2001).

4.2 Introduction and Aim; Adult ewe ovaries

As discussed earlier, the current understanding is that during fetal life or shortly after birth, female mammals acquire a finite number of oocytes which is not renewed throughout the remainder of life (Rodgers *et al.*, 2003). Recently, this theory has been challenged (Johnson *et al.*, 2004), nevertheless the prevailing view is in favour of fixed population that declines throughout reproductive life. For the present study ovaries from ewes (age 6-7) years and confirmed by pedigree analysis to carry the FecX2^{W} gene were collected for these studies. Using identical techniques to that described for 4-week old lambs, the aim was to determine if a similar pattern of follicular development had been preserved through development of the animal; and whether there was a higher number of Type 5 follicles in the W+ animals.

4.2.1 Results

The results for the total numbers of non-atretic follicles from Types 1/1a and Types 2-5 and the overall atretic follicle numbers are shown in Figure 28 and 29 respectively. These data show that the FecX2^{W} animals have significantly more Type 5 follicles ($p<0.01$) but that for all other follicle types the numbers between each genotype were not statistically significant. Moreover no differences were noted in the number of atretic follicles (Figure 29).

The lower mean number of Type 4 follicles in the FecX2^{W} animals, although not significantly different ($p=0.423$), infers the possibility that some developed an early antrum and therefore were classified as Type 5 follicles. This is further supported by the difference in Type 5's being only in the smaller (<1mm) size caterogry (Figure 30). The Woodlands carrier ewes had at least twice as many small Type 5 follicles (< 1mm)

when compared to wild-type contemporaries although no difference was seen in the numbers of antral follicles greater than 1mm in diameter (Figure 30).

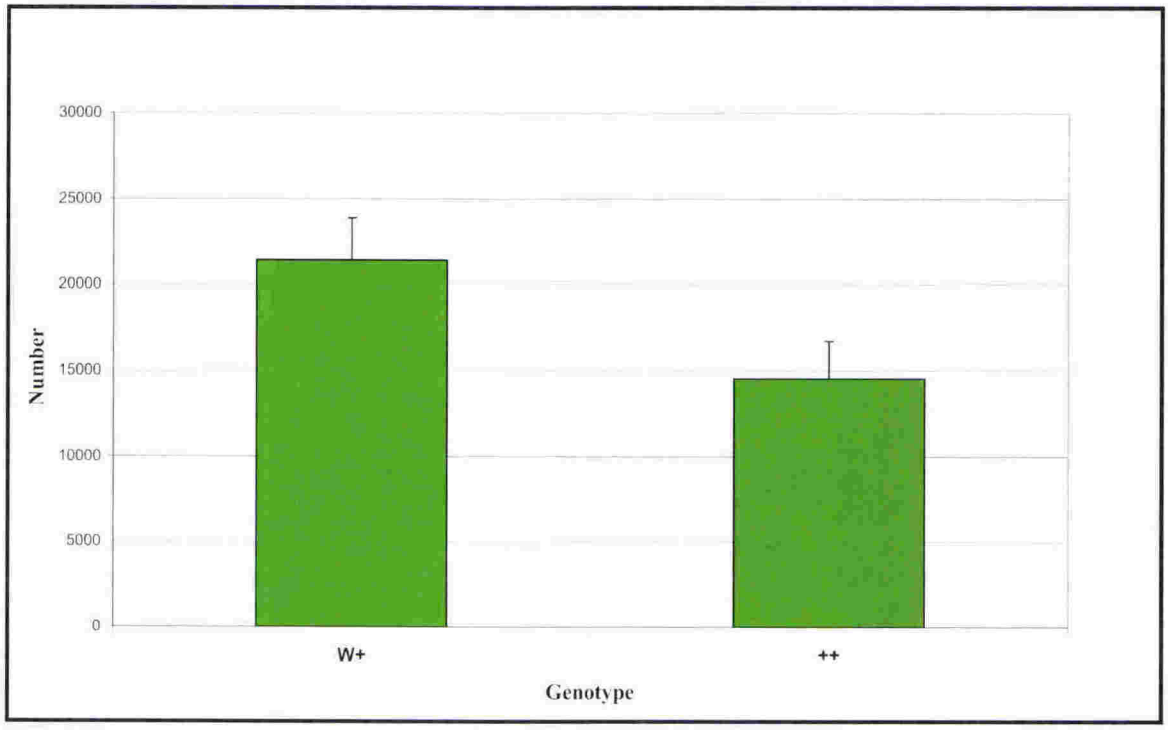


Figure 28: Mean number (+sem) of Type 1 and 1a follicles.

Type 1 and 1a follicles combined for Woodlands (W+) vs. control (++), no significant difference.

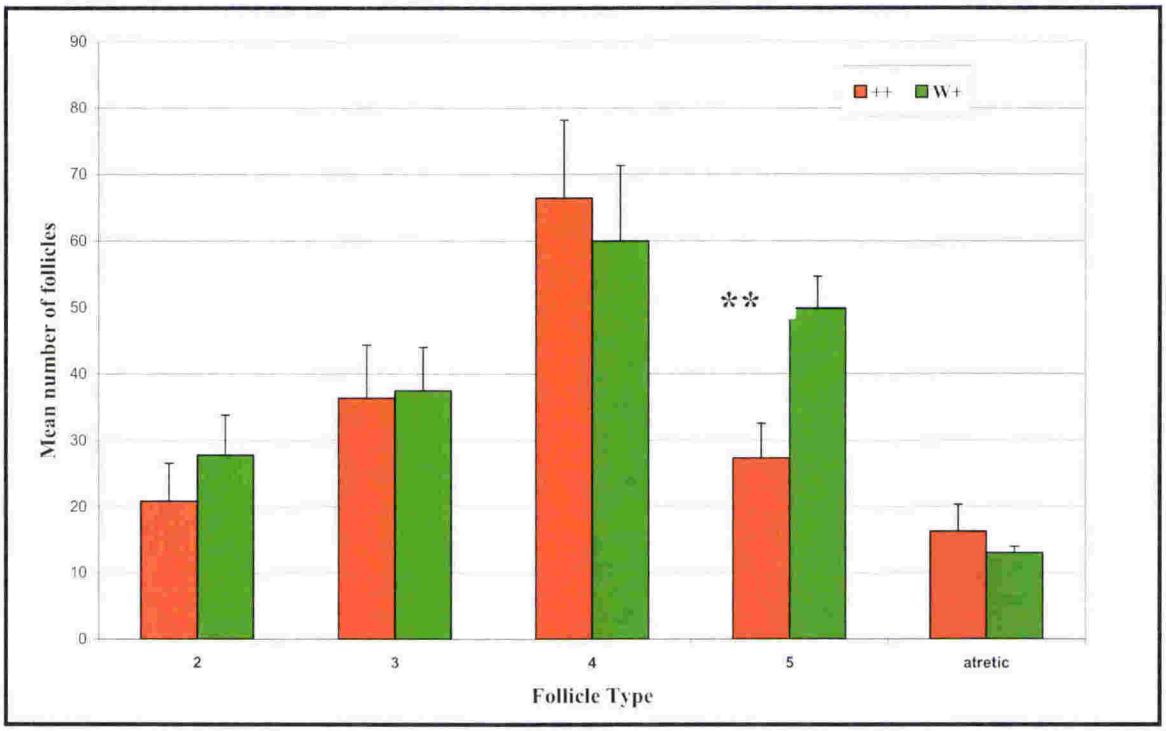


Figure 29: Mean numbers of follicles in Woodlands carriers (W+) vs. control (++) ewes with respect to follicle Type.

The Type 5 but not the Type 4 ($p=0.423$) follicular population in W+ animals were significantly different compared to ++ (**$p<0.01$).

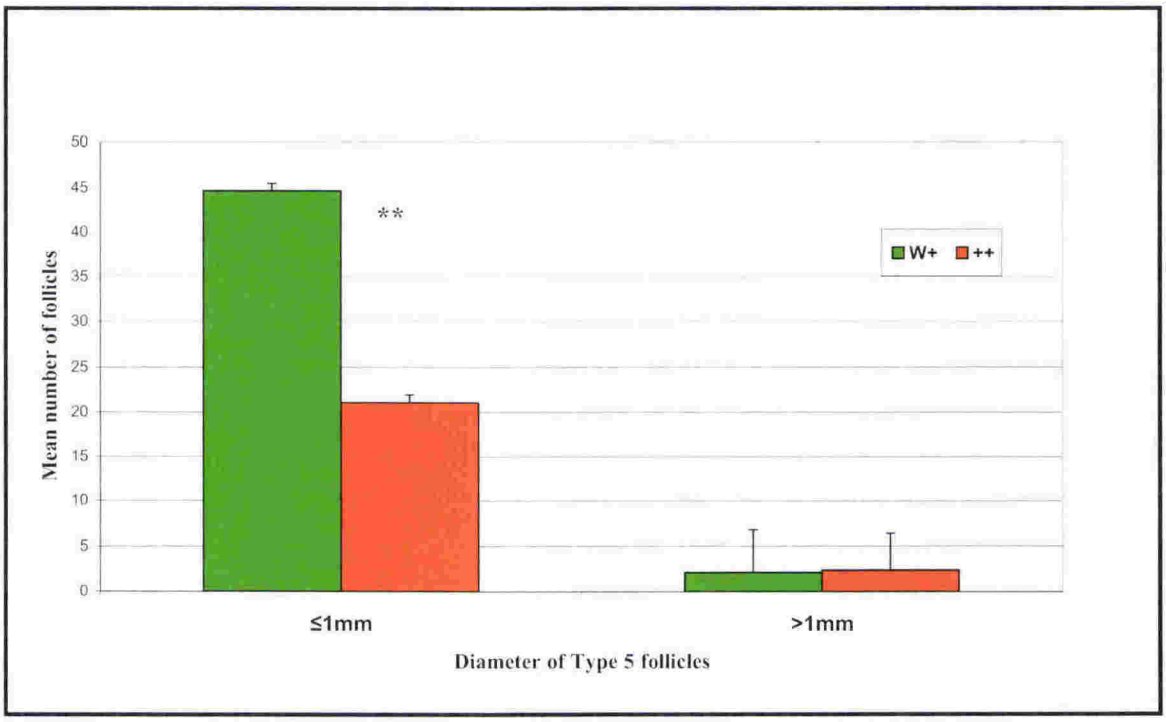


Figure 30: Mean number (+sem) of Type 5 follicles ≤ 1 or > 1mm diameter in the Woodlands (W+) vs. control ewes (++).

The Type 5 follicles ≤ 1mm were statistically different to the control group ($p<0.001$).

Both 4-week old lambs and adult ewes had a significantly larger number of Type 5 follicles compared to wild-type suggesting that the cause of this increase may play a role in the increase in ovulation rate. The mean diameters of follicles and oocytes with respect to follicular types are shown in Figure 31a and b. For Type 5 follicles the overall mean diameter was significantly smaller in the $FecX2^{W}$ animals relative to the controls.

The overall difference in mean diameter of Type 5 follicles between Woodland and control ewes was due to the significantly greater number of follicles ≤ 1mm diameter in the Woodlands (W+) ewes. The diameters of the largest non-atretic Type 5 follicles in Woodlands (W+) ewes was between 1172-1393 μm and for the controls (++) between 1376-1592 μm, suggesting that the sizes to which W+ follicles grew was not necessarily different from that in the ++. For all other follicular types there were also no differences noted in the mean diameters (Figure 31a). Moreover, no differences in mean oocyte diameter (Figure 31b) were noted for any follicular type. However, some differences were noted in the morphology of Type 4 and 5 follicles in the ovaries of the $FecX2^{W}$ ewes. Figure 32 shows a typical Type 5 follicle in control ewes as a reference. Many of the smaller Type 5 follicles (i.e. ≤ 1mm diameter) found in the Woodlands ovaries were unusual in their shape. The antrum did not display the usual pattern of development (see Figure 33) and the number of granulosa cell layers was more consistent with that of a Type 4 follicle. The unusual small (≤ 1mm) Type 5 follicles had the normal array of granulosa cells seen in a Type 4 follicle as described by Lundy et al (1999) but also include an antrum (Figures 34-36). Around 36% of the small Type 5 follicles (≤1mm) counted in this study contained this unusual antrum arrangement in the $FecX2^{W}$ animals whereas in the non-carriers this was rare (<1%). Moreover, using the previously described criteria for atresia there was not difference in the incidence of atresia between the carrier and non carrier animals. No differences in the mean diameter of the oocyte was found between the W+ and ++ animals when the follicular antrum formed even though the mean diameters of the follicles were significantly smaller on W+ compared to the ++ for those ≤ 1mm.

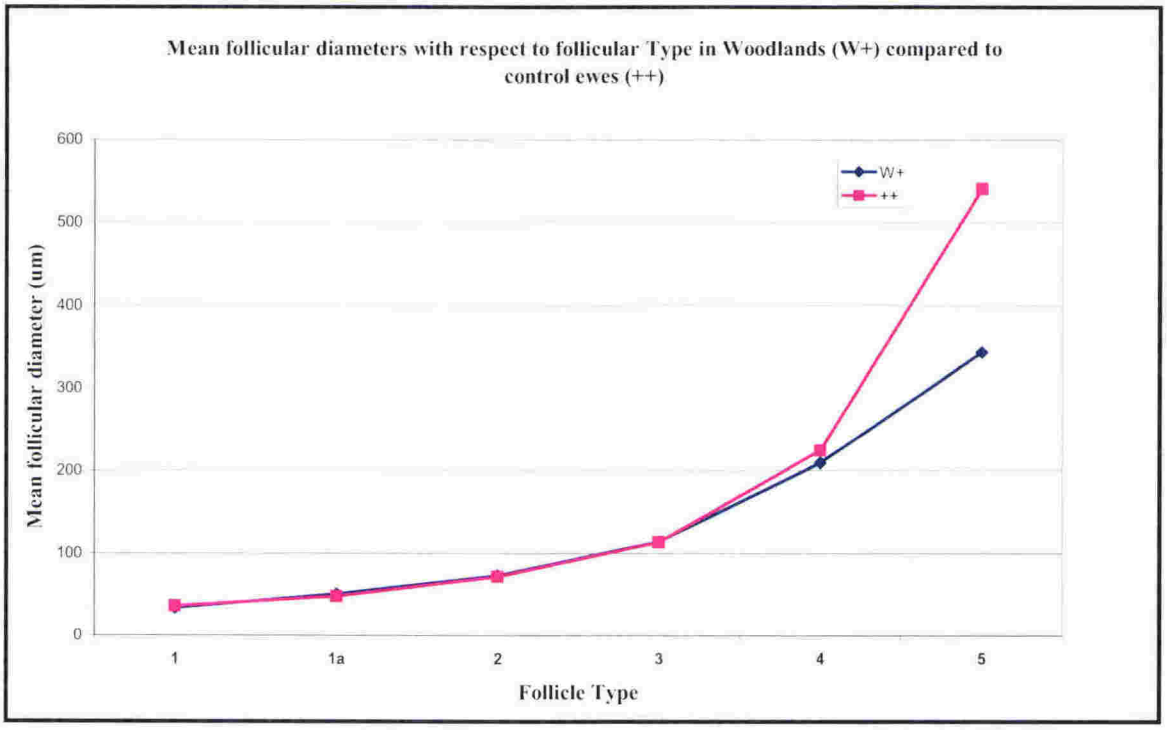


Figure 31a: The mean follicular diameter in ++ for the Type 5 follicles was greater than in W+.

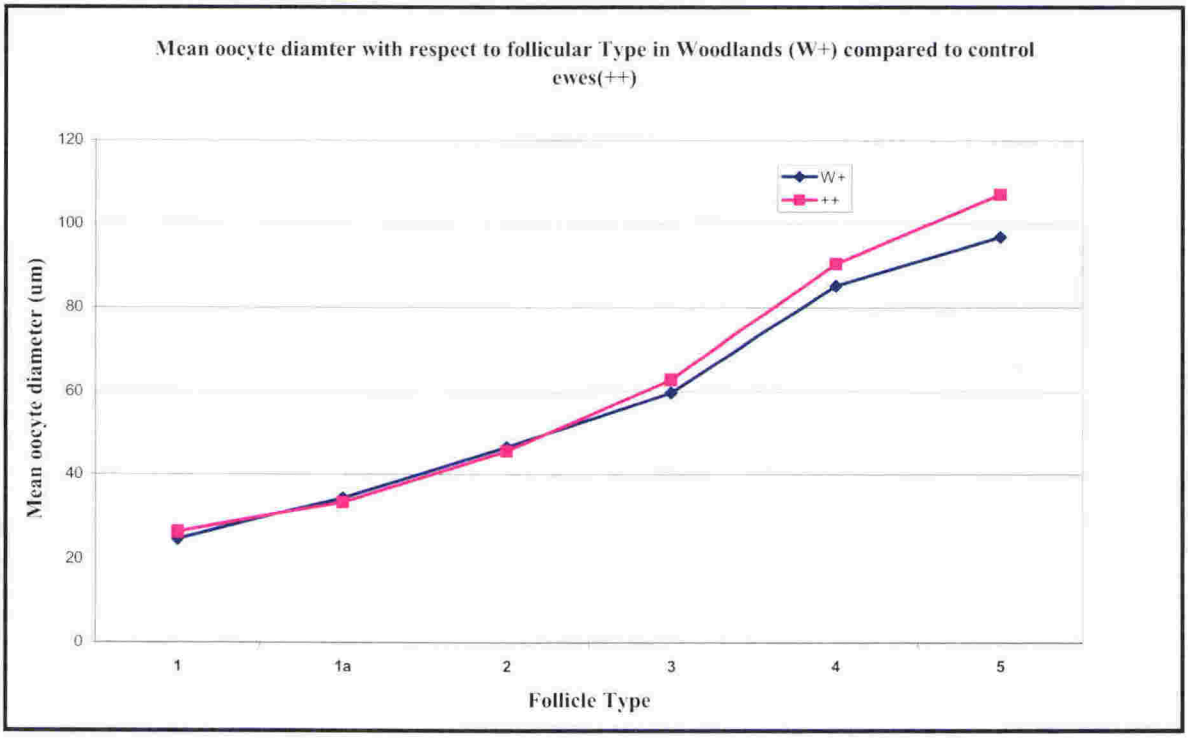


Figure 31b: There were no statistically significant differences in the mean diameters of the oocytes between the W+ and control ewes for each follicle Type.

In Booroola ewes, the oocyte diameter was larger in smaller-sized follicles. In this study, the difference was not statistically different in the size of the oocyte at any stage of growth, although there was a trend for the Woodlands after the Type 3 to be slightly smaller. The possibility exists that these minor changes in size of the oocyte could be indirect and due to altered function in the granulosa cells as a consequence of the FecX2^{W} mutation. Because the change in ovulation rate is small (i.e. 0.4) the changes in folliculogenesis could also be minor.

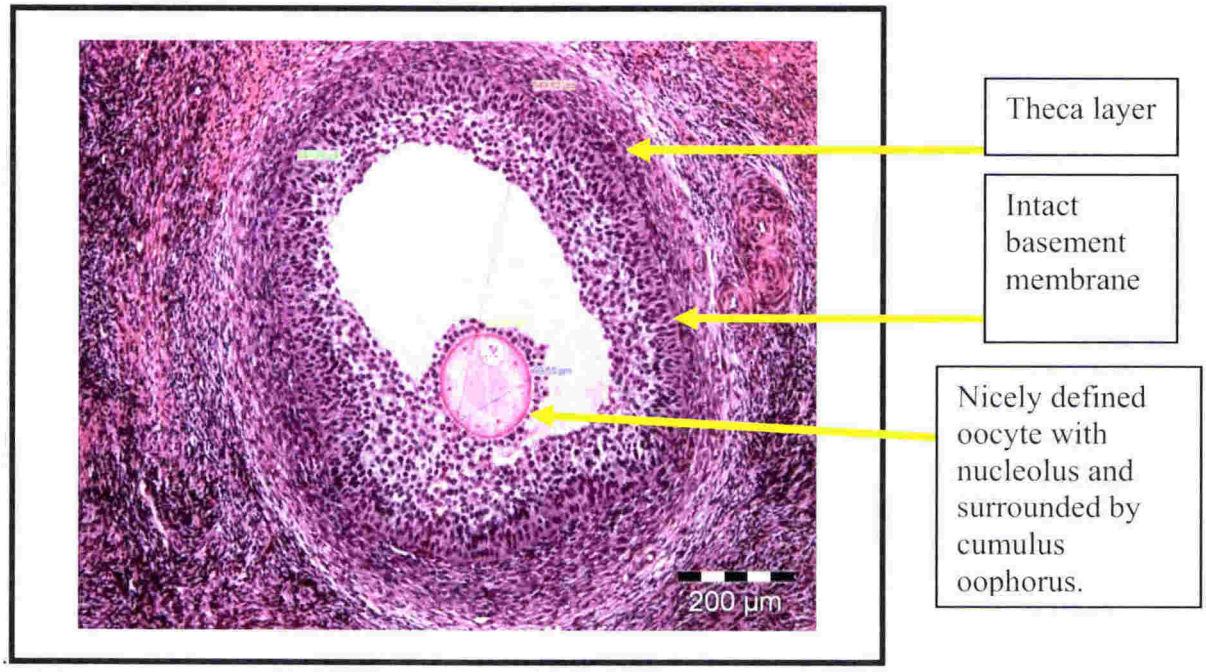


Figure 32: A small Type 5 antral follicle in a ewe not carrying the FecX2^{W} mutation.

Note the distinct granulosa layers of cells (>6), intact basement membrane, uniform theca and oocyte with cumulus oophorus. The lines on the photo are indicative of measurements taken of the follicle and the oocyte.

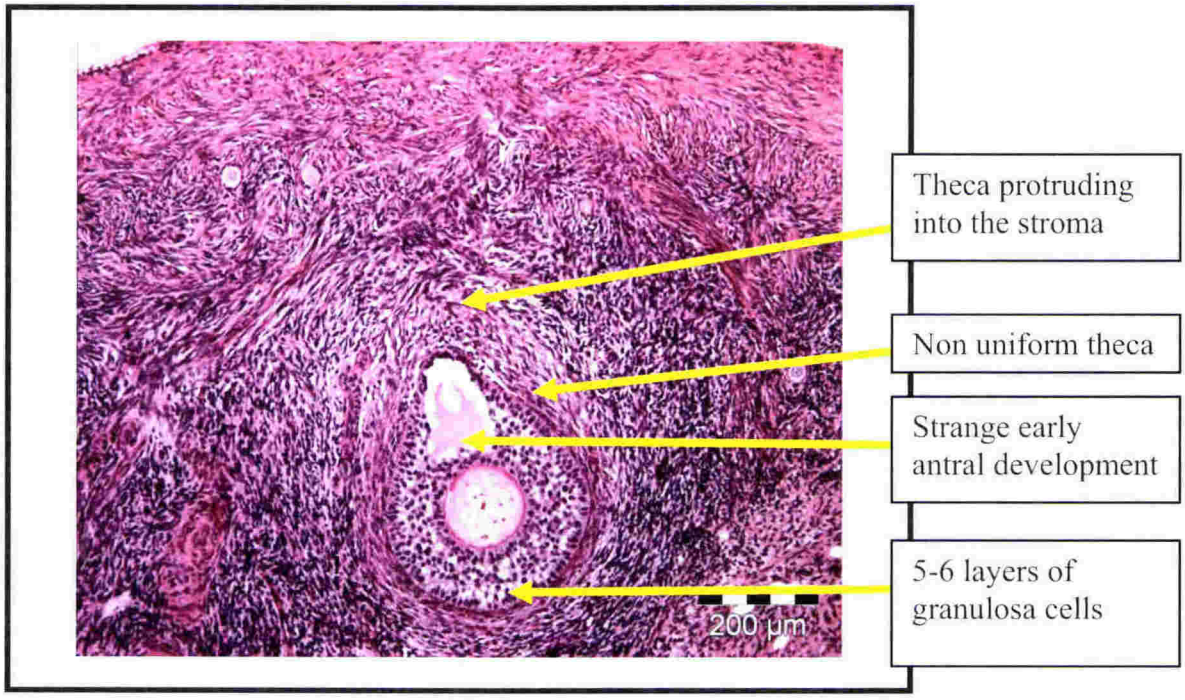


Figure 33: A small antral follicle in a Woodlands ewe

Note that the oocyte is surrounded by 5-6 layers of granulosa cells, with an unusual atypical antrum which appears to be pushing out into the theca which is also not a uniform concentric layer around the granulosa cells.

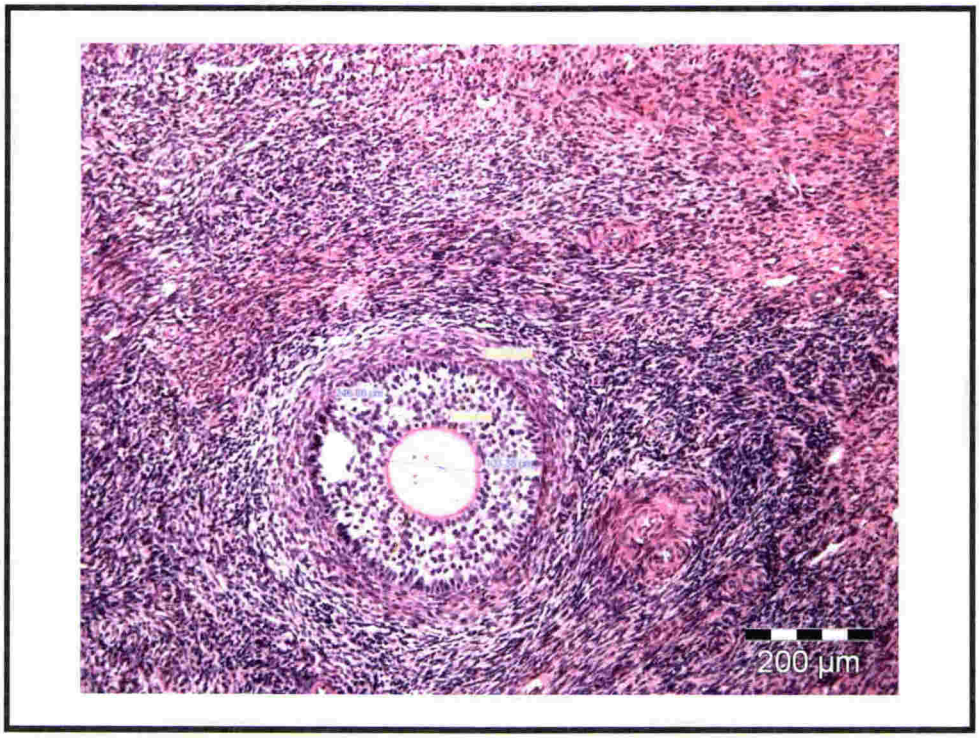


Figure 34: Early antral (Type 5) follicle in Woodlands adult ewe.

Note a well organised theca but an unusual pattern of antrum development.

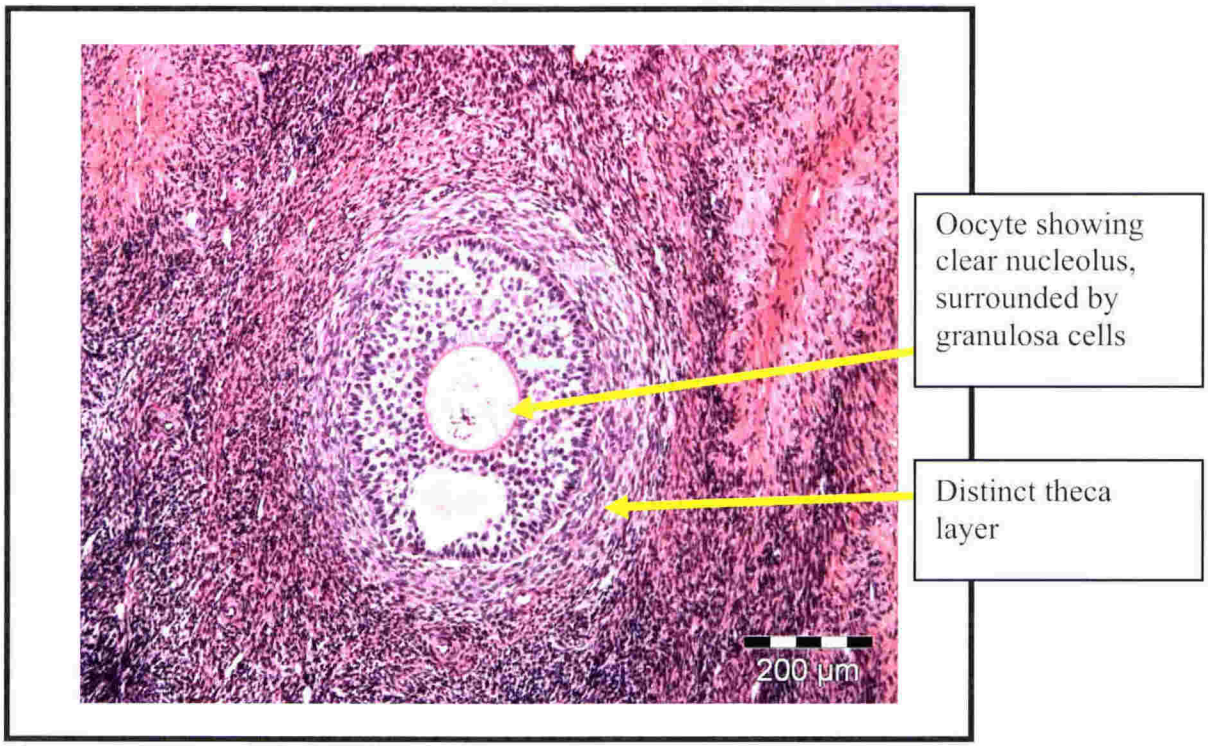


Figure 35: Early antral (Type 5) follicle in a Woodlands ewe with an unusual; antrum.

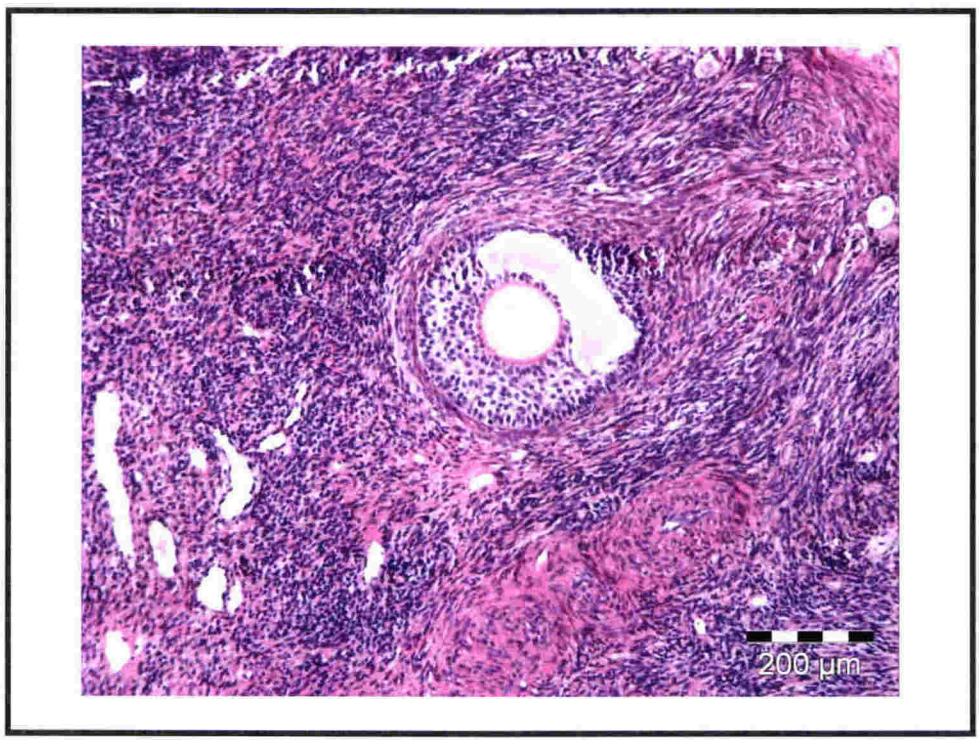


Figure 36: A small Type 5 follicle in a Woodlands ewe with an eccentric formation of the antrum.

This unusual, small Woodlands Type 5 follicle and the often eccentric and early formation of the antrum may be a consequence of the mutation. This early antrum formation could also account for the higher number of Type 5 follicles (≤ 1mm) as without the antrum most of the follicles would have fallen into the category of a Type 4. The confounding issue is that the difference in the number of Type 5 and Type 4 follicles did not equate.

4.3 Hormone and Cyclic AMP studies of the large ovary phenotype: a confounding issue in studying the Woodlands mutation.

4.3.1 Introduction and aim

As discussed previously, the cAMP response of granulosa cells gives an indication of the sensitivity or responsiveness to FSH and LH. Woodlands sheep had been identified in some cases by pedigree analysis but also in some instances by the large ovary phenotype in 4-week old lambs. Studies by Dr George Davis, (Invermay AgResearch Centre) subsequently showed that the large ovary phenotype was not linked to the Woodlands $FecX2^{W}$ gene. Consequently, as the cAMP experiments were performed on lambs with the large ovary phenotype, the results were focussed on determining whether granulosa cells from animals with the large ovary phenotype had a different LH and FSH responsiveness compared to cells from normal ovaries.

4.3.2 Results

The range in ovarian weights and numbers of follicles from which granulosa cells were isolated in these studies are shown in Table 5. Animals (i.e. Numbers 423, 388, 419, 373 and 382) with ovarian weights ≥ 0.6 g were arbitrarily considered to have the large ovary phenotype (LOP) whereas those with ovaries <0.6 g were considered to have the normal ovary phenotype (NOP). Animals with the LOP have a mean ±sem follicular population of 110 ± 35 whereas those with NOP have a mean ±sem follicular population of 29 ± 9 (i.e. for follicles ≥ 1mm diameter; LOP vs. NOP; $p< 0.05$ on log-transformed data). Thus animals with LOP had more developing antral (Type 5) follicles than their

NOP counterparts. Consequently, it would be expected that the animals with the LOP have a higher overall population of granulosa cells. Since the plasma concentration of inhibin are directly correlated with the total population of granulosa cells in antral follicles (McNatty *et al.*, 1993), it might be anticipated that the plasma concentrations of inhibin would be higher in the LOP compared to the NOP. If this was the case then the plasma FSH concentrations might also be expected to be reduced as there is normally an inverse relationship between biologically active inhibin and FSH concentrations.

Table 5: Lamb number, left ovary weight and total number of antral follicles, and numbers at different follicular diameters.

lamb	left ovary	Total follicle	Follicle number with respect to follicular diameter (mm)		
			1-3 mm	>3-4.5 mm	>4.5 mm

prerequisite before investigating the cAMP responses of granulosa cells. Collectively, these data are consistent with results from previous studies showing a relationship between the number of antral follicles or ovarian weight and plasma inhibin concentrations. However the finding that there was no relationship between inhibin and FSH (Figure 40) and that there was no difference in FSH concentrations between animals with LOP and NOP suggests that at least some of the forms of immuno-reactive inhibin were not biologically active. The inhibin radioimmunoassay that was used (McNatty et al., 1992) detects 31 kDa inhibin and the pro-αC subunit (Robertson et al., 1989). Therefore, from the plasma data for inhibin and FSH it is not possible to predict what the likely responsiveness of the granulosa cells would be in lambs with LOP condition. The LOP was found not to be linked to the Woodlands FecX2^{W} mutation but some lambs in this study may in retrospect be carriers of the FecX2^{W} gene. Thus it was difficult to be sure that putative differences in inhibin were not, in part, due to the FecX2^{W} mutation. Opportunistically however, when the ovaries were collected from the adult ewes for morphometric analysis, blood samples were taken at the time the ovaries were recovered. These samples were analysed by Norma Hudson and Stan Lun at Wallaceville Animal Research Centre for FSH and inhibin respectively. These animals were known to be carriers (W+) or non carriers (++) of the FecX2^{W} mutation by pedigree analysis and no genotype differences were noted in ovarian size between the genotypes. In these animals no differences were noted either in plasma inhibin concentration or in FSH between the W+ and the ++ ewes (Figure 41 and 42). Importantly, no differences in inhibin were found notwithstanding the fact that there were significantly more small (i.e. $\leq$ 1mm diameter) but not larger (i.e.> 1mm diameter) follicles in the W+ ewes (Figure 37). While there were no genotype differences in FSH concentration, the FSH concentrations in the W+ ewes were much more variable as can be noted by the large standard errors of the means. Presumably, the increased overall numbers of granulosa cells from approximately 25 more follicles $\leq$ 1mm in diameter were insufficient to raise the levels of inhibin secretion above that generated by the larger antral follicles. Thus, the inhibin differences found in the lambs is more likely to be related to the LOP rather than the FecX2^{W} mutation. Consequently, it follows that any potential differences in FSH or LH/hCG responses in granulosa cells are more likely to be related to the LOP. The overall mean FSH dose effects on cAMP responses of granulosa cells from different- sized follicles from lambs with the large ovary phenotype (LOP) or with normal ovarian phenotype (NOP) are shown in Figures 43, 44

- 46. Also shown are the LH/hCG responses (Figure 47). For the NOP, increasing doses of FSH caused dose-related increases in cAMP synthesis in 1-1.25, >1.25-2.5 and >2.5-4 mm but not >4 mm diameter follicles and the maximum responses were 8-10pmol/10^6 cells at either 10 or 100 ng/ml FSH. A markedly reduced (if any) dose response for the LOP was observed for FSH with follicles < 4 mm. The hCG responses from cells from all follicle sizes were not different from the controls for both LOP and NOP. In LOP, the dose response effect to FSH was either modest or absent and less than from the NOP. Similarly, no hCG response was noted. Thus overall, cells from follicles of the NOP were generally responsive to FSH up to 4mm in diameter whereas the responsiveness of cells to FSH from follicles of the LOP were less responsive until the 4mm follicle, and no evidence of an hCG response was noted for either phenotype except in granulosa cells from some >1.25-2.5 mm follicles from the NOP. The reasons why cells from all of these follicles had a greater hCG response than the average NOP is not known.

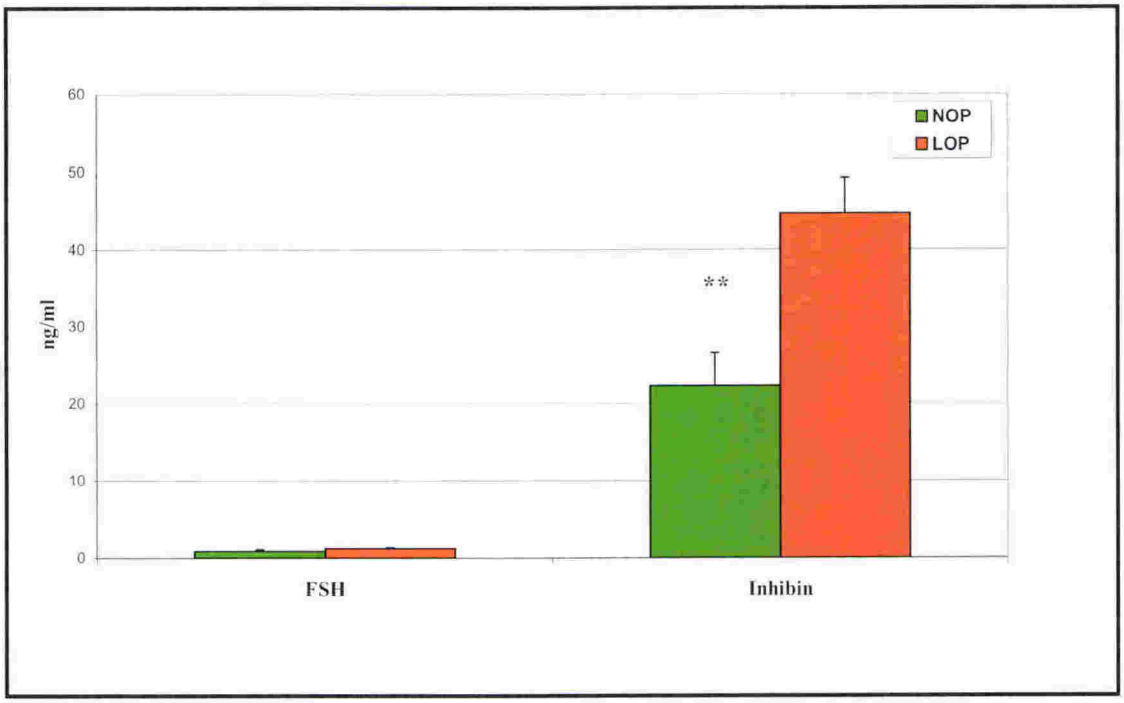


Figure 37: Plasma concentration of Inhibin and FSH in lambs with the large ovary phenotype (LOP) and those with normal ovarian phenotype (NOP) ** p<0.01

(Data from Juengel, J.J, Feary, L, Lun, S., Hudson, N.L and McNatty, K.P, unpublished)

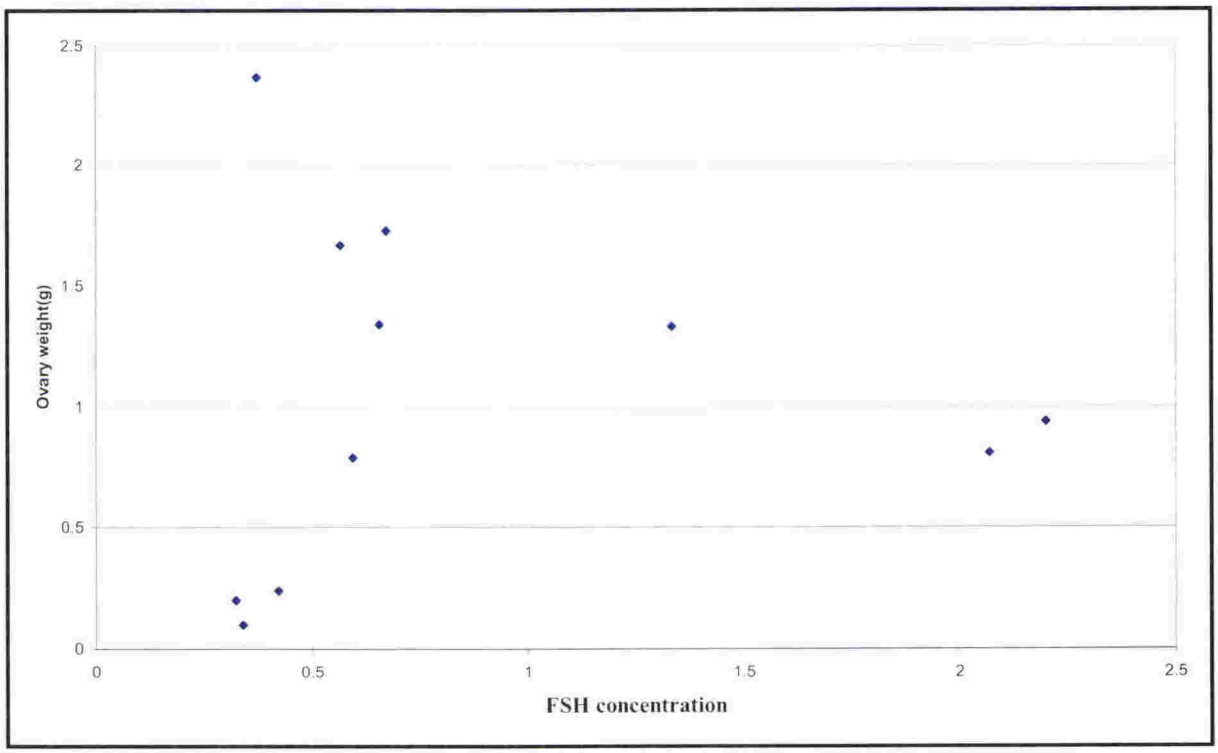


Figure 38: Ovary weight vs. FSH concentration in 4-week old lambs.

No relationship was found between ovarian weight and plasma FSH concentration (p=0.9).

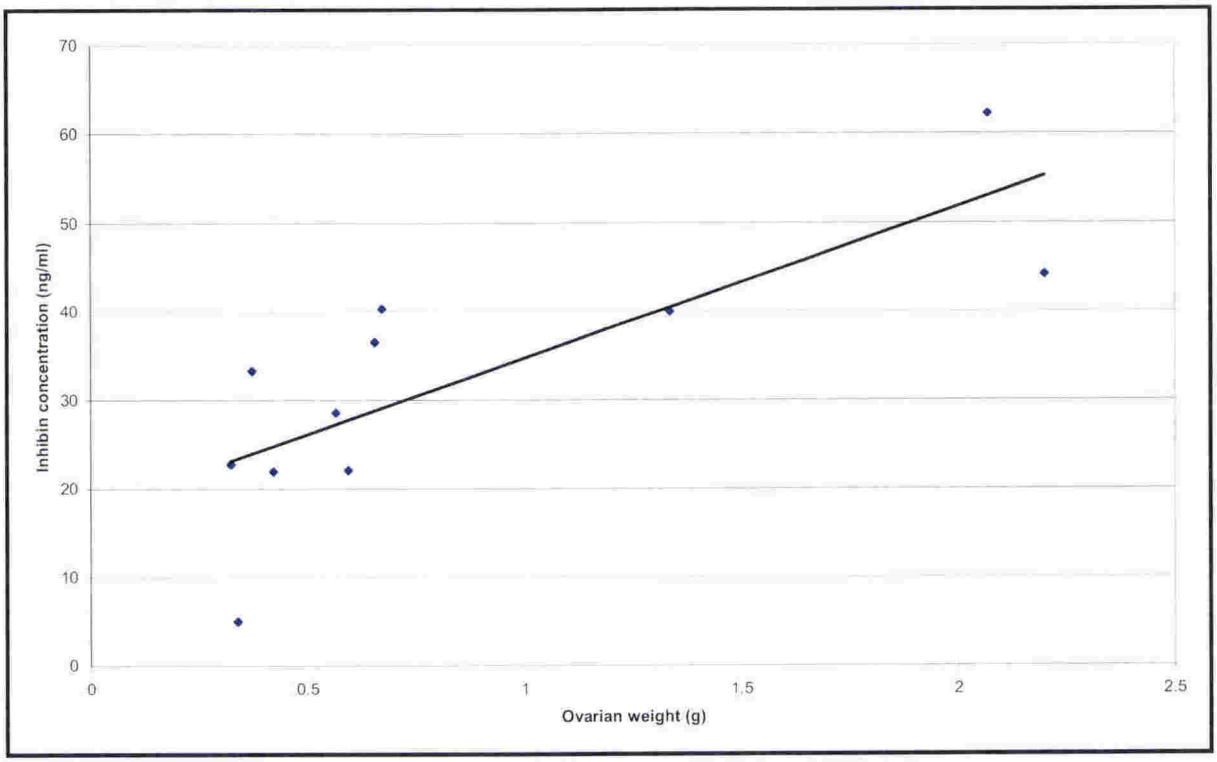


Figure 39: Inhibin vs. ovarian weight in the 4-week old lambs.

There was a significant linear relationship between plasma inhibin concentrations and ovarian weight (R2 =0.6163; $p<0.01$).

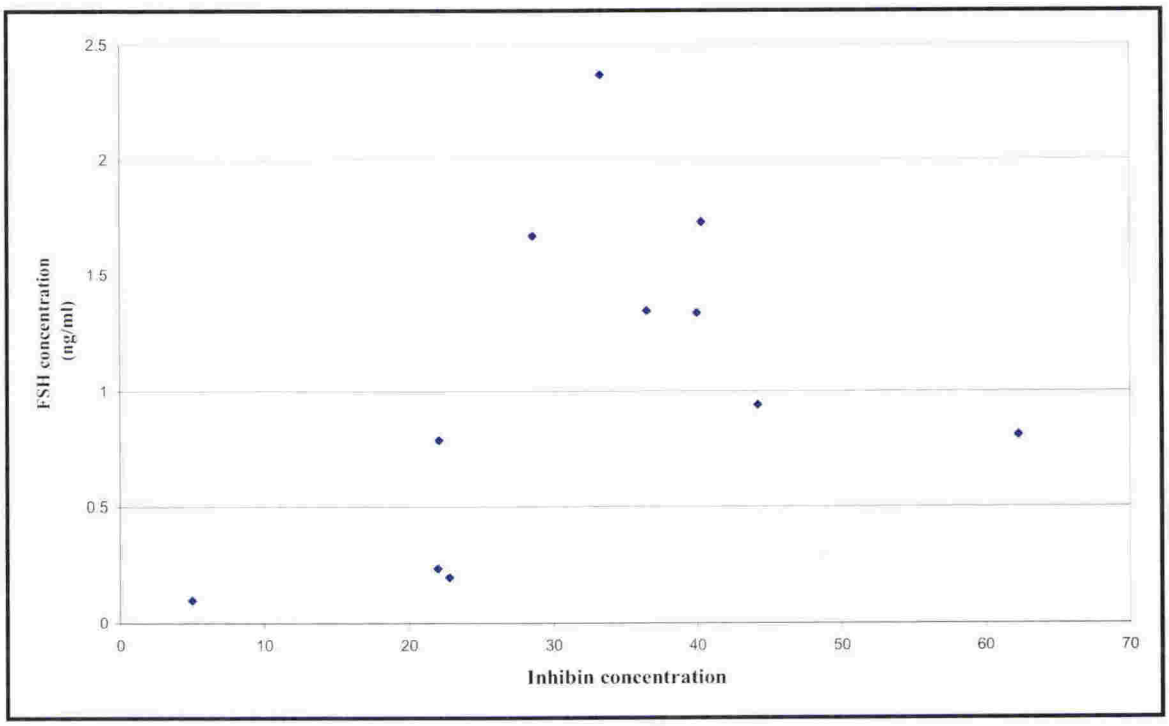


Figure 40: Inhibin vs. FSH in the 4-week old lambs.

There was no significant linear relationship between plasma FSH and inhibin (R2 = 0.1696).

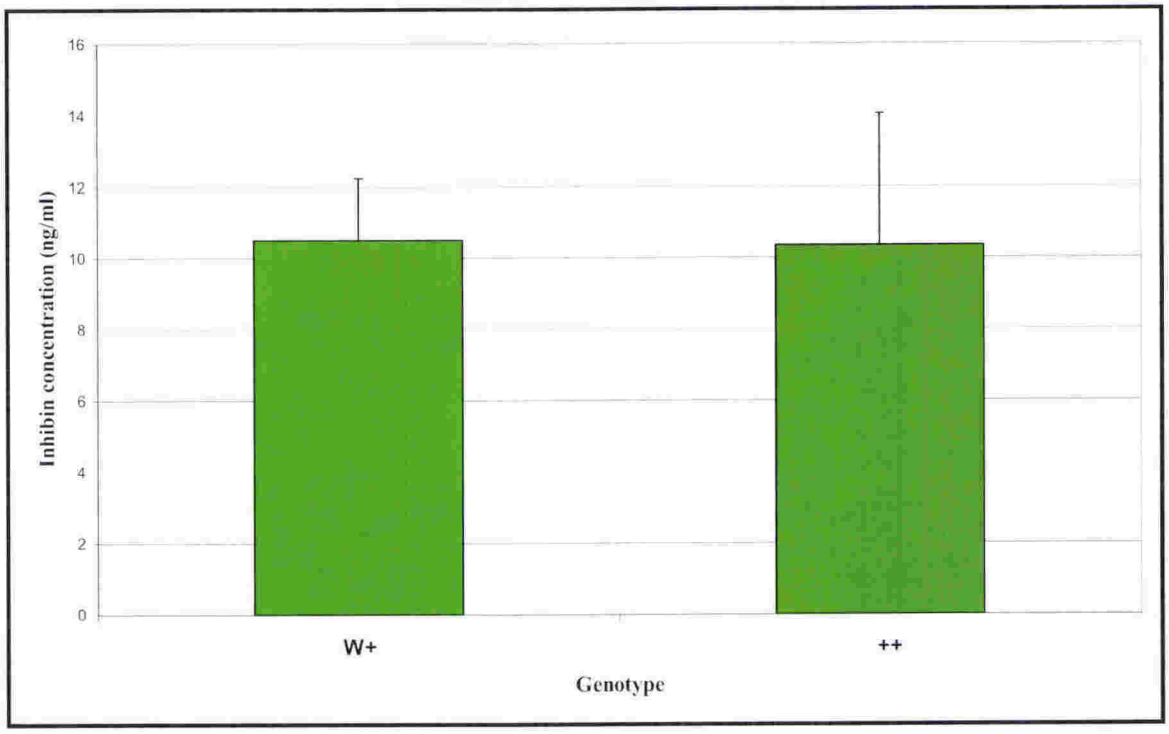


Figure 41: Mean plasma concentrations (+ sem) of inhibin in Woodlands ewes that were carriers (W+) or non-carriers (++) of the FecX2W mutations.

(Data supplied by S. Lun, Wallaceville Animal research Centre, unpublished)

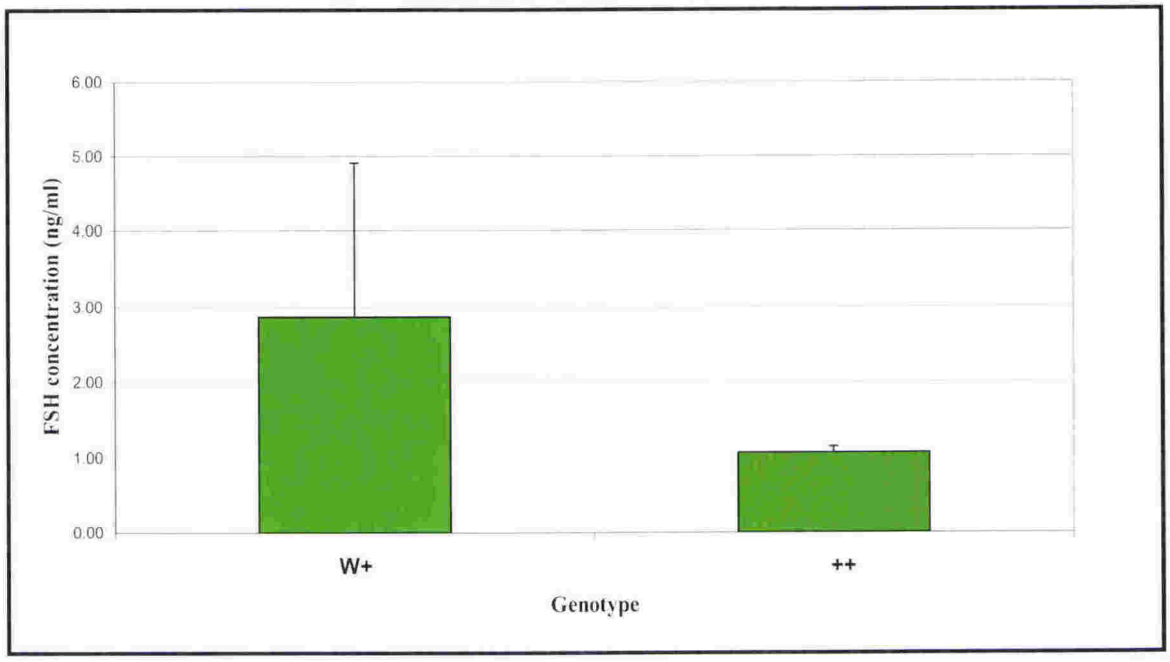


Figure 42: Mean plasma FSH concentrations (+ sem) in Woodlands ewes that were carriers (W+) or non-carriers (++) of the FecX2^{W} mutation

Data supplied by N. Hudson, Wallaceville Animal Research Centre, unpublished).

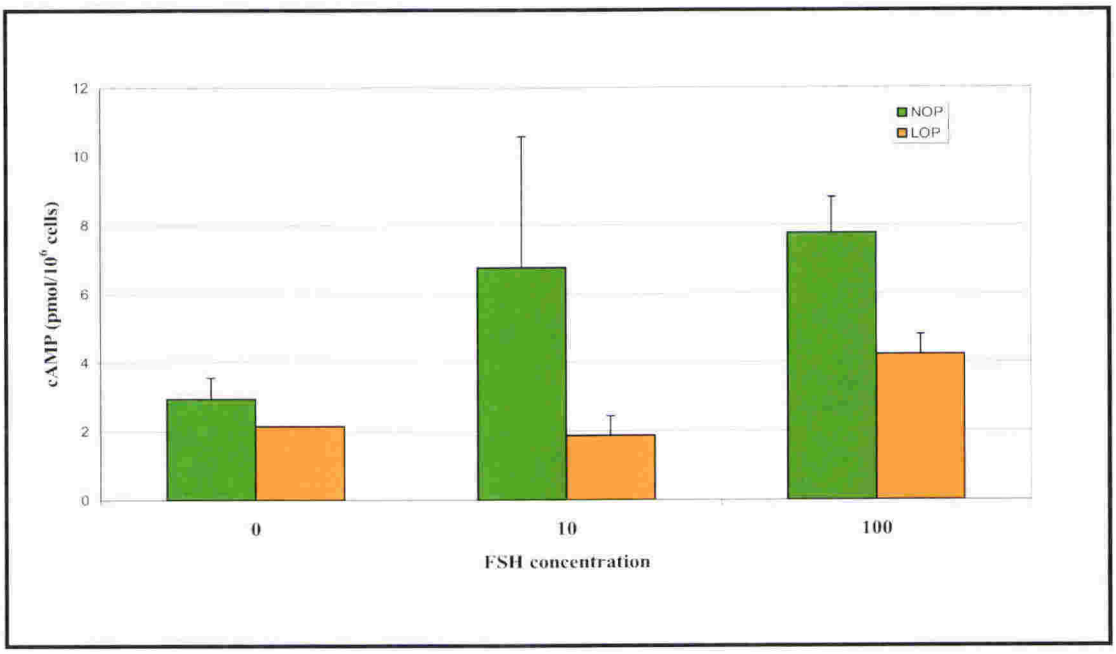


Figure 43: Mean (+sem) cAMP response of granulosa cells from 1-1.25 mm diameter follicles with respect to dose of normal ovarian phenotype (NOP) and large ovarian phenotype (LOP) for follicles 1-1.25 mm.

There were significant dose responses ($p<0.05$) found for both genotypes but the mean overall response in NOP tended to be lower.

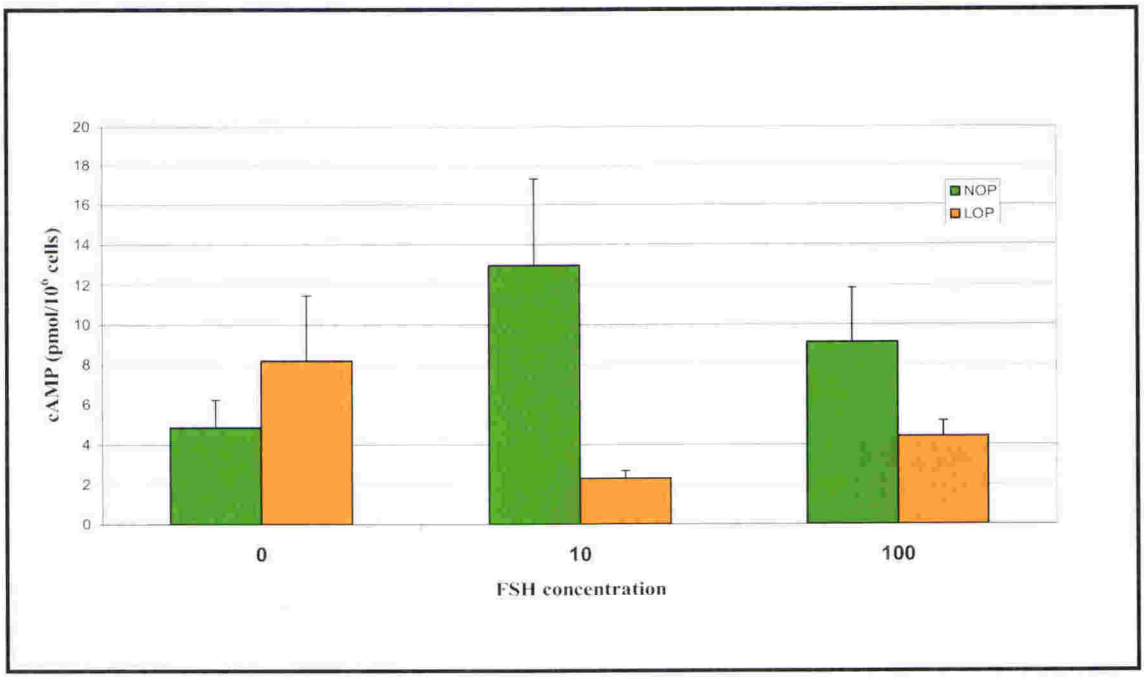


Figure 44: : Mean (+ sem) cAMP response of granulosa cells from >1.25-2.5 mm diameter follicles with respect to dose of FSH and of normal ovarian phenotype (NOP) and large ovarian phenotype (LOP) For follicles >1.25-2.5 mm

Variable increases in cAMP responses were observed in response to FSH in LOP but not in cells from NOP.

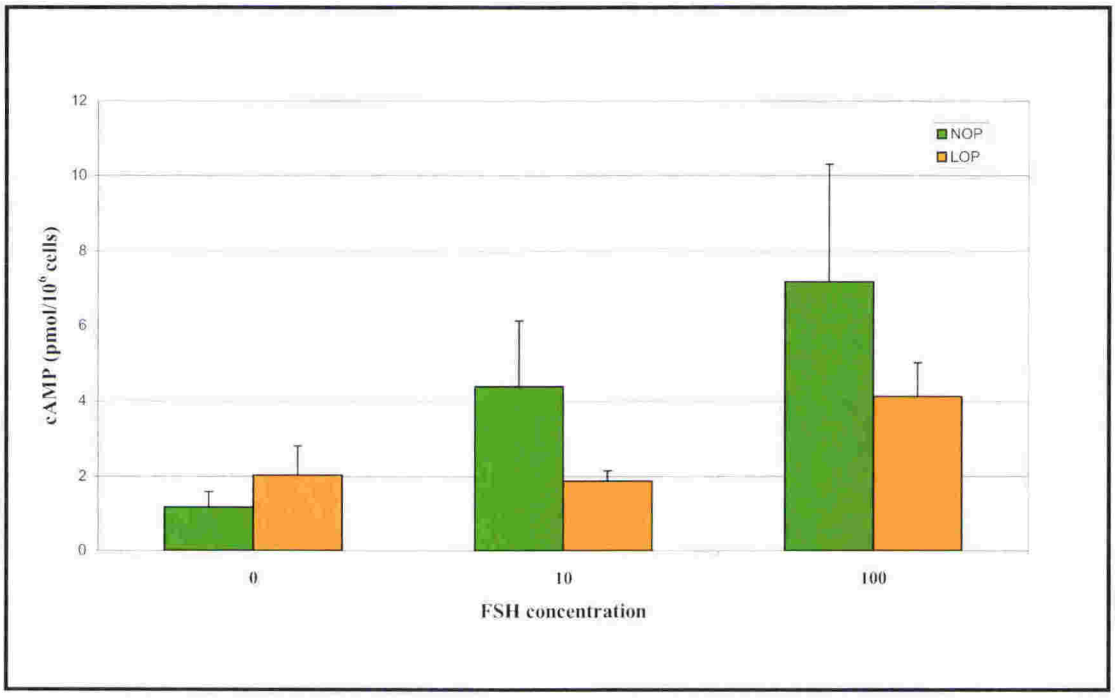


Figure 45: Mean (+sem) cAMP response of granulosa cells from >2.5-4 mm diameter with respect to dose of FSH and normal ovarian phenotype (NOP) or large ovarian phenotype (LOP) For follicles >2.5-4 mm.

Significant dose response were found for both genotypes ($p<0.01$) but the overall mean response in LOP were lower.

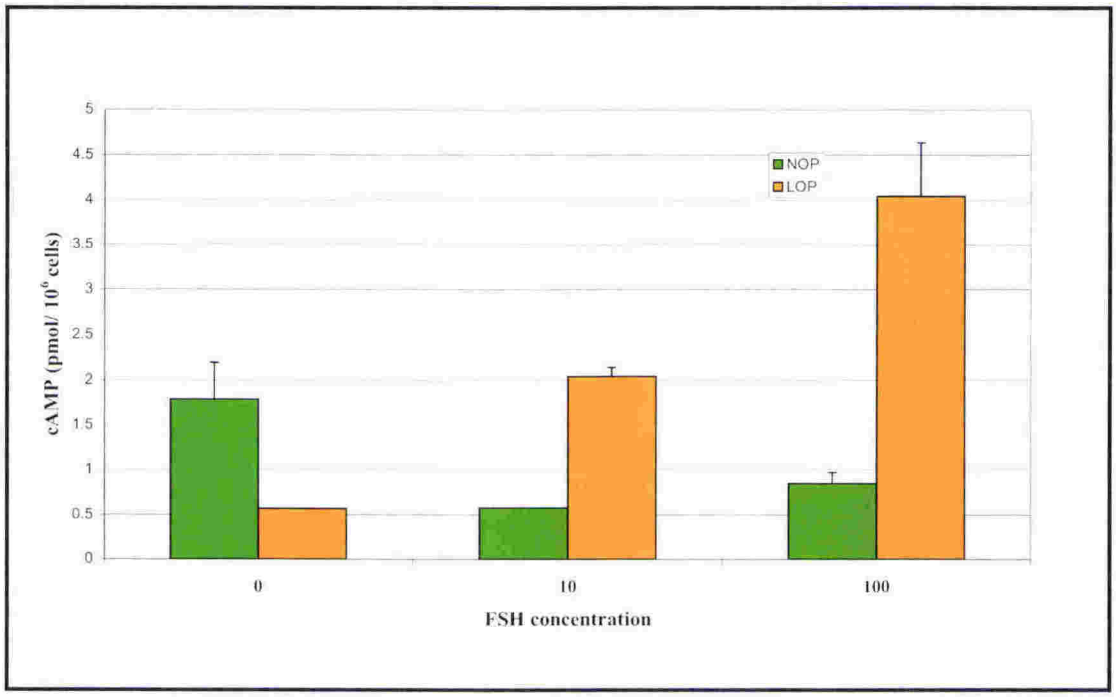


Figure 46: Mean (+sem) cAMP response of granulosa cells from >4 mm diameter follicles with respect to the NOP or LOP for follicles >4 mm.

For dose=0, the NOP had a higher response ($p < 0.05$) than the LOP, but for the other 2 dose levels the LOP had a higher response ($p < 0.01$) than the NOP.

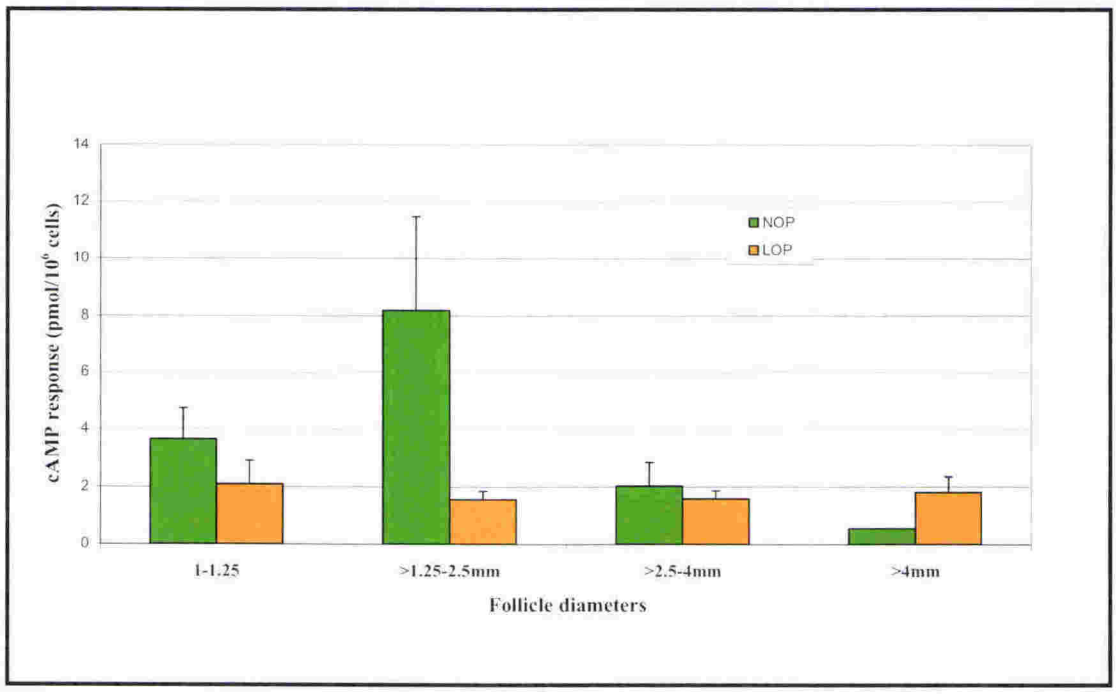


Figure 47: Mean (+sem) cAMP response of granulosa cells over all follicle diameters with respect to the dose of hCG/LH and (NOP) or the (LOP), The dose of hCG used was 1000 ng/ml.

The higher mean response by cells in NOP for the >1.25-2.5 mm follicles was not significantly different to cells from LOP and was due to 2 follicles (1.9 mm and 2.3 mm) which responded the hCG challenge.

In these series of studies the aim was to elucidate if there was a different sensitivity to both FSH and LH in the granulosa cells of the LOP compared to the NOP independent of the Woodlands mutation. Differences between the LOP and NOP were difficult to relate to other findings because it is not known if the LOP causes an increase in ovulation rate. Findings from the Booroola sheep found that the highest mean amounts of cAMP produced by cells challenged with FSH or LH did not differ between genotypes but were achieved in follicles at a smaller diameter (Henderson *et al.*, 1987). For the FSH doses, the cells from the LOP had a higher response ($p < 0.01$) than the NOP, which may be just a consequence of having more Type 5 follicles with more granulosa cells.

4.4 In-situ hybridisations

4.4.1 Introduction and aim

The aim was to determine if there were any differences in the ontogeny or patterns of expression of a selection of genes thought to be important for ovarian follicular development and/or ovulation. The genes chosen were GDF9, BMP15, ERα, ERβ, and FSHr, β_A inhibin, β_B inhibin and α inhibin with GDF9 and BMP15 singled out for further analyses because previous mutations found in these genes were found to affect ovulation rate in sheep (see McNatty et al, 2005b for review).

4.4.2 Results

No differences in the ontogeny or cell types expressing GDF9, BMP15, ERα, ERβ, FSHR, β_A inhibin, β_B inhibin and α inhibin were noted between carrier or non-carriers of $FecX2^W$ gene. Therefore the findings for both genotypes were pooled and results for ligand, cell type and ontogeny of expression are shown in Table 6.

Photomicrographs showing the patterns of expression of α inhibin, β_A inhibin, β_B inhibin, ERβ, ERα, FSHR and GDF9 and BMP15 mRNA are shown in Figures 48-54 respectively.

Table 6:

Ligand	Ovarian cell type	Ontogeny of expression with respect to follicle type				
		1/1a	2	3	4	5
GDF9	Oocyte	•	•	•	•	•
BMP15	Oocyte		•	•	•	•
ERα	Granulosa			•	•	•
	Surface epithelium	•	•	•	•	•
ERβ	Granulosa			•	•	•
	Oocyte			•	•	•
FSHR	Granulosa			•	•	•
$ß_A$ inhibin	Granulosa			•	•	•
$ß_B$ inhibin	Granulosa				•	•
α inhibin	Granulosa			•	•	•

• signifies gene expression was evident.

GDF9 was first seen in the Type 1 and 1a follicles through to the Type 5 whereas BMP15 was only seen from a Type 2 or committed stage onwards (Figure 54; Table 6). For the genes BMP 15 and GDF9 further studies were done to determine whether any quantitative differences could be determined for the level of expression (Figure 55). However, no significant differences between the genotypes in any of the follicle types analysed were noted (Figure 56a and b).

For GDF9 there was an increase in signal intensity to Type 3 and then an apparent decline to Type 5 (Figure 57). For BMP 15 there was a progressive increase in signal intensity to the Type 4 stage followed by a decline (Figure 58).

BMP15 and GDF9 are known to be essential oocyte regulatory factors in the control of follicular growth. No differences in these factors were observed in this study with Woodlands ewes. This suggests that the $FecX2^W$ mutation is unlikely to have downstream effects on GDF9 or BMP15 expression. However, as the $FecX2^W$ mutation only caused a very small increase in mean ovulation rate compared to controls, the

possibility remains that there may have been a slight increase in these factors that was below the limit of detection for differences in grain counting.

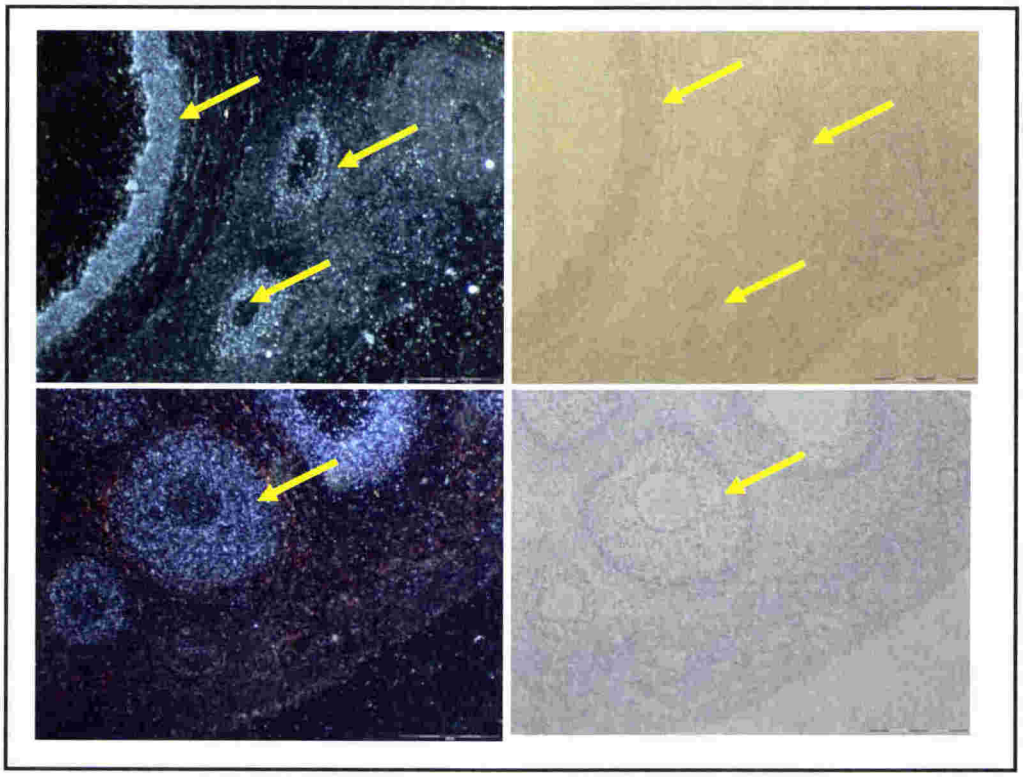


Figure 48:

Corresponding darkfield and brightfield views of ovarian sections from W+ (top panels) and ++ (bottom panels) following hybridization with α inhibin. Expression was limited to the granulosa cells of Types 3, 4 and Type 5 follicles. All slides showed similar patterns of expression, with no signal in the surrounding tissue or oocytes. The yellow arrows indicate expression of granulosa cells. The top panels show expression in granulosa cells in one Type 5 (i.e. the larger follicle) and two Type 4 follicles and in the bottom panels, in granulosa cells of a Type 4 follicle.

The cell specific expression of mRNA encoding α inhibin during follicular development was similar to that reported for other sheep breeds (Juengel *et al.*, 2000).

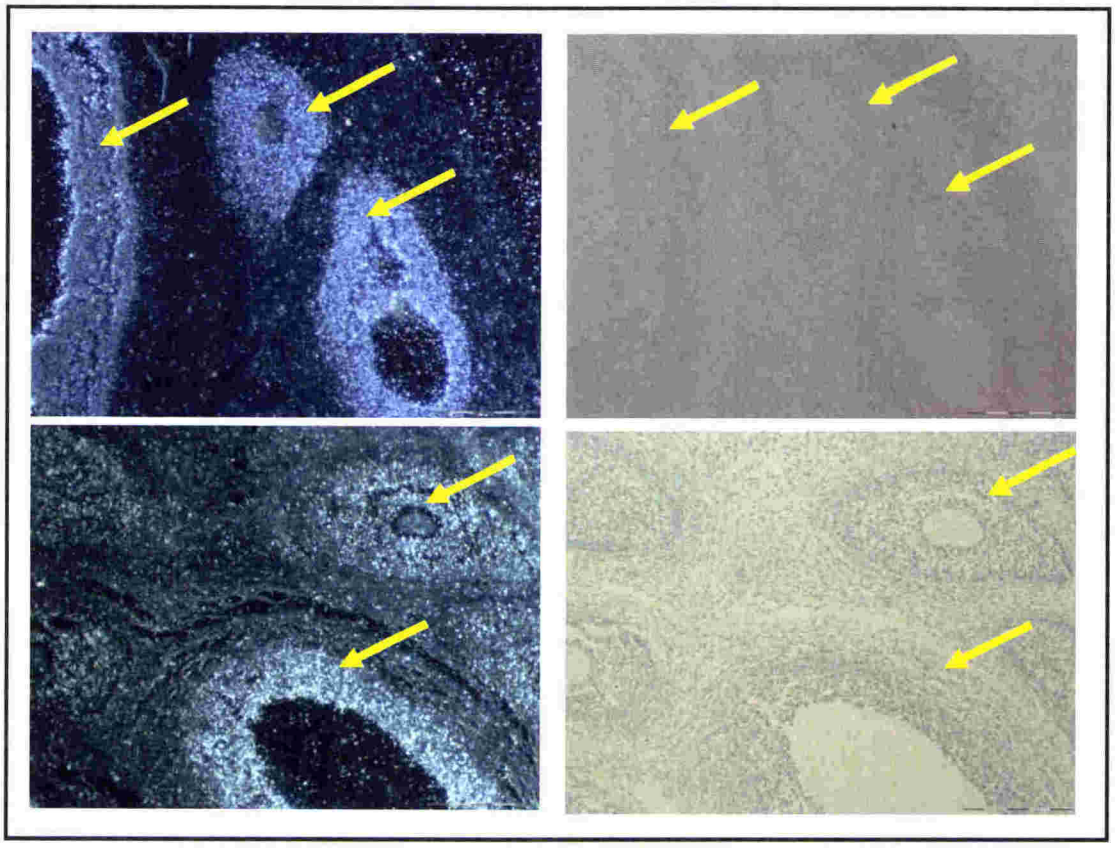


Figure 49:

Corresponding darkfield and brightfield views of ovarian sections, from W+ (top panels) lambs following hybridization with α inhibin mRNA. Also W+ (bottom panels). Also shown is $ß_A$ inhibin mRNA expression in lambs' ovaries from W+ ewe's bottom two slides. Expression was limited to the granulosa cells of Types 3, 4 and type 5 follicles for the α inhibin and to Type 4 and 5 follicles for $ß_A$ inhibin. All slides showed similar patterns of expression, with no signal in the surrounding tissue or oocytes. The yellow arrows indicate the expression of granulosa cells in a Type 5 and two Type 4 follicles.

Expression of mRNA encoding β_A inhibin was seen at Type 3 stage of development which is earlier than previously observed in other sheep (e.g. Inverdale) where expression was not detected until a later stage of development, i.e. Type 5. This is most likely due to differences in sensitivity of the hybridisations as the same probe was used as in previously reported experiments (Juengel *et al.*, 2000).

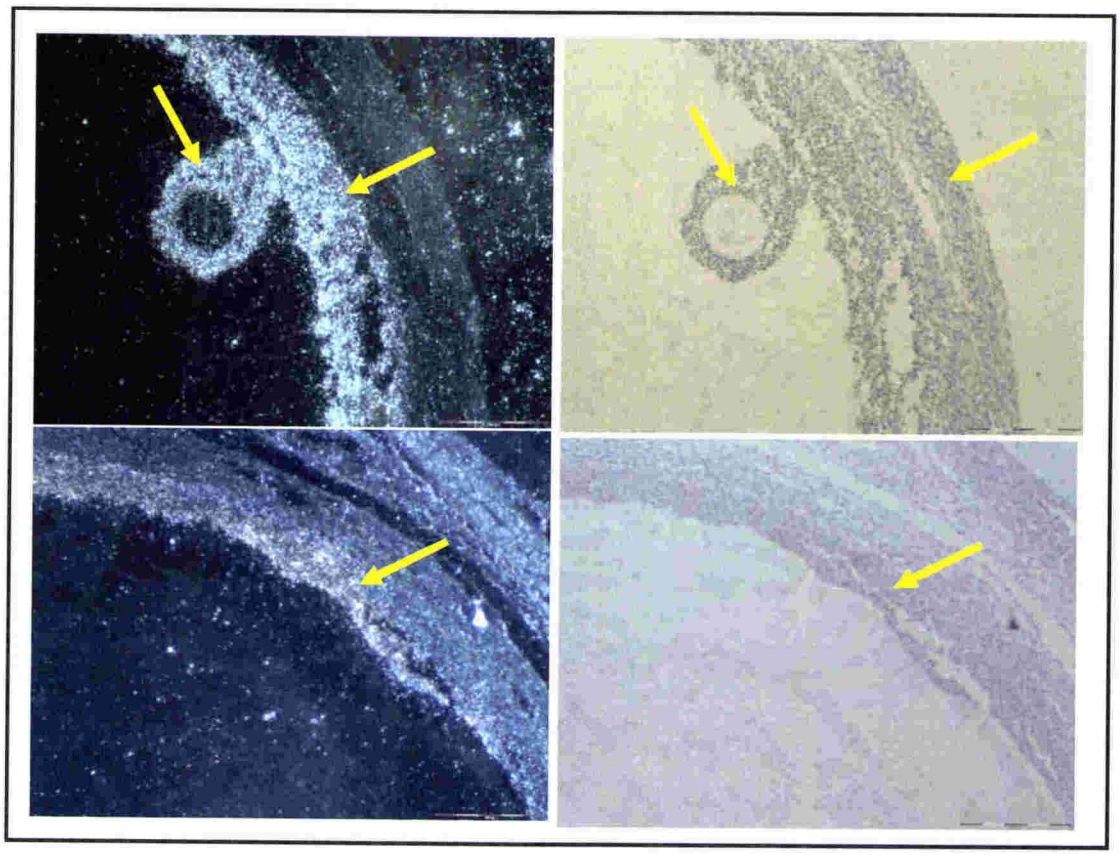


Figure 50:

Corresponding darkfield and brightfield of view of ovarian sections from W+ (top panels) and ++ (lower panels) lambs showing expression of expression of β_B inhibin mRNA in granulosa cells (arrows) Expression of β_B inhibin following was limited to granulosa cells of Type 4 and Type 5 follicles for both genotypes.

Expression of mRNA encoding β_B inhibin was seen at Type 4 stage of development which was later than seen on other sheep (e.g. Inverdale: Juengel *et al.*, 2000) where expression was at the Type 3 stage. Again this may have been a product of the sensitivity of the in-situ hybridisation method and probe.

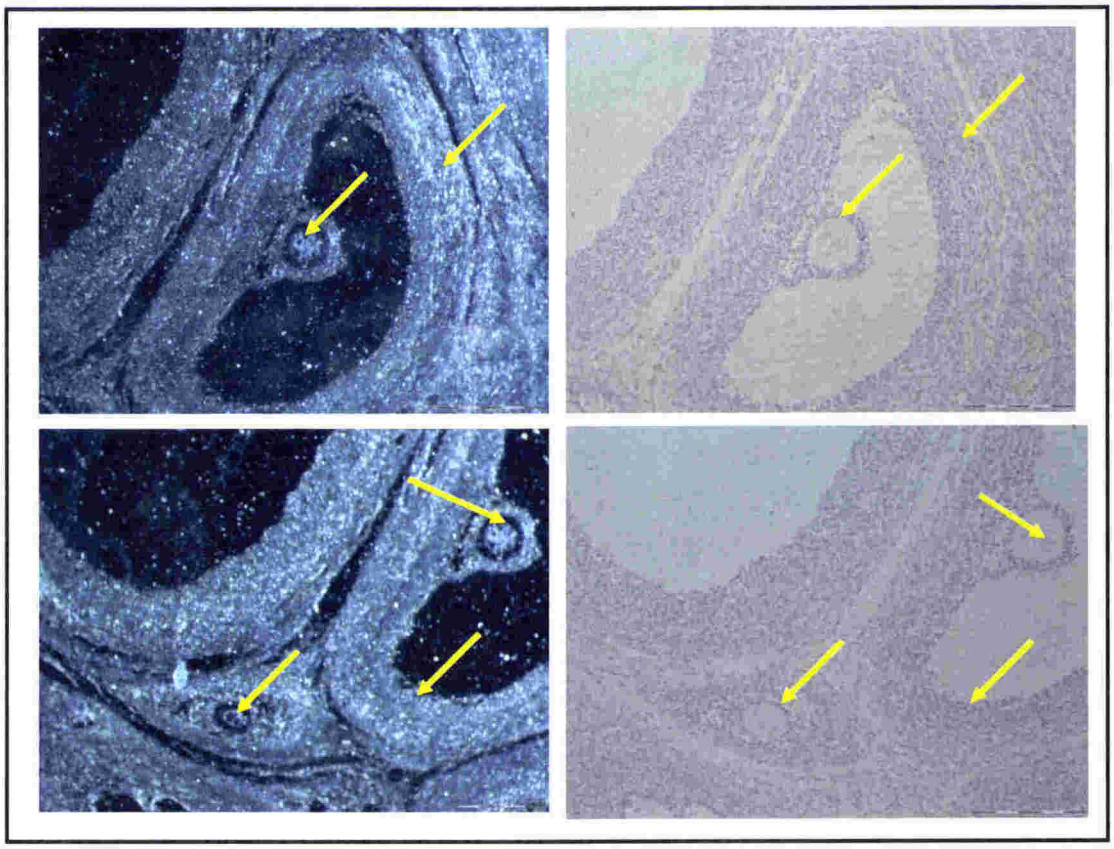


Figure 51:

Corresponding darkfield and brightfield of views of ovarian sections from W+ (top panels) and ++ (lower panels) lambs showing expression of ERβ mRNA. Expression ERβ mRNA was limited to granulosa cells of Types 3, 4 and type 5 follicles for both genotypes, and also to the oocyte of these follicles. The top panels shows the arrows pointing to expression in the oocyte and granulosa cells of a Type 5 follicle and the bottom panels show expression in the oocyte of a Type 4 and a Type 5 follicle with expression also in the granulosa cells.

The cell-specific expression of mRNA encoding ERβ and ERα were similar to that reported for other breeds of sheep

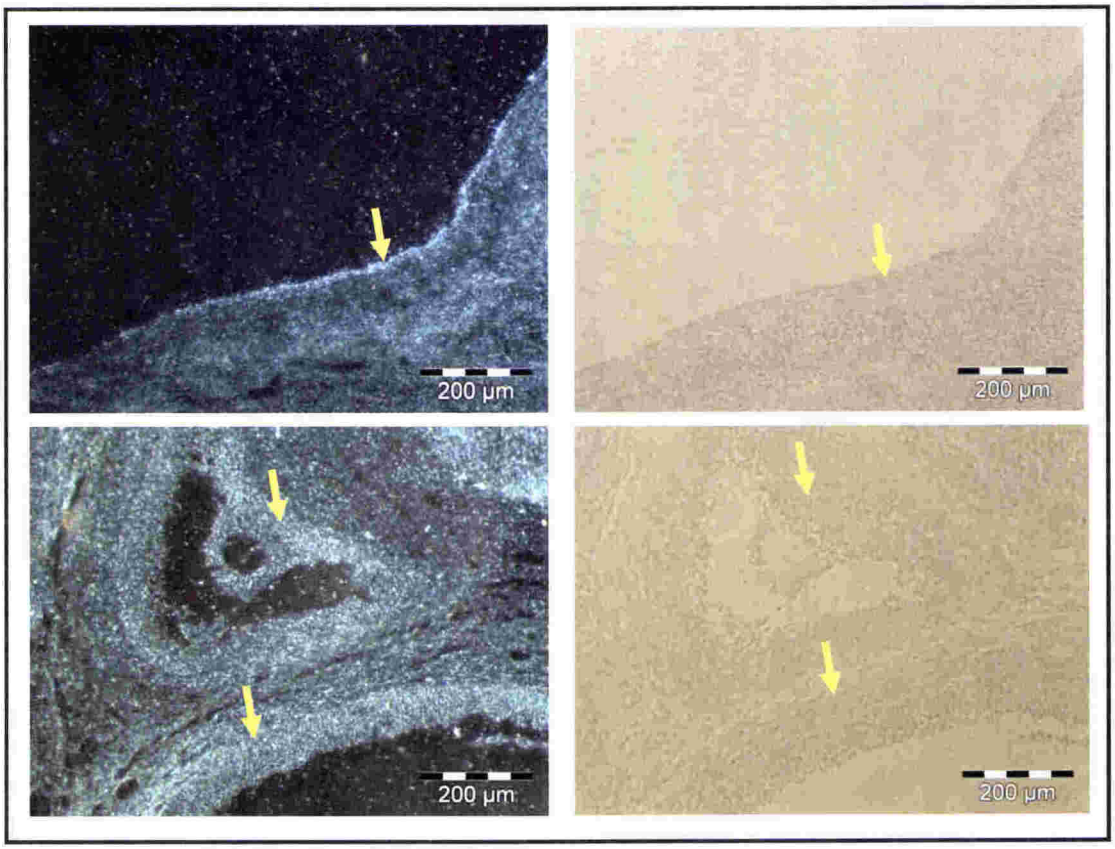


Figure 52:

Corresponding darkfield and brightfield views of ovarian sections from W+ lambs following hybridization with ERα mRNA. The top panels show the dark and light field for expression in the epithelium. The bottom panels show the expression in the granulosa cells of type 5 follicles. Expression was limited to granulosa cells of Types 3, 4 and 5 follicles.

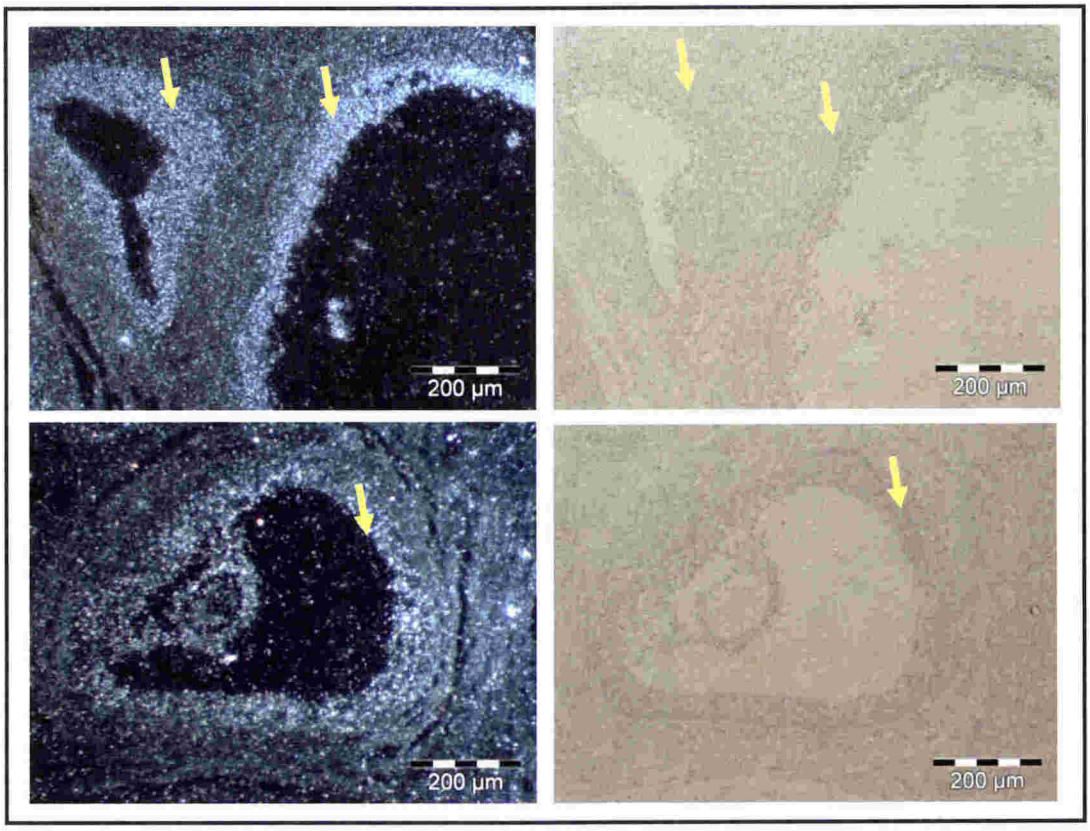


Figure 53:

Corresponding darkfield and brightfield views of ovarian sections, from W+ lambs showing expression of FSHR mRNA in the top panels . The bottom panels show the expression in the granulosa cells of a Type 5 follicle for a ++ lamb. Expression was limited to the granulosa cells of Types 3, 4 and 5 follicles.

The FSHR is expressed in a stage-specific manner, and the first expression observed in the Woodlands animals was at a Type 3 which is consistent with that reported for other sheep breeds (Juengel *et al.*, 2000).

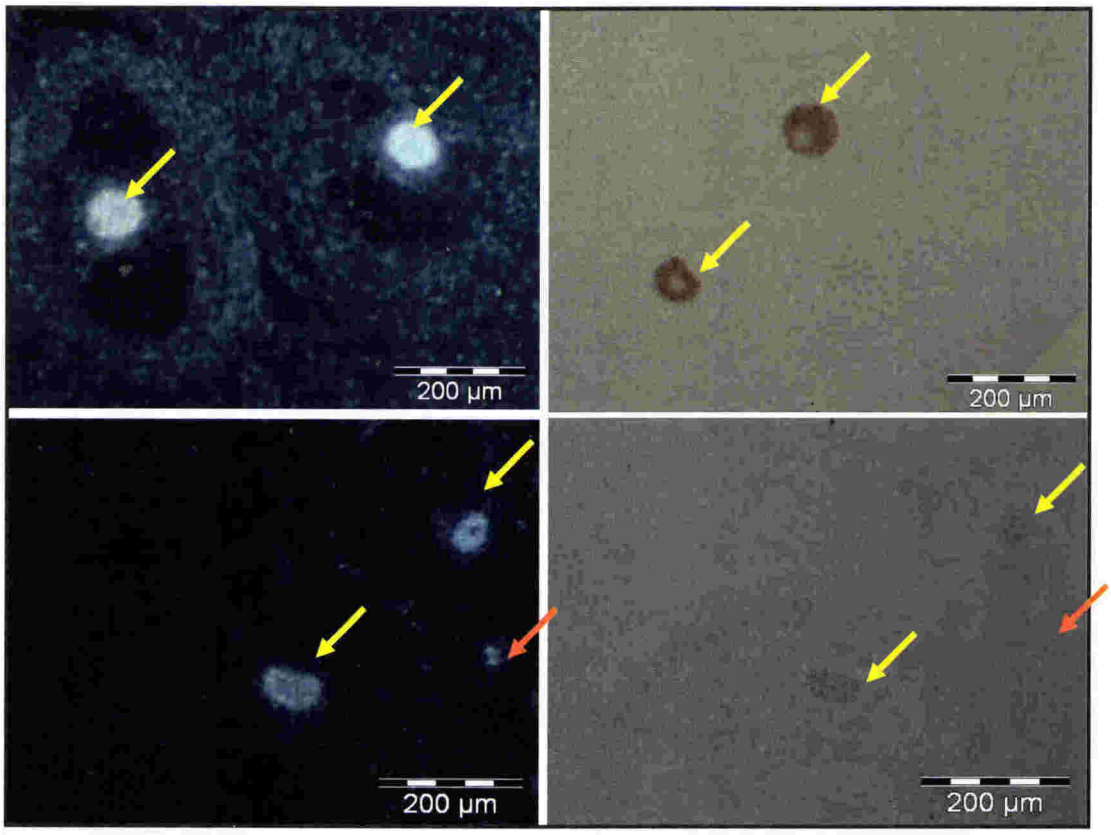


Figure 54:

Corresponding darkfield and brightfield views of ovarian sections, from W+ lambs showing expression of GDF9 mRNA in two Type 5 follicles in the top panels. The bottom panels show the expression in the oocyte of a Type 5 follicle and a Type 4 follicle for BMP15 in a ++ lamb. The yellow arrows in both pictures indicate the expression of the oocyte and the orange arrow points to an oocyte in a Type 3 follicle.

These results are consistent with expression of GDF9 and BMP15 in other prolific breeds with GDF9 being expressed from the Type 1/1a stage of follicular growth through to the Type 5 stage and BMP15 expressed from the Type 2 stage through to the Type 5 stage (McNatty *et al.*, 2005).

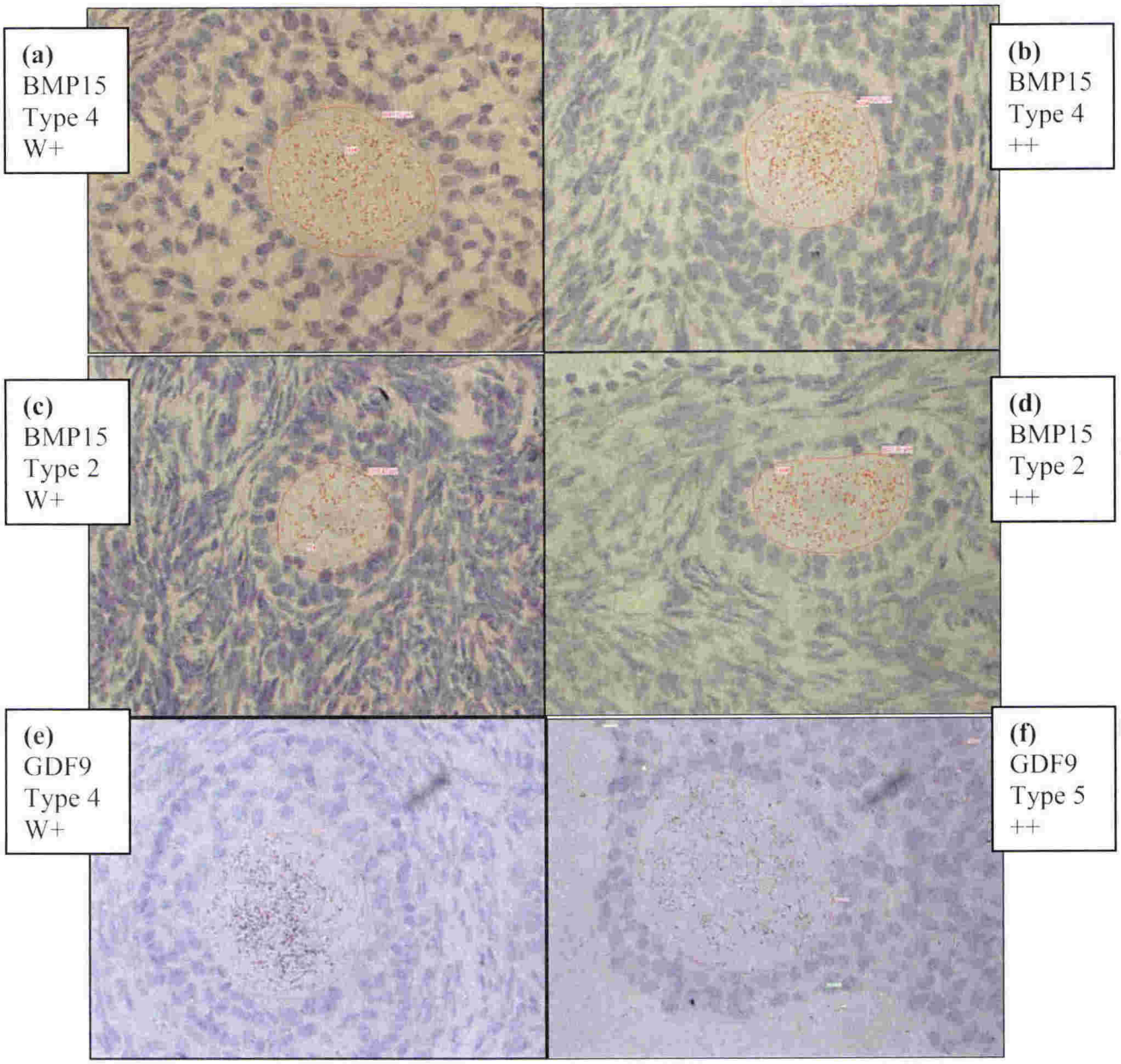


Figure 55: Photomicrographs of different follicle types with silver grains counted in the oocyte where both BMP15 and GDF9 are expressed.

(W+ = FecX2^{W} carrier; ++ = non – carrier).

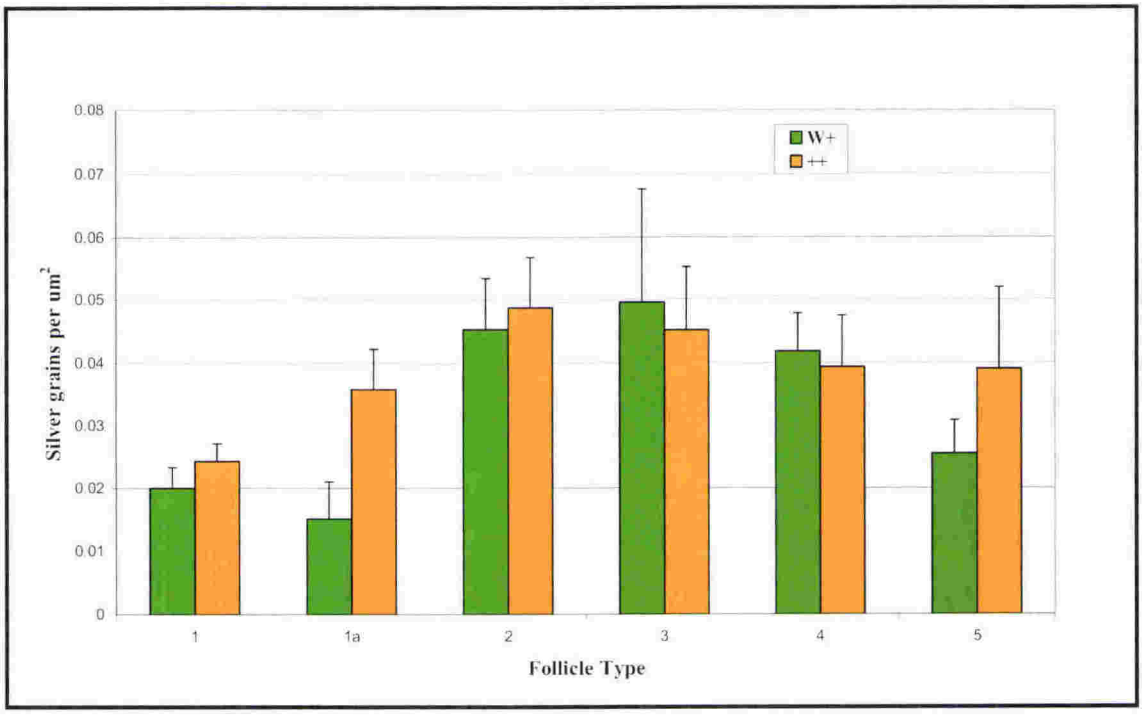


Figure 56a: Mean GDF9 silver grain counts (+ sem) for Woodlands (W+) vs. control (++) ewes.

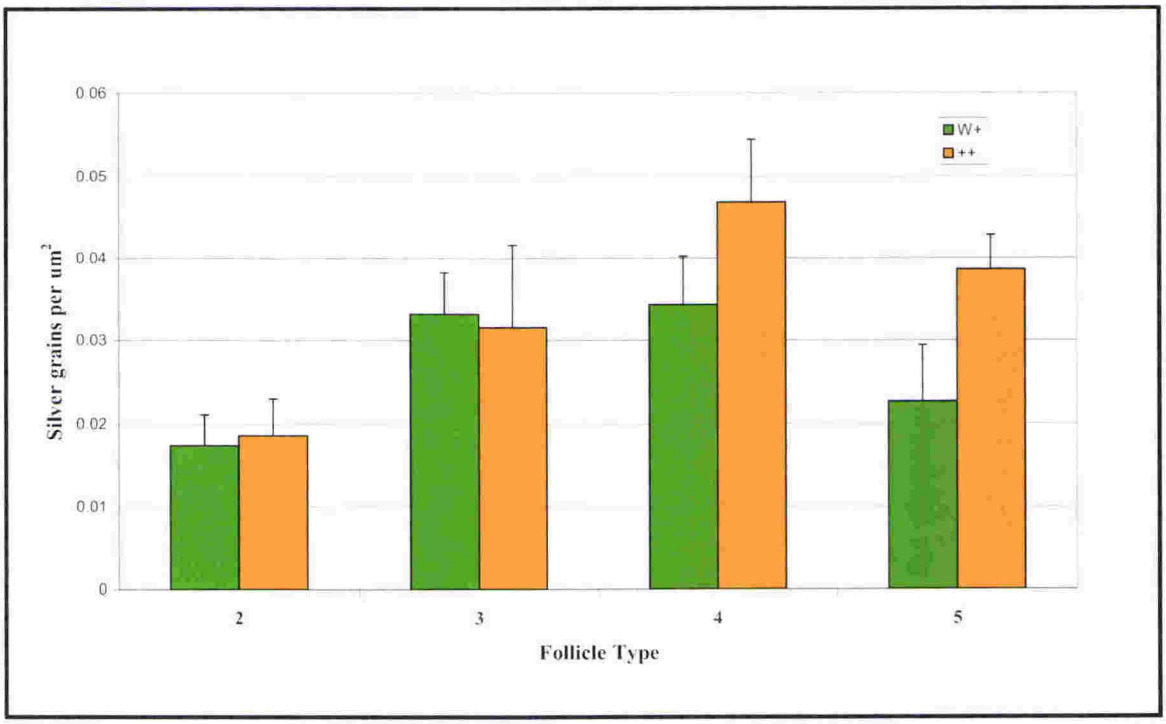


Figure 56b: Mean BMP 15 silver grain counts (+ sem) for Woodlands (W+) vs. control (++) ewes.

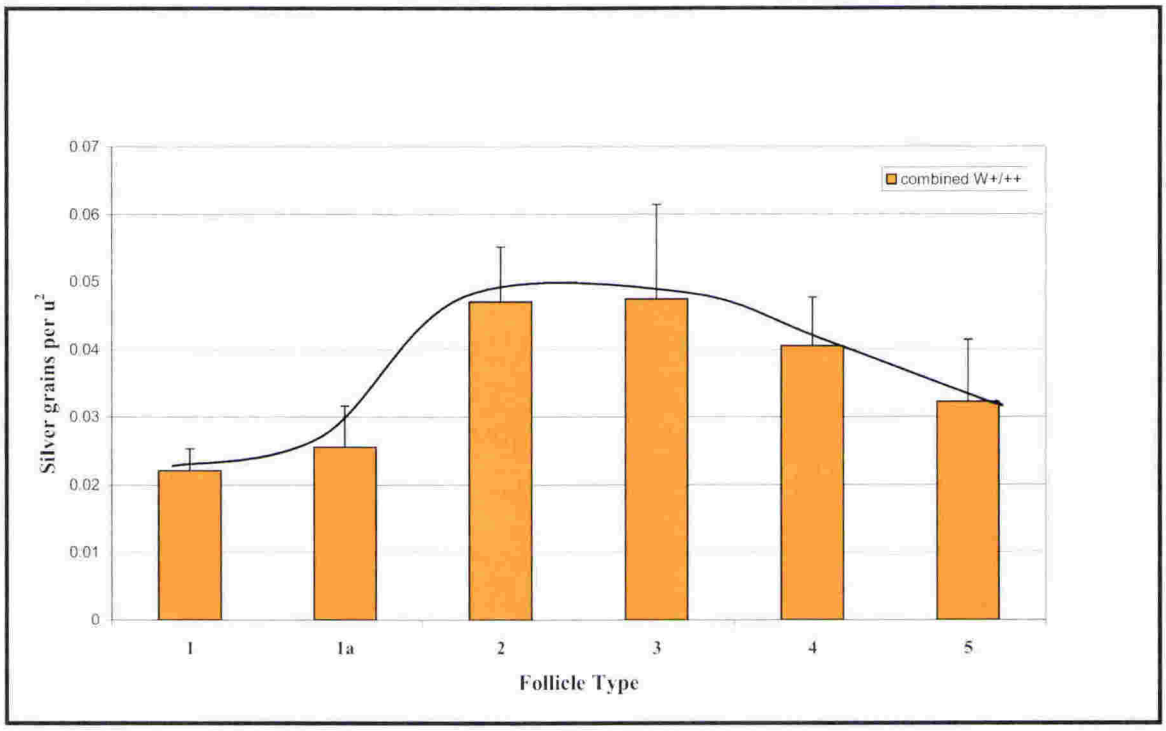


Figure 57: Combined mean + sem GDF9 silver grain counts, from W+ and ++ ewes showing the changing pattern of gene expression in oocytes during follicle growth.

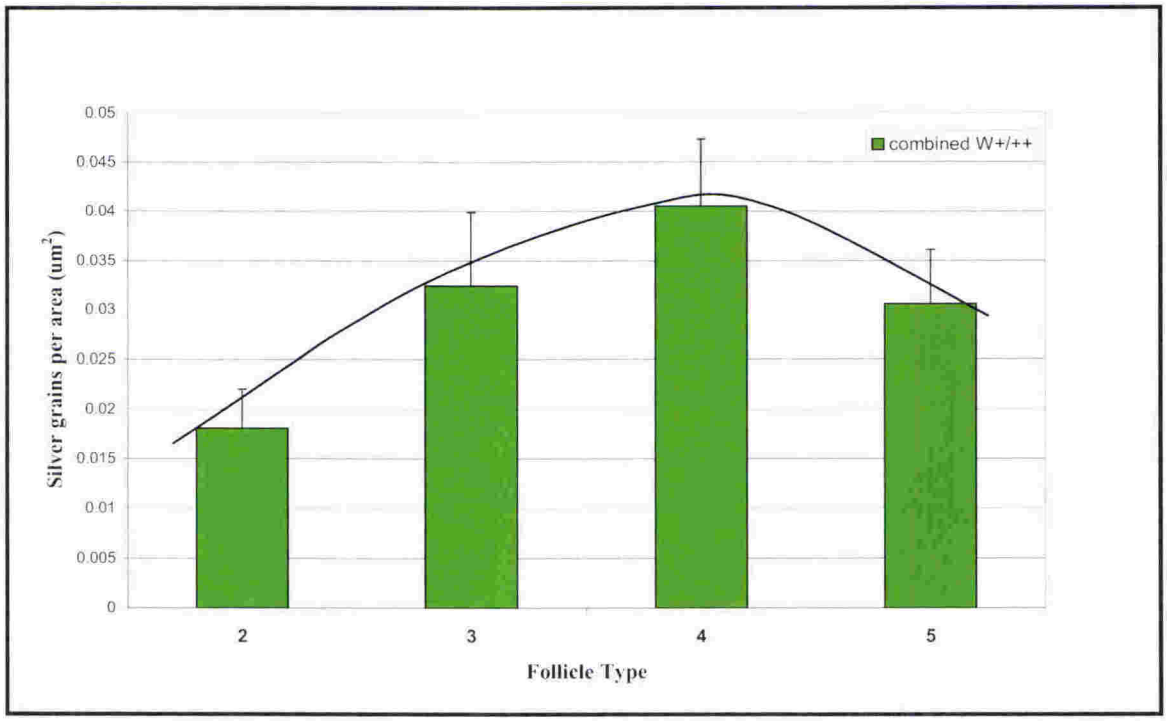


Figure 58: Combined mean + sem BMP15 silver grain counts, from W+ and ++ animals showing the changing pattern of gene expression in oocytes during follicular growth.

4.5 Follicular fluid from large and small ovaries

4.5.1 Introduction and Aim

During follicular growth an antrum develops. This antrum is filled with follicular fluid containing plasma-derived proteins and other products as well as secretory factors or metabolites from follicular cells including the oocyte (McNatty, 1981). This fluid is also likely to contain some products essential for follicular growth. The aim of investigating the follicular fluid of follicles from lambs with the large (LOP) or normal ovarian phenotype (NOP) was to determine if there were any major differences in the type and quantity of proteins that could be used to characterise the follicular phenotype given the extreme differences in follicular development found between the LOP and NOP. In previous experiments with prolific Finn sheep, proteins that were identified included albumin, transferrin, immunoglobulins and clusterin (Driancourt *et al.*, 1996). In mature human follicles, proteins that have been identified included serotransferrin precursor, apolipoprotein A-1, thioredoxin peroxidase, Ig heavy chain and transthyretin, hormone sensitive lipase, apolipoproteins A-IV precursor and 2 unnamed proteins (Lee *et al.*, 2005). Most, if not all of the aforementioned proteins were thought to come from plasma and therefore not directly useful as markers of follicular growth. However, the techniques employed in these studies were not sufficiently sensitive to detect secretory proteins in low concentration from follicular cells. To identify proteins in the present study, the follicular fluid was extracted from follicles of different sized ovaries and was processed for 1DE and 2DE.

4.5.2 Results

The follicular fluid samples had been stored in PBS and 0.1% BSA The amount of BSA added prior to storage completely dominated the 1DE protein profiles of follicular fluid. An albumin removal kit was therefore used to deplete albumin prior to 2DE. Follicular fluids from the follicles of an animal with the large ovary phenotype (LOP) and from an animal with the normal ovary phenotype (NOP) were separately pooled. Both sets of

fluid were processed for depletion of albumin and IgG using an Amersham® Albumin Removal Kit. This kit utilises antibody affinity resin to remove albumin and IgG.

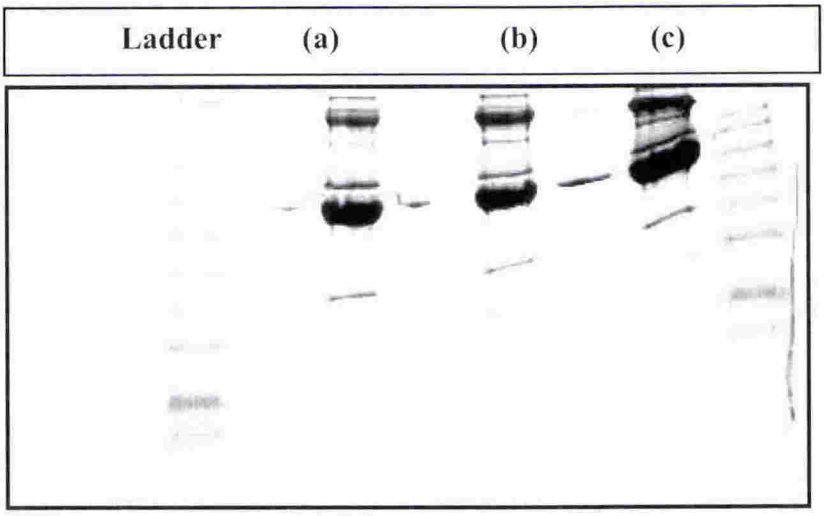
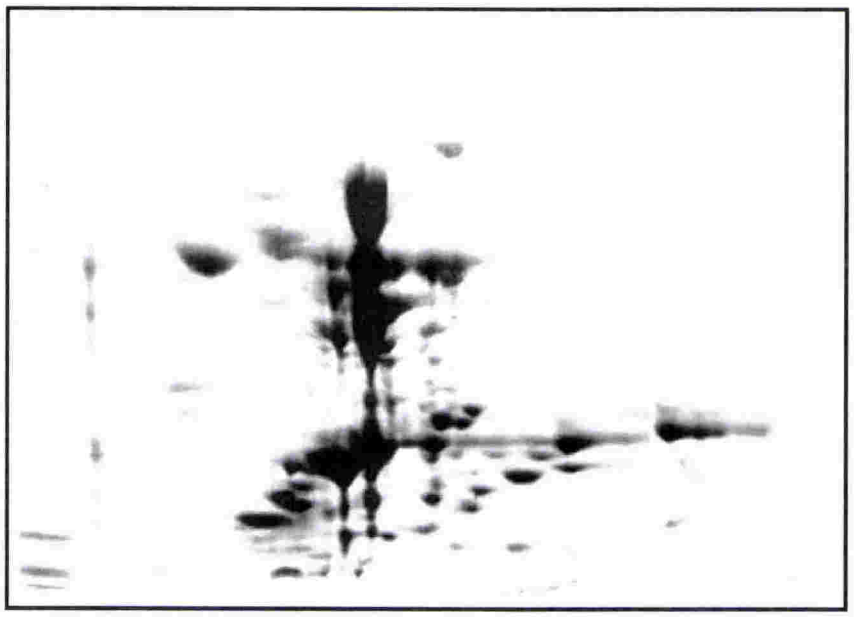


Figure 59: One dimensional gel of comparison of follicular fluid through the albumin and IgG kit.

One dimension gel showing from left to right a protein standard ladder, pooled follicular fluid treated with the albumin removal kit (a), follicular fluid from sheep number 384 (0 .284g ovary) treated with the albumin removal kit (b) and follicular fluid not processed through the albumin removal kit (c). Albumin and Ig depleted samples were then used for protein separation by 2DE.

(a)



(b)

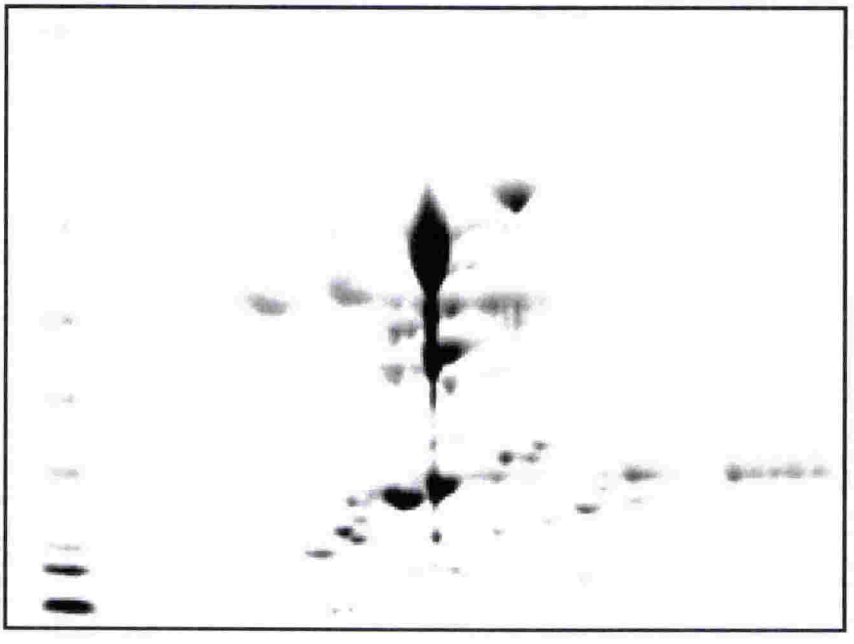


Figure 60: a and b show 2D gels of the same follicular fluid.

(a) Follicular fluid (b) Follicular fluid through the albumin and IgG removal kit.

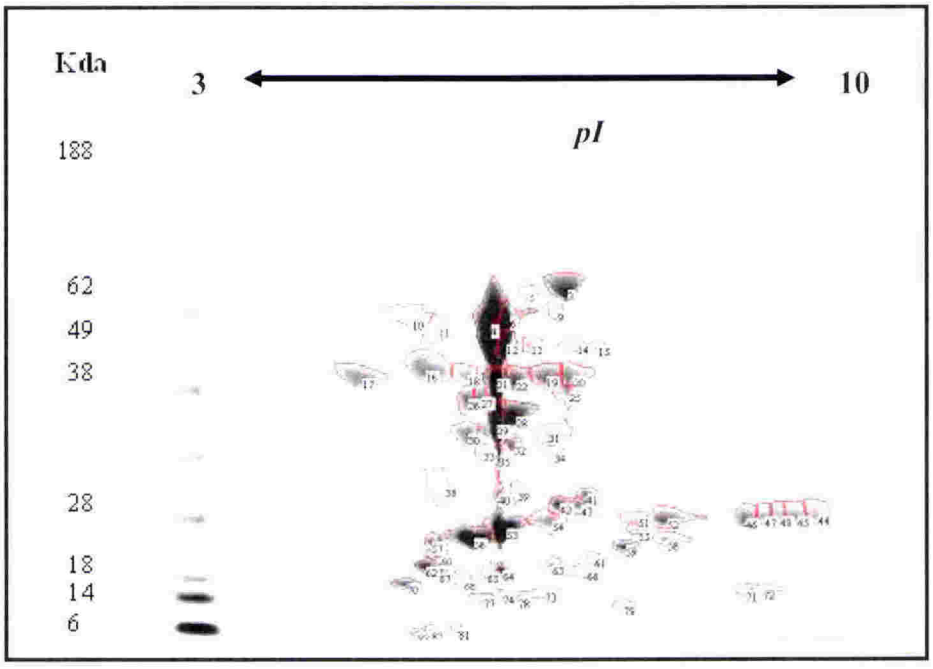


Figure 61: 2DE of large ovary phenotype follicular fluid.

Fluid was pooled from the left ovary (weight 1.4 g) of Sheep #398. Numbered protein regions were detected using ImageMaster software. Molecular weight markers are in the lane on the left side of the gel.

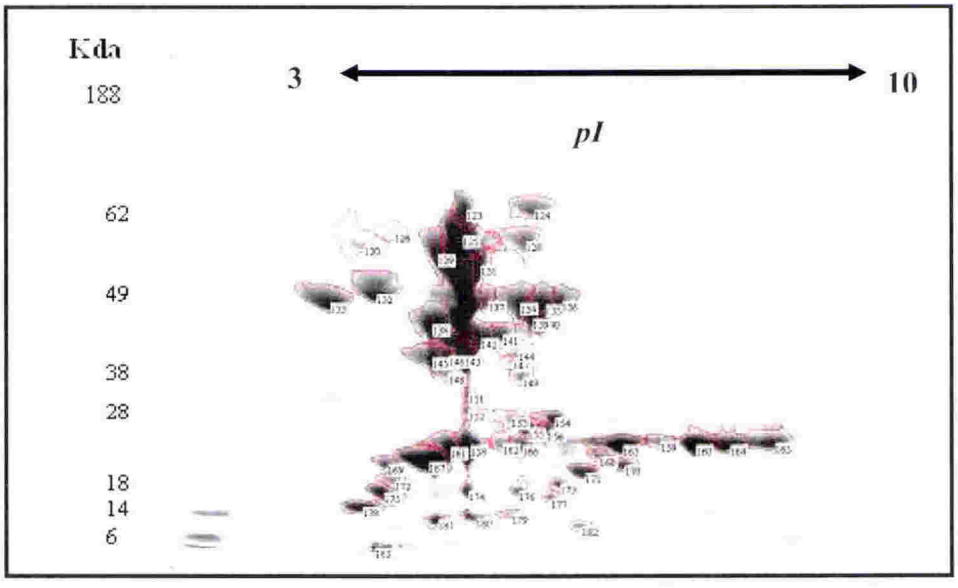


Figure 62: 2DE of normal ovary phenotype follicular fluid.

Fluid was pooled from the left ovary (weight 0.28 g) of Sheep #384. Numbered protein regions were detected using ImageMaster software. Molecular weight markers are in the lane on the left side of the gel.

Figures 61 and 62 show 2DE gels stained with Sypro® Ruby and Figure 63 shows a false colour overlay image of the two gels .When the two gels, one from the largest ovary (LOP) and another from a smaller ovary (NOP) were over-laid, some distinct proteins spots were dominant in one or other ovary were identified and then were excised from the gel for more detailed analysis (see Figure 63).

The numbered proteins spots in Figure 63 were cut out and subjected to digestion with trypsin and MALDI mass spectrometry.

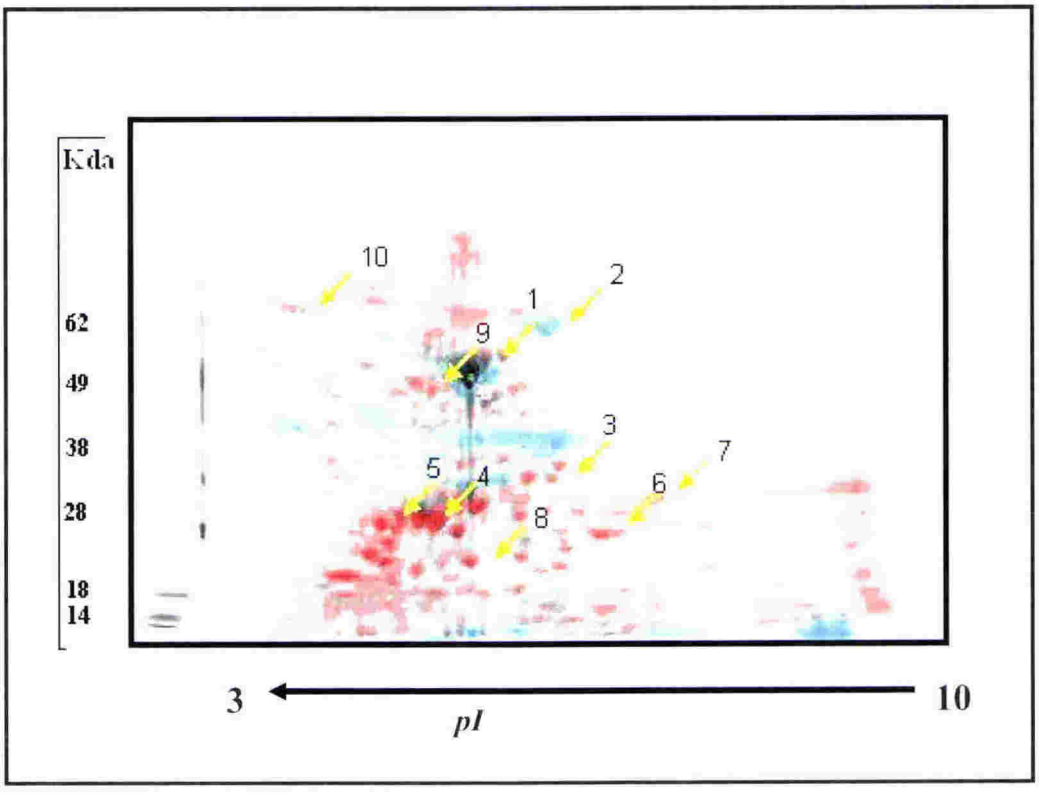


Figure 63: ImageMaster overlay of the images of large (Figure 61) and normal (Figure 62) ovary phenotype gels.

Arrows indicate protein spots that were excised for tryptic digestion and MALDI mass fingerprinting. Blue regions are spots from the small ovary phenotype follicular fluid and red regions are spots from the large ovary phenotype follicular.

Figure 63 shows numbered protein regions that were excised from the gels for tryptic digestion and MALDI mass fingerprinting. The image overlay technique can also be used to compare relative abundances of proteins between two protein samples but the current gels were difficult to match because of differences in total protein abundance between the two samples.

Protein identification was also difficult because of the small amount of protein present in follicular fluid. The only protein spot that could be identified was Spot 2 whose tryptic digest mass fingerprint matched to ovine serum albumin precursor using the ProFound protein search tool (Table 7). The theoretical pI 5.8 and predicted size 71.2 kDa of this matched protein sequence are consistent with the pI and size position of spot 2 on the 2DE gels. None of the other excised protein spots gave spectra of sufficient intensity for peptide mass matching. In an attempt to predict gel positions of some proteins of interest a small database of predicted pI and mass values of several proteins

was compiled using tools and sequences from the EXPASY web site (Table 8). Proteins of interest including BMP15, GDF9 and inhibin A and B could be expected to separate on the 2D gels but were probably too low in abundance to be detected or identified in the present work.

Table7:

Protein Candidates for search B65B7198-0574-77C8A465 [260661 sequences searched]							
Rank	Probability	Est'd Z	Protein Information and Sequence Analyse Tools (T)	%	*pI*	kDa	®
1	**1.0e+000**	**1.30**	**T gi|113582|sp|P14639|ALBU_SHEEP Serum albumin precursor**	**11**	**5.8**	**71.17**	®

Input Summary							
Date & Time	Mon Apr 03 02:31:17 2006 UTC (Search Time: 1.98 sec.)						
Sample ID							
Database	NCBInr (2005/06/01)						
Taxonomy Category	Mammalia (mammals)						
Protein Mass Range	10 - 100 kDa						
Protein pI Range	3.0 -10.0						
Search for	Single protein only						
Digest Chemistry	Trypsin						
Max Missed Cut	1						
Modifications	+C2H3ON@C(Complete);						
Charge State	MH+						
Peptide Masses (Da,Average)							
Tolerance(AVG)	1.00 Da						
Peptide Masses (Da,Monoisotopic)	1283.735	1439.861	1440.856	1441.870	1453.880	1454.872	1471.860
	1503.820	1504.814	1505.830	1506.835	1517.836	1518.824	1519.805
	1595.825	1596.833	1597.835	1598.840	1610.849	1612.834	1625.979
	1626.980						
Tolerance(MON)	0.06 Da						
Number of Peptides	22						

Table 7 continued

Details for rank 1 candidate in search B65B7198-0574-77C8A465 </TD< TR>

gi|113582|sp|P14639|ALBU_SHEEP Serum albumin precursor

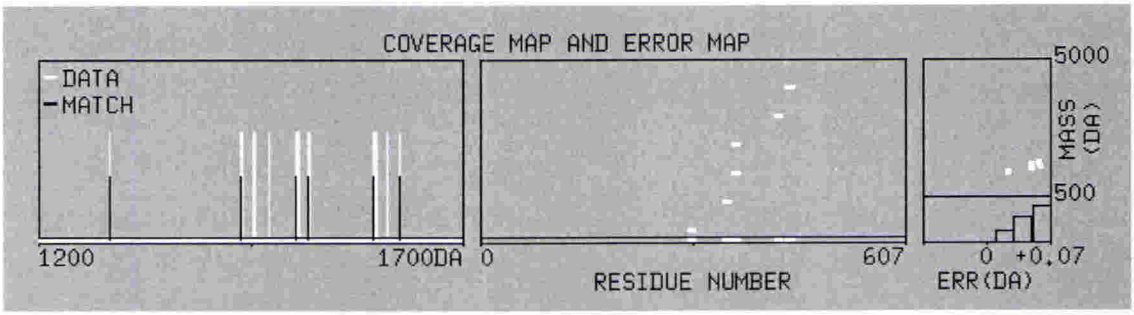
gi|476383|pir||ABSHS serum albumin precursor - sheep

gi|1387|emb|CAA34903.1| pre-pro serum albumin [Ovis aries]

Sample ID : [Pass:0]

Measured peptides : 22 peptides that are measured

Matched peptides : 6 only 6 were matched



Measured Mass(M)	Avg/ Mono	Computed Mass	Error (Da)	Residues Start	To	Missed Cut	Peptide sequence
1282.727	M	1282.703	0.025	361	371	0	HPEYAVSVLLR
1438.854	M	1438.804	0.050	360	371	1	RHPEYAVSVLLR
1502.812	M	1502.762	0.050	421	433	0	HGEYGFQNALIVR
1517.817	M	1517.757	0.059	298	309	1	LKECCDKPVLEK
1594.817	M	1594.766	0.051	347	359	0	DVFLGSFLYEYSR
1624.971	M	1624.914	0.057	437	451	1	KAPQVSTPTLVEISR

Unmatched Monoisotopic Masses:

1440.856 1441.870 1453.880 1454.872 1471.860 1504.814 1505.830 1506.835 1517.836 1519.805 1596.833 1597.835 1598.840 1610.849 1612.834 1626.980

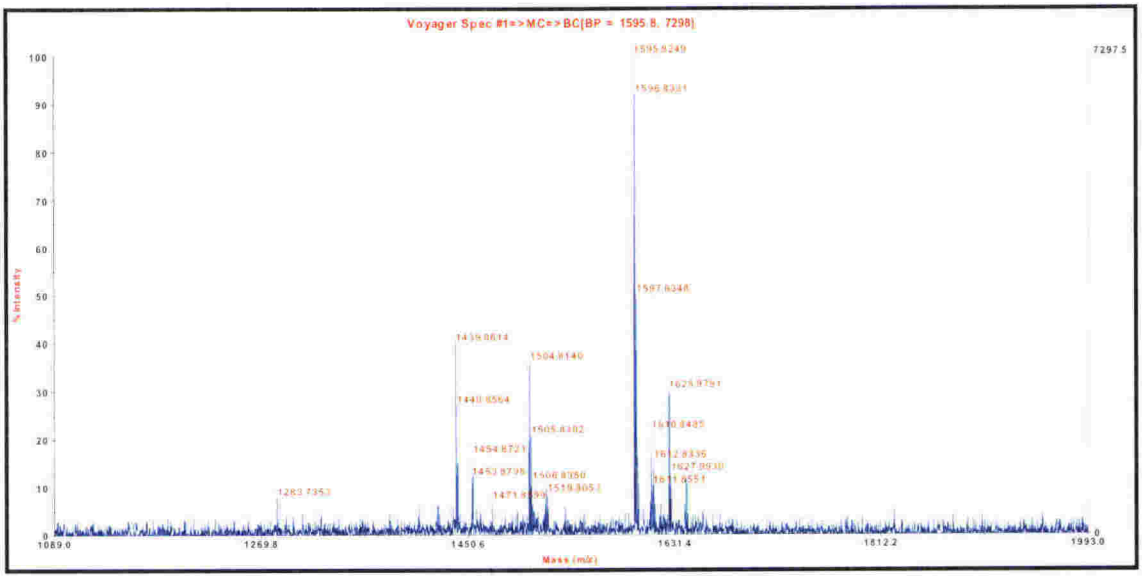


Figure 64: The spectra from which the spot was matched from the 'ProFound' search.

Table 8: Shows some of the expected proteins

Expected proteins	molecular weight (Da)	aa length	subunits	predicted pI
BMP15	44900	393	homodimers or heterodimers	9.46
GDF9	51776	453	Homodimers or heterodimers But, in contrast to other members of this family, cannot be disulfide-linked	8.96
Inhibin A	28754	265	Dimeric, linked by one or more disulfide bonds, a dimer of alpha and beta-A.	8.12
Inhibin B	47565	425	Inhibin B is a dimer of alpha and beta-B.	7.87
Albumin	69188	607		5.8
Transferrin	48956	44		9.23

Some of the expected proteins that could be seen in the gel spots taken from the 2D gel. The molecular weight, predicted pI, amino acid length and possible subunits were identified using http://ca.expasy.org/uniprot .

Chapter 5 - Discussion

Morphology and Morphometry of the Woodlands animals

Some of the unusual morphology and morphometry of the Type 5 (antral) follicles in Woodlands lambs and ewes suggests that the mutation is either a contributing or causal factor. The larger numbers of early developing Type 5 follicles and their unusual shape in the ewes were not characteristic features in the control sheep. The question arises as to whether these unusual follicles are capable of developing through to ovulation. There were no obvious signs of atresia (as described in the methods 3.3 page 49) in these follicles so it could be assumed that these follicles have a potential to continue to grow as the oocyte appeared normal and of a similar size as those in the larger ovulatory Type 5 follicles.

The unusual morphology of these small Type 5 follicles could be the result of a complex of interactions between many genes. There are no other reports of these unusual Type 5 follicles in the literature and more studies would need to be completed to see if this is a unique finding in this particular flock of Woodland ewes or whether the Woodlands ewes have a unique process that permits some or all small follicles to develop an antrum earlier in development or to have a different process of developing an antrum. Interestingly, both the Woodlands lambs and ewes had increased numbers of Type 5 follicles, although in the 4-week old lambs it was evident in follicles > 1mm rather than in the smaller antral follicles. This difference between the lambs and ewes may have been complicated by the fact that the lambs also expressed the large ovary phenotype which was not evident in the ewes. It is possible that the larger number of small antral follicles in the ewes is related to the higher ovulation rate in these animals but further studies would be required to prove this.

The mechanisms of actions of some mutations known to cause an increase in ovulation rate in sheep have been elucidated. The common feature of the pre-ovulatory follicles of the prolific Booroola sheep is that their oocytes enlarge earlier in development and follicular diameter at ovulation is smaller and at each stage of antral development they have fewer granulosa cells after ovulation, and smaller corpora lutea (McNatty *et al.*, 1984). In this context, Woodlands ewes appear not to fulfil these characteristics as there

are no statistically significant differences in oocyte diameter at any stage of follicular development. Also the granulosa cell populations at the Type 1-2 stage of development, as judged by the number of cells in the largest cross section were not different and there were no obvious differences in the granulosa cell population at any stage of antral follicular development or in the diameters to which follicles could develop to. However, detailed studies of the follicular cell populations in preovulatory follicles or corpora lutea have not yet been performed.

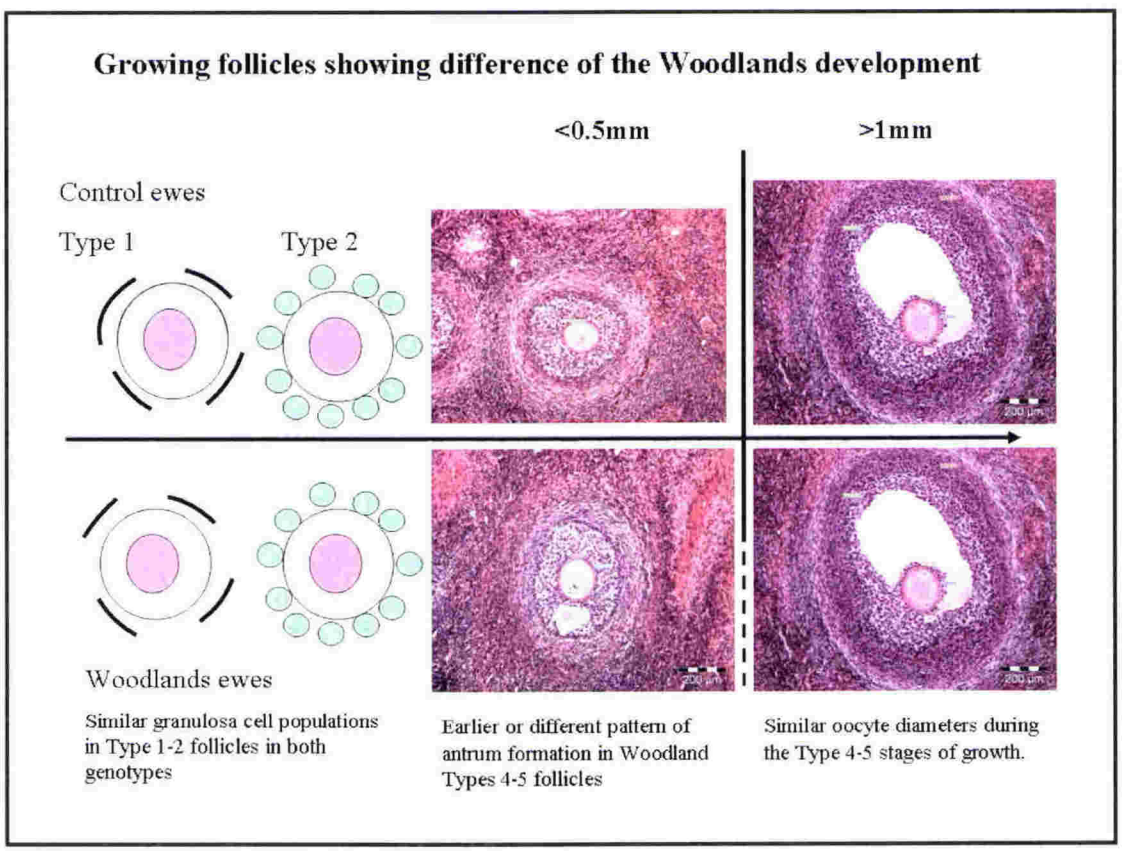


Figure 65: Shows the development of a follicle in Woodlands (W+) and a control ewe (++) from the Type 1 stage of growth.

The Type 4-5 transition in the control ewes develops with increasing layers of granulosa cells and the antrum develops with 5-7 layers of granulosa cells. At least 36% of the Woodlands follicles develop an antrum at an earlier stage with only 3-6 layers of granulosa cells but with a similar oocyte diameter to that in the control. Moreover, the antrum development appeared unusual in that it started from close to the basement membrane rather than at random sites within the granulosa cell matrix.

One of the observations made but not investigated in this study of the unusual Type 5 follicles was that the stroma around the theca layer looks congested (photos A and D, Chapter 4). It appears that the follicular wall is pushing out into the theca which also appears irregular. It is difficult to ascertain what is changing first but the basement

membrane is still intact and there are no obvious signs of atresia. A possible explanation for the unusual architecture of these follicles may be found in the adjacent stroma, and a more detailed evaluation of the stromal-follicular morphology and function may be worthy of future study.

Cyclic AMP and inhibin

The results from the study of the cyclic AMP responsiveness of granulosa cells from animals with the LOP revealed that up to the 4mm diameter follicle there was a rather modest or poor overall dose response to FSH compared to that observed in the NOP ewes. However, follicles that were > 4mm in LOP had granulosa cells showing a stepwise increase in response compared to cells from the NOP which did not respond. In neither the NOP or LOP ovaries was there convincing evidence for a consistent increasing hCG/LH induced cAMP response with increasing follicular diameter.

In this study the aim was to elucidate if there was a different sensitivity to both FSH and LH in the granulosa cells of the LOP compared to the NOP independent of the Woodlands mutation. It is possible that the proliferation rate of granulosa cells in the large ovary phenotype was different from that in normal ovaries and this may have been reflected in the cAMP response. This would have to be determined in vivo perhaps using BrdU labelling studies. The results of the cAMP were interesting because of the reduced FSH induced cAMP dose related response in the LOP until follicles reached >4mm when a dose related increase was evident. Moreover, cells from the NOP had a higher basal cAMP output ($p < 0.05$) than from the LOP in the absence of FSH, but for the FSH doses, the cells from the LOP had a higher response ($p < 0.01$) than the NOP. This may be in some way related to the increase in number of Type 5 follicles and the subsequent increase in the size of the ovary seen in the LOP compared to the NOP. Normally an increase in FSH induced cAMP response would be expected as the follicle develops beyond 1mm in diameter, as the cells increasingly change from a proliferative state to a differentiative state (Henderson *et al.*, 1987). At the antral stage, negative feedback effects of steroids and inhibin, produced by the developing follicles, causes a reduction in FSH response (Henderson *et al.*, 1987) . One interpretation of the results in the present study is that the granulosa cells in the LOP were immature relative to the

cells from the NOP. Thus it is possible that the cells in the NOP develop relatively normally but are never exposed to normal adult levels of LH so that the follicles are unable to secrete substantive levels of steroids or able to acquire LH receptors in the granulosa cells. By contrast the cells in follicles from the LOP might be more immature and unable to develop an adequate FSH responsiveness until the follicles are at a later stage of growth. However, because LH secretion in lambs is invariably low until after puberty, neither the LOP or NOP animals were unable to develop follicles with a significant LH induced cAMP responsiveness in the granulosa cells. It may also be interesting retrospectively to identify if any of these lambs were Woodlands lambs, as although they were chosen based on the large ovary phenotype some may still be found to carry the Woodlands mutation. However their pedigrees will not be certain until further progeny studies are completed in subsequent years. Also, it would be interesting to determine whether lambs that experience the LOP have as adults an ovulation rate and/or level of fertility that differs from that from animals with NOP.

The results from the measurements of plasma inhibin and FSH concentrations showed there was a direct correlation between ovarian weight and inhibin but no such correlation with FSH. The mean plasma inhibin concentrations from the large ovaries (>0.6g) was significantly higher than from the small ovary (<0.6g) ($p<0.001$), but there was no difference in FSH concentrations. This raises the question why the higher level of inhibin did not suppress FSH to a greater extent than from the small ovaries. Results from another 4-week old Woodlands lamb study (Dr Juengel personal communication) also showed a similar pattern of inhibin and FSH plasma concentrations, confirming these data. So, why are the FSH concentrations not suppressed? The most likely result is that most of the inhibin was predominantly α subunit and not biologically active at the level of the pituitary gland. However, it is possible that the α inhibin subunit may have a local effect in the ovary. Because inhibins act as an antagonist to activins which promote granulosa cell proliferation, the higher levels may be causing the change from a proliferative state to a differentiative state which may include the development of the antrum. However, in vitro studies on mouse and rat ovaries have shown activins have no effect on antrum formation or follicle survival, but instead promote oocyte and preantral follicular growth (Matzuk *et al.*, 1996; Thomas *et al.*, 2003). However, the roles of activin in these processes in sheep are not known

In Booroola sheep it was found that the highest mean amounts of cAMP produced by cells challenged with FSH or LH did not differ between genotypes. However, the granulosa cells in BB ewes developed a greater sensitivity to FSH with respect to cAMP synthesis in-vitro and acquired LH receptors and developed aromatase activity at a smaller follicular diameter compared with these functions in control ewes (Henderson *et al.*, 1987; Wilson *et al.*, 2001). This suggested that the Booroola mutation caused an earlier maturation of follicles (Henderson *et al.*, 1987) allowing more to be available for ovulation. Although there is no evidence in Woodlands ewes for advanced maturation of the oocyte or in the size of the largest non-atretic antral follicles, the follicles did appear to undergo antrum formation earlier in development. It is possible that these changes seen at a discrete stage of follicular development in the W+ ewes might be a direct or indirect result of the changes in the levels of expression of the ALK6 or BMP15 gene. The reason for this suggestion is that Woodlands sheep crossed with sheep heterozygous for the Booroola (ALK6) or Inverdale (BMP15) mutation have ovulation rates that are additive for each of these mutations (Davis *et al.*,1999). The BMP receptors are known to form heteromeric and homomeric complexes which permit a flexibility and diversity in response to different ligands. The mRNA encoding ALK6 has been observed in granulosa cells of follicles once they have entered the growing phase (i.e. Type 2). It has also been observed in oocytes from the Type 1 through to Type 5 stage of growth (Wilson *et al.*, 2001). Another interesting observation made for the Booroola mutation was that the decrease in proliferative activity and the increase in the expression of the main markers of final follicular maturation predominantly the luteinising hormone receptor (LHR) and aromatase appeared in granulosa cells (GCs) from $FecB^{B}$ earlier in development than in GCs from FecB+ wild type animals (McNatty *et al.*, 1989). As a result, there are fewer GCs in $FecB^{B}$ than in FecB+ pre-ovulatory follicles but the total number of GCs in pre-ovulatory follicles per animal, as well as ovarian secretion rate of steroids and inhibin which were similar in both genotypes (McNatty *et al.*, 1975; McNatty *et al.*, 1989; Fabre *et al.*, 2003).

The mutations in other prolific sheep have been in the oocyte-expressed growth factors GDF9 and BMP15 with the difference being, that in homozygous carriers the result is infertility, not an increase in ovulation rate (Galloway *et al.*, 2000; Hanrahan *et al.*, 2004). Current evidence suggests that homozygous carriers of the $FecX2^{W}$ mutation are fertile as no evidence for infertility has been observed in the Woodlands sheep

(G.Davis, personal communication). When heterozygous Booroola and heterozygous Inverdale sheep are crossed all the daughters have fully functioning ovaries and high ovulation rates, an average of 4.4 (Davis *et al.*,1999). It is believed that these two mutations also share a common pathway because the heterozygous crossbred ewes show that the effect of the two genes in combinations is higher than the sum of the effects of each gene alone (Davis *et al,* 1999.). Combinations of the prolific sheep, Booroola, Inverdale and Woodlands have also been bred, and the daughters carried one copy of each of three mutations (BMP15, ALK6 and $FecX2^{W}$). The ovaries appeared normal and the ovulation rates were 5 and 8 at 1.5yrs and 12 at 2.5years (G.Davis personal communication). Importantly this indicates again that there is an additive effect with these genes, and that Woodlands is potentiating or adding to the effect of the other two genes.

In-situ hybridisation and concentration studies

The in-situ hybridisations completed on GDF9, BMP 15, βA and βB inhibin, α inhibin, ERα, ERβ and FSHR did not demonstrate any differences in the ontogeny or pattern of expression. The onsets of expression of all these genes were similar to those reviewed by Juengel & McNatty (2005) except ERβ. In this study ERβ is not observed in oocytes until the Type 3 stage of follicular development. In contrast in a study recently reported by others in the Wallaceville laboratory (Juengel *et al.*, 2006) ERβ was first observed at the Type 1 stage of growth. Given that I was not able to observe this gene being expressed until later suggests a difference in sensitivity between the two studies as the same sequence of riboprobe was used in both studies. Further investigation with respect to the expression levels of BMP15 and GDF9, found no significant difference between Woodlands ewes and their wild type contempories. An interesting observation from these results which has not been reported before is that the increases in expression for both GDF9 and BMP15 were observed. The Types 3 and 4 stages respectively followed either by no change or a modest decline during the remainder of follicular growth. The decline was not statistically significant but these findings indicate that for both BMP15 and GDF9 the highest levels are found during the preantral stage of growth and preceding steroidogenic activity by the follicles (Logan *et al.*, 2002).

It is now well understood that the TGFß growth factors BMP15 and GDF9 are essential in the follicular growth and maturation (for review see McNatty et al 2005b). However no observable differences in these two genes were found in the present study. In Booroola sheep ($FecB^B$) there is a mutation in BMP receptor 1B (ALK6), one copy of this gene increases ovulation rate by about 1.5 and two copies increases it by 3.0 (Davis *et al.*1999; Souza *et al.*, 2001). Recent work by Juengel et al (2006 unpublished) suggest that ALK6 may be a contributing factor in the Woodlands mutation, as although in-situ hybridisation did not highlight any differences, further comparisons of the intensity of signal indicated that the Woodlands had lower levels of expression when compared to its wild type contempories. The question from this finding is what is the interaction? The Woodlands mutation is understood to be X-linked which suggests that there is still an unknown factor/interaction which is causing the decrease expression of the ALK6 if the latter finding is confirmed. We know that the BMP15 gene is located on the X chromosome so it is possible that there is a relationship between the X-linked expression of BMP15 and the reduced expression of ALK6 but the complicating factor is that the Woodlands mutation inheritance appears to be imprinted (Davis *et al.*, 2001) The Booroola mutation is not imprinted so that the observation of different levels of ALK6 may be a downstream effect of the Woodland mutation.

Imprinting

There has been no further evidence uncovered that the gene is maternally imprinted but many imprinted loci appear to be involved in growth control and most are expressed in the placenta (Reik *et al.*, 2003). Interestingly the oocyte contains a large number of stored maternal gene products and therefore it may be that the maternal effect loci play an essential role in interpreting and /or maintaining these epigenetic marks. There are also unidentified factors in the oocyte which are considered to be responsible for the rapid demethylation of incoming paternal genome shortly after fertilization. (Duselis *et al.*, 2005).The imprinted gene which has been studied the most is IGF2. Fetal mice heterozygous for the IGF2 gene inherited from their fathers displayed excessive growth but when received from the mother, the offspring showed a normal phenotype (Wrzeska & Rejduch, 2004). This supports the gametic conflict theory first described by Haig and Westoby (1989) described in the introduction (page 36). The growth-stimulating genes

should be subject to maternal imprinting and growth suppressing genes only the maternal allele should be expressed. It may also be an explanation for the large ovary phenotype with excessive follicles, but this needs further investigation.

Analysis of follicular fluid proteins

A challenge in this part of the experiment was to remove the PBS and BSA from follicular fluid. Both of these additives are contaminants for 2D gels and Maldi and needed to be removed as much as possible in order to identify putative novel proteins in the fluid. In hindsight the addition of PBS and BSA compromised the subsequent experiments. The albumin and IgG removal kit that was used was designed to remove human albumin and IgG and therefore was not specific for sheep. Nevertheless these reagents were able to remove a large percentage of the albumin. The resulting spectra from the Maldi TOF were clear, although other than the albumin precursor protein, no other proteins were identified. This may be because of the very low abundance of the locally-produced proteins and also because the lower molecular weight proteins may have been lost following use of the albumin and IgG clean up kit. In future studies it would be prudent not to preserve the sample in BSA. Recently a method was developed (Colantonio *et al.*, 2005) whereby the depletion of albumin component (DAC) was successful with samples of between 100 µl and 1ml and this method may be able to be used more effectively on the small quantities of fluid utilised in the present experiment. Pooled follicular fluid of around 100 µl volume could also be used. A method for future experiments of follicular fluid would be to eliminate the 2D gel step and use reverse-phase liquid chromatography electrospray ionization in tandem mass spectrometry (LC/MS/MS). In this method the proteins are digested with a proteolytic enzyme to produce a large collection of peptides, which are then separated on line with a tandem mass spectrometer. The molecules are separated by their chemical properties in the chromatographic step and then separated by m/z value with subsequent structural characterization in an MS/MS mode.

Chapter 6 - Conclusions and future directions

This thesis investigated a highly novel line of sheep that have an X-linked maternally imprinted gene that is associated with an increased ovulation rate. After three years we have not been successful in elucidating the physiological basis of the Woodlands ($FecX2^W$) mutation. Nevertheless the results herein suggest a novel gene or combination of genes is responsible for the increase in the Woodlands ovulation rate.

The key findings from the studies from the ewes and/or lambs that were carriers (W+) or non-carriers (++) of the $FecX2^W$ gene were:

- No genotype differences were present either in the numbers of primordial (Types 1/1a)or developing preantral (i.e. Types 2-4 follicles)
- Significant genotype differences in the numbers of small antral (Type 5) follicles (W+>++)
- An earlier onset of antral follicular development in W+ vs. ++ ewes with a high proportion of W+ follicles having irregularities in morphology between the basement membrane and stroma in the former.
- No genotype differences in the onset of gene expression during follicular development or in the cell-types expressing GDF9, BMP15, α inhibin, β_A inhibin, β_B inhibin, FSHR, ERα, ERβ.
- No genotype differences in the levels of GDF9 or BMP15 gene expression in oocytes throughout follicular growth.
- No genotype difference in largest diameters that follicles reached in the present study

A confounding issue during these studies was the discovery that a large ovary phenotype (LOP) which was present in many of the W+ lambs but not ++ lambs at 4-weeks of age was in fact a coincidence and not linked to the $FecX2^W$ mutation. Initially it was thought that LOP was strongly linked with or a result of the $FecX2^W$ mutation. Studies on lambs with the LOP independent of the $FecX2^W$ mutation revealed that the ovaries were 6 fold larger in volume, contained 10 times more type follicles even though there were no differences in the numbers of Type 1, 2, 3 or 4 follicles. Moreover, lambs with the LOP contained significantly higher concentrations of immunoreactive inhibin but not FSH consistent with the notion that immunoreactive

inhibin is a correlate of ovarian weight, the numbers of Type 5 follicles and specifically the total numbers of granulosa cells in Type 5 follicles. These results also indicate that many of the forms of inhibin present were not biologically active since no differences in the plasma FSH concentration were observed. It is likely the higher concentrations of immunoreactive inhibin were due to the LOP and not to the FecX2^{W} mutation since no genotype differences were noted for FSH in W+ and ++ ewes.

An assessment of the cyclic AMP responses were lower overall in LOP vs. the NOP when cells were recovered from follicles between 1-4 mm diameter. These findings infer that granulosa cells in the LOP may experience a delay in maturation relative to cells from NOP.

The overall conclusion is that the FecX2^{W} mutation affects ovarian follicular development in W+ sheep differently from that in ewes with either the Booroola, Inverdale or Hanna mutations and that the key differences appears to be that W+ ewes show evidence of earlier and altered pattern of antrum formation and greater number of small antral follicles. The underlying causes for these differences remain to be elucidated. It will be interesting to establish whether this difference persists with FecX2^{W} ewes crossed with either the FecB^{B} or FecX^{I} mutations or both. The LOP is a phenomenon previously identified in pigs (Grasso *et al.*, 1988) but has not to our knowledge been reported for sheep. Its cause remains unknown but is hypothesised to be associated with delayed antral follicular maturation.

Future directions

One of the differences in the Woodland phenotype observed was the pattern of early antrum formation and disturbed general nature of the stroma. To recover granulosa cells from the unusual follicles poses technical problems because presently the only way of identifying the follicles is after the tissues have been cut and stained. However, statistically there were significantly more of the small Type 5 follicles in the Woodlands ewes and more of the larger Type 5 follicles in the Woodlands lambs, so dissecting out follicles and culturing the granulosa cells from each Type 5 follicle and testing for secretions of proteins and steroids, should theoretically help us identify what differences

there are of the abnormal Type 5 follicles. Biochemical markers for antrum formation may be another way of assessing if the smaller Type 5 follicles are getting an earlier signal to develop an antrum. A differential gene expression profile in Woodlands (W+) vs. controls (++) at the Type 4/5 stage of growth would be an area to investigate because of the early antrum formation in the W+, and the higher number of smaller type 5 follicles. A careful characterization of the granulosa cell numbers and FSH responsiveness during preovulatory follicle maturation may also help us elucidate potential function differences between the genotypes shortly before ovulation. It is possible that the granulosa cells in the Woodlands follicles $\geq$ 4mm diameter have an increased response to either FSH or LH relative to the control animals.

There are two proteomic techniques which may be of use in further analysing follicular fluid Imaging mass spectrometry as reported by Caprioli and co workers (Chaurand *et al.*, 2003), allows direct analysis of thin tissue sections, mounted on a MALDI target coated with MALDI matrix. The tissue is then subjected to MALDI-TOF-MS and may give some insight into any differences in the follicular fluid or stroma of the smaller type five follicles or the stroma of the large and small ovaries. The imaging mass spectrometry (IMS) utilizes the MALDI-TOF-MS to profile and map proteins present in thin tissue sections. For an ovary containing the unusual shaped Type 5 follicles it may also allow one to pinpoint the expression of any unusual proteins. Secondly there is the 2-D Fluorescence Difference Gel Electrophoresis (2D-DIGE); this is a technique that labels complex protein mixtures with fluorescent dyes prior to undertaking 2D electrophoretic separation. This would mean that follicular fluid from a LOP and NOP or from W+ and ++ ewes could be mixed and then separated out based on the differences.

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Appendix 1

Abstracts

Identifying the mechanism of a novel genetic mutation affecting fecundity in Sheep

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The objective of this study was to examine ovarian function in a line of Coopworth sheep called Woodlands which have ovulation rates about 0.4 higher than wild-type sheep. These animals have a novel, imprinted, X-linked fecundity gene called $FecX2^{W}$ where Fec = fecundity, X = X chromosome, 2 = 2^{nd} mutation identified on X chromosome and W = Woodlands.

Standard methods of morphometric and histological analysis showed that the ovaries of 4 week-old lambs with the natural mutation are approximately 6 times heavier and have 10 times more antral follicles than wild-type ovaries. The cortical and ovarian volumes were larger than those in the wild-type animals. No differences were observed in mean numbers of types 1, 1a, 2, 3 and 4 follicles between the genotypes. Moreover, no differences were observed between genotypes in follicle or oocyte diameters for any follicle type. Using established *in-situ* hybridisation techniques, the genes bone morphogenic protein 15, growth differentiating factor 9, estrogen receptor α and β, inhibin α, inhibin/activin β_A and β_B, follicle stimulating hormone receptor, bone morphogenic protein receptor I and II, showed no observable differences of expression patterns between genotypes.

Thus, the Woodlands mutation $FecX2^{W}$ not only affects ovulation rate in adults but also appears associated with a polycystic ovary phenotype in lambs. However, the pathways that the mutation is utilising to affect follicular development has not yet been identified

Acknowledgements

Foundation for Research Science and Technology and Ovita, for supporting an enterprise scholarship

Morphometric and histological analysis of ovaries from sheep heterozygous for the prolific Woodlands allele

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Woodlands are a line of Coopworth sheep with a novel, imprinted X-linked fecundity allele resulting in ovulation rates about 0.40 higher than wild-type animals. Daughters of progeny tested sires with and without the gene were studied.

Previously, lambs heterozygous for the Woodlands allele were found to have larger ovaries and more antral (i.e. type 5) but not preantral (i.e. types 1-4) follicles than in wild-type contemporaries. The large ovary phenotype was found to be transient and was absent after puberty. However, based on follow-up studies it was evident that the large ovary phenotype was not strongly associated with the Woodlands fecundity allele. Thus, it was uncertain whether animals carrying the Woodlands gene had different follicular populations compared to wild-type controls. To address this question, follicular populations were compared in adult ewes heterozygous for the Woodlands allele with age matched controls. Using standard morphometric methods and histological analysis, no differences were observed in the mean numbers of types 1, 1a, 2, 3 and 4 preantral follicles between the genotypes. Furthermore, no differences were observed between genotypes in follicular or oocyte diameters for any follicular type. The adult Woodlands carrier ewes had twice as many small type 5 follicles (< 1mm) when compared to wild-type contemporaries although no difference was seen in the numbers of antral follicles > 1mm in diameter. In addition, antrum formation occurred at a smaller follicular diameter in the heterozygous Woodlands animals.

Therefore, the increased number of antral follicles observed in both lambs and adult ewes suggests that this difference in pattern of follicular development is associated with the X-linked fecundity allele. This novel phenotype of early antrum formation and larger number of small antral follicles differs from that observed in sheep with the Inverdale or Booroola mutations, suggesting that a different mechanistic pathway is involved.

Acknowledgements. The Marsden Fund and Foundation for Research Science and Technology and Ovita, for supporting an enterprise scholarship

Expression of Growth Differentiation Factor (GDF) 9, Bone Morphogenetic Protein (BMP) 15, Activin Receptor like kinase 5 (ALK5) BMP receptor (BMPR) type IB and BMPRII mRNA in ewes carrying the Woodlands FecX2W gene.

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Woodlands sheep have a major genetic mutation ($FecX2^W$) that increases ovulation rate. At present the identity of the Woodlands genetic mutation is unknown. While X-linked, this gene does not appear to be related directly to any of the known mutations in BMP15, GDF9 or BMPRIB that affect ovulation rate in sheep. Nevertheless, the $FecX2^W$ mutation may be affecting ovulation rate by changing the expression patterns of the BMPRIB, BMP15 or GDF9 genes. In addition, since BMPRII is involved in signal transduction for both BMP15 and GDF9, and ALK5 is involved in signal transduction for GDF9, changes in expression pattern of these genes would also be likely to affect ovulation rate. Thus, the objective of this experiment was to examine the patterns of expression of mRNAs encoding GDF9, BMP15, BMPRIB, ALK 5 and BMPRII during follicular development in ewes heterozygous for the Woodlands mutation and their wild-type contemporaries.

Expression patterns of selected genes were determined following in situ hybridisation. Intensity of the signal was established by quantification of silver grains in the cell(s) of interest. As GDF9 and BMP15 are expressed exclusively in oocytes, the signal was only quantified in this cell-type. Silver grain counts for BMPRIB, ALK5 and BMPRII, were made separately in the oocyte, granulosa and theca. Between 1-3 slides were analysed per animal and each follicle type (i.e. types 1-5) was observed in at least 3 animals of each assigned genotype.

Expression of GDF9 was observed to increase in oocytes from type 1 to 3 follicles and then decreased, whereas BMP15 increased in follicles from type 2 to 4 and then decreased. However, there were no differences between genotypes for expression of these two genes. The theca cells showed no statistically significant difference between

follicle types or genotype for any of the receptor genes. There was a significant effect of follicular follicle type in the granulosa cells for all 3 genes ($p<0.01$) and a significant genotype effect for BMPRIB, where overall, the expression in the wild-type was consistently higher ($p<0.01$). In the oocyte, there was a significant effect of follicle type and genotype on expression of ALK5 and BMPRIB, whereas for BMPRII no significant effect was noted on follicle type. Expression of ALK5 was increased in oocytes of Woodlands ewes whereas expression of BMPRIB was decreased.

Mutations in BMPRIB result in increased ovulation rates in sheep, and therefore the differences in expression level of BMPRIB in both the oocyte and granulosa cells may play a role in the increase in ovulation rate observed in Woodlands ewes.

Acknowledgements Marsden fund, Foundation for research, science and technology and Ovita for an enterprise scholarship

Appendix 2

Table 9: Cells plasmids and vectors used:

Gene	ßb inhibin	α inhibin	ßa inhibin	FSH(receptor)	LH(receptor)	GDF9-b (BMP-15)	GDF9
Cell ID	WR306	WR299	WR298	WR308	WR301	WR340	WR352
Cell strain	DH 5a	DH 5a	DH 5a	DH 5a	DH 5a	DH 5a	DH 5a
Plasmid ID	WRPS201	WRPS194	WRPS193	WRPS203	WRPS196	WRPS225	WRPS228
Vector ID	pGem7zf	pGem3z	pGem3z	pGemT	pBS11KS	pGemT	pGemT
Species	ovine	ovine	ovine	ovine	ovine	ovine	ovine
Antisense	Apa 1	EcoR1	Xba1	EcoR1	Bam H1	Sal 1	Sph1
Promoter(as)	SP6	SP6	T7	SP6	T3	T7	SP6
Sense	Bam H1	Xba1	EcoR1	Pst 1	EcoR1	Nco 1	Sal 1
Promoter(s)	T7	T7	SP6	T7	T7	SP6	T7
Release insert	Bam H1 & Apa1	EcoR1 & Xba1	EcoR1 & Xba1	EcoR1 & Pst 1	Bam H1 & EcoR1	EcoR1	EcoR1
Size(bp)	400	384	322	500	278	499	450
Plasmid comment	Subcloned from WRPS200 R Rodgers beta B inhibin subunit DNA	n/c	n/c		From G.Niswender, Colorado State university	From O.Ritvos lab c/o Mika Laitinen	

Gene	Er α inhibin	Er β inhibin
Cell ID	WR286	WR376
Cell strain	DH 5a	DH 5a
Plasmid ID	WRPS181	WRPS252
Vector ID	Bluescript SK	pGemT
Species	Ovine	Ovine
Antisense	Hind 111	Spe 1
Promoter(as)	T3	T7
Sense	Bam H1	EcoR1
Promoter(s)	SP6	T7
Release insert	Bam H1 & Hind 111	EcoR1
Size(bp)	485	415
Plasmid comment	From Mohan Manikkam University of Missoure, Dept Animal Science	n/c

Information from the Reproduction database Wallaceville Agresearch (2004)

Appendix 3

In Situ Hybridisation Solutions

DEPC Water

1 Litre of milliQ water

Add 1 ml of DEPC

Stir o/n with lid loose

Autoclave

7.5 M Ammonium Acetate pH 7.0

Ammonium acetate 57.81 g

Make up to 100 ml with unautoclaved DEPC water

pH to 7.0

Autoclave

1 M Tris

Tris 60.55 g

Autoclaved DEPC water 400 ml

pH to 6.8 , 7.2 or 7.5

Autoclaved DEPC water to 500 ml

Autoclave

500 mM EDTA pH 8.0

EDTA 73.06 g

Make up to 400 ml with unautoclaved DEPC water

pH to 8.0.

Note that EDTA will not dissolve until it has reached a certain pH (do the pH first)

Make volume up to 500 ml.

Autoclave.

20xSSC

3 M NaCl 175.3 g

0.3 M Na Citrate.$2H_20$ 88.2 g

Make up to 1 Litre with unautoclaved DEPC water (use MQ water and add DEPC while stirring overnight).pH to 7.0 and autoclave.

Proteinase K 10 mg/ml

Make up to required concentration with 50 mM TrisHCl pH 8.0 (made in DEPC) ON ICE.

Filter sterilise through 0.22 um filter.

Store in aliquots at –20 °C.

2 M Triethanolamine pH 8.0

Triethanolamine 53 ml

pH to 8.0 with cHCl

Make up to 200 ml with unautoclaved DEPC treated water

Wrap bottle in tin foil and autoclave

Store at room temp.

5 M NaCl

NaCl 146.1 g

Add unautoclaved DEPC water up to 500 ml

Autoclave

0.5 M $NaPO_4$ pH 6.8

Na_2HPO_4 7.09 g Make up to 100 ml with unautoclaved DEPC water

NaH_2PO_4 7.8 g Make up to 100 ml with unautoclaved DEPC water - autoclave

pH 0.5 M Na_2HPO_4 with 0.5 M NaH_2PO_4 to pH 6.8.

0.5 M Na_2HPO_4 alone cannot be autoclaved because it precipitates out.

Heat to 68 °C to drive off the DEPC and then filter sterilise the solution.

Note that the solution should not be autoclaved as precipitation occurs.

Denhardts 50x

PVP 0.2 g

BSA 0.2 g

Ficoll 400 0.2 g

Add autoclaved DEPC water to 20ml.Filter sterilise through 0.22 um filter.

Store in aliquots at –20 °C.

1.0 M DTT

DTT 1.54 g

Make up to 10ml with Autoclaved DEPC water

Filter sterilise through 0.22 um filter

Store in aliquots at –20 °C

tRNA 10mg/ml

Make up the whole vial to the required concentration with autoclaved DEPC water.

Filter sterilise through 0.22 um filter.

Store in aliquots at –20 °C

Dextran Sulphate 50%

Use only Pharmacia brand

Dextran sulphate 10 g

Make up to 20 ml with autoclaved DEPC water.

Heat to help dissolve – very viscous solution!

Filter through 0.22 um filter

Store in aliquots at –20 °C

Rnase A 20 mg/ml

To the little vial of unopened RnaseA (100 mg), add 5 ml of sterile DEPC milliQ.

Vortex, filter sterilise through 0.22 filter and pipette aliquots of 110ul.

Cap and boil for 2 min in boiling water bath.

Cool to room temperature and freeze at –20 °C

0.3 Ammonium Acetate / Ethanol solutions

For 500 ml per solution;

30% Ethanol = 20 ml of 7.5 M Ammonium Acetate + 150 ml Ethanol , and make up to 500 ml with Autoclaved DEPC water

60% Ethanol = 20 ml of 7.5 M Ammonium Acetate + 300 ml Ethanol , and make up to 500 ml with Autoclaved DEPC water

80% Ethanol = 2 0ml of 7.5 M Ammonium Acetate + 400 ml Ethanol , and make up to 500 ml with Autoclaved DEPC water

95% Ethanol = 20 ml of 7.5 M Ammonium Acetate + 475 ml Ethanol , and make up to 500 ml with Autoclaved DEPC water

1% Acetic Acid

1 Litre of MilliQ water + 10 ml of glacial acetic acid

Scotts Tap Water

Potassium Bicarbonate (Potassium hydrogen carbonate)	2.0 g
Magnesium Sulphate ($7H_20$)	20.0 g
Formalin as a preservative	1.0 ml

Make up to 1000 ml with distilled water

Gills Haematoxylin

Haematoxylin	6.0 g
Distilled H20	690 ml
Ethanediol	25 0ml
Sodium Iodate	0.6 g
Aluminium Sulphate	52.8 g

Dissolve all solids

Add 60 ml Glacial Acetic Acid

Leave on windowsill for at least 1 month before using.

Equilibration Buffer

For	200ml	1L
NaCl 5 M	12 ml	60 ml
1 M Tris pH 6.8	2 ml	10 ml
0.5 M NaP pH 6.8	4 ml	20 ml
0.5 M EDTA pH 8.0	2 ml	10 ml
Formamide	100 ml	500 ml
Sterile water	80 ml	400 ml

Ilford Fixer - 5 L

Dissolve contents of Ilford Ilfofix II in 3750 ml unautoclaved MQ water, stir.

Make up to 5 L. Filter through 2 pieces of Whatman filter paper no:1 into 2 x 2.5 L dark glass bottles. Store at 4 °C.

Kodak Developer D-19, 3.8L

Heat 3.8 L unautoclaved MQ water to 52 °C. With stirring, slowly add the developer until all dissolved. Filter through 2 pieces of Whatman filter paper no:1 into 2 x 2.5 L dark glass bottles

Solutions for sample preparation & protein extraction

All buffers and solutions are to be made with nanopure (MilliQ) water. All solutions can be stored at ambient temperature unless stated otherwise.

* Use up to 2% ampholytes for IEF with Multiphor. For IEF using the IPGphor, use only 0.5%.

** In this case, the author used the Multiphor for IEF. Reduce IPG Buffer to 0.5% when using IPGphor.

*** Add 1 Roche's Complete Mini – EDTA Free Protease Cocktail Inhibitor tablet (Cat# 1836170) per 50ml solution. Alternatively, use 500µL Calbiochem Protease Inhibitor Cocktail Set III (Cat# 539134) per 50ml (this is a 1 / 100 dilution).

**** Add DTT only prior to using the Lysis Buffer.

Rehydration Solution	
	Per 4ml
8 M Urea	1.92 g
2% Detergent	0.08 g
0.15% DTT	6 mg (1.5 mg per 1 ml aliquot)
2% ampholytes *	- - (See note below)
Bromophenol Blue	8 µL of 1% stock

This solution can be made fresh on the day of use or stored frozen (max temperature – 20 °C) in 0.5 ml aliquots. Make up to 4 ml using an accurate measuring system. Storage

best not exceed 3 months. DTT should only be added prior to strip rehydration, as DTT tends to break down forming cyanate ions that interacts with protein amino groups. Add ampholytes in 2% of total Rehydration Solution + protein sample volume, not of the actual volume of Rehydration Solution alone.

Modified Rehydration Solution III	
	Per 4 ml
7M Urea	1.68 g
2M Thiourea	0.60896 g
2% Detergent	0.08 g
0.15% DTT	6 mg (1.5 mg per 1 ml aliquot)
0.5% ampholytes	- - (0.5% of total volume)
Bromophenol Blue	8 µL of 1% stock

This method was obtained from Sjourke Hoving et. al. 2002 (Proteomics Vol 2; p127 – 134), which was adapted and modified specifically to improve IEF of pH 6 – 11 basic, narrow range strips. The author uses the Multiphor. Cup loading applied on the anodic end. At the cathodic end, the paper wick was immersed in the aforementioned modified buffer containing 3.5% DTT (i.e.: 140mg per 4ml aliquot) but omitting the IPG buffer.

Sample Solution	
	Per 10 ml
8M Urea	4.8 g
3M Thiourea	2.3 g
4% Detergent	0.4 g
65mM DTT	0.1 g (0.01 g per 1 ml aliquot)

As with previous solutions, dissolve using sonication, in minimal amount of liquid before making up to desired volume. To be stored frozen in 1ml aliquots for up to 3 months. Add 0.01g DTT to each aliquot prior to use. Another modified version of the Sample Solution contains 2M Thiourea, used commonly in AgResearch proteomics lab.

Bromophenol Blue 1% Stock Solution	
	Per 10 ml
Bromophenol Blue	100 mg
Tris – Base	60 mg

Make up to 10 ml.

10mM PBS Solution	
	Per 500 ml
$Na_2HPO_4 : 2H_2O$	0.53397 g
$Na_2H_2PO_4 : 2H_2O$	0.46803 g

0.1M Na_2CO_3	
	Per 500 ml
Na_2CO_3	5.2995 g
For Sodium Carbonate washing	

10% TCA in Acetone	
	Per 100 ml Acetone
10% TCA	10 g

Caution: TCA is corrosive. Avoid contact with skin.

Dissolve in Acetone and store solution in – 20°C.

Solutions for equilibration of focussed IPG Strips

All buffers and solutions are to be made with nanopure (MilliQ) water. All solutions can be stored at ambient temperature unless stated otherwise.

50 mM Tris – HCL Stock Solution	
	Per 100 ml
Tris	0.61 g

Dissolve Tris in a third of the total volume and adjust pH to 6.8 with concentrated HCL acid. Make up to 5 0ml and check pH.

Equilibration Solution	
	Per 500 ml
50mM Tris – HCL Stock Solution	50 ml
Urea	181.8 g
Glycerol	150 ml
SDS	10 g

Make up to 500 ml and store frozen in 10 ml aliquots.

Equilibration Solution #1

Equilibration Solution

1% DTT [w/v]; (i.e.: 0.1g per 10 ml). Add DTT prior to strip equilibration

Equilibration Solution #2

Equilibration Solution

2.5% Iodoacetamide [w/v]; (i.e.: 0.25g per 10 ml). Add Iodoacetamide prior to use.

SDS PAGE solutions

All buffers and solutions are to be made with nanopure (MilliQ) water. All solutions can be stored at ambient temperature unless stated otherwise.

4 × SDS Buffer	
	Per 9.5 ml
1M Tris – HCL (pH 6.8)	2.5 ml
Glycerol	4 ml
SDS	0.8 g
1% Bromophenol Blue Stock	200 µl
ddH_2O	2.8 ml
400mM DTT *	--

Store frozen in 475 µl aliquots.

* Before using, add 25 µl of 8M DTT stock to give 400 mM DTT and a 500 µL total in volume.

8M DTT Stock Solution	
	Per 200 µl
DTT	247 mg

Calculation based on DTT $_{MW}$ of 154.2; 1233.6 g per litre. Store frozen. Discard unused solution within a week.

1M Tris (pH 6.8) Stock Solution	
	Per 100 ml
Tris	12.11 g

Dissolve in 80ml and adjust pH to 6.8 before topping up to 100ml with an accurate measuring cylinder. Re-check pH again.

10% Ammonium persulphate solution	
	Per 1 ml
Ammonium persulphate	0.1 g

Made fresh on the day it is required and can be stored at 4 °C up to 3 days. Fresh chemical should crackle when water is added.

3 M Tris Stock Buffer (pH 8.8)	
	Per 100 ml
Tris	26.12 g
Tris – HCL	13.26 g

Check pH after dissolving.

1.5 M Tris – HCL Stock Solution	
	Per 200 ml
Tris – HCL	47.28 g

1M Tris – HCL Buffer (pH 6.8)	
	Per 100 ml
Tris	12.11 g

Adjust pH as per usual.

50% Glycerol	
	Per 100 ml
Glycerol	50 ml
Water	50 ml

Resolving (Separating) Gel Buffer (1.5 M Tris – HCL, pH 8.8)	
	Per 300 ml
Tris	54.5g

Dissolve Tris in 150ml water and add up to 81ml of 1.5M Tris – HCL stock. Continue to adjust the pH using the Tris – HCL before making up to 300ml with water. Check pH after addition of water.

Stacking Gel Buffer (0.5M Tris – HCL, pH 6.8)	
	Per 100 ml
Tris	6 g

Dissolve Tris in 60 ml water and adjust pH to 6.8 with concentrated HCL. Make up to 100 ml with a measuring cylinder. Check the pH again and store in – 4 °C or ambient temperature.

10% SDS	
	Per 100 ml
SDS	10 g

Overlay Solution	
	Per 100 ml
SDS	0.1 g

0.5% Agarose Sealing Solution	
	Per 10 ml
Agarose (electrophoresis quality)	0.05 g
1 × Electrophoresis Tank Buffer	10 ml
1% Bromophenol Blue Stock solution	20 µL

Store in 2 ml aliquots and re-dissolve at 100 °C.

15% Resolving gel casting mix	
	Per 2 × 15% mini gels / 1 × 20×20cm gel
30% Acrylamide stock	15 ml
Resolving gel buffer	7.5 ml
10% SDS	300 µl
Nanopure water	7 ml
TEMED**	15 µl
10% Ammonium persulphate**	150 µl

**Degas the solution for 20 min before adding TEMED and 10% Ammonium persulphate solution. Work quickly after adding the solutions.

Add Overlay solution or 70% ethanol over the top layer to cut off solution contact with oxygen and allow for polymerization.

12% Resolving gel casting mix	
	Per 2 × BioRad Protean II xi large, *1DE* format, wide spacers, 1.5 mm
30% Acrylamide stock	40 ml
Resolving gel buffer	25 ml
10% SDS	1 ml
Nanopure water	33 ml
TEMED**	40 µl
10% Ammonium persulphate**	400 µl

4% Stacking gel casting mix	
	Per 2 × BioRad Protean II xi; Large, 1DE format, wide spacers, 1.5 mm
30% Acrylamide stock	3.9 ml
Stacking gel buffer	7.5 ml

10% SDS	300 µl
Nanopure water	18.3 ml
TEMED**	30 µl
10% Ammonium persulphate**	150 µl

This stacking layer is important for 1D electrophoresis system and enables proteins to better enter the resolving gel layer. Ensure the pH of the stacking buffer is accurate.

Tricine Gel System

All buffers and solutions are to be made with nanopure (MilliQ) water. All solutions can be stored at ambient temperature unless stated otherwise.

5 × Anode Buffer (Lower tank buffer; pH 8.9)	Inaccurate pH; pH ≈ 9 – 10
	Per Litre
Tris – HCL	30.4 g
Tris (base)	97.7 g

Add water to a final volume of a litre. When the solution is diluted to 1 ×, the pH should be about 8.9. To dilute, add 600ml of 5× to 2400ml of water per BioRad Protean II tank or 2 of Amersham miniVE electrophoresis tanks (note: miniVE tanks need about 2 litre for complete topping up).

1 × Anode Buffer (Schägger's; pH 8.9)	
0.2 M Tris	Correct to pH 8.9 using HCL

10 × Anode Buffer (Recommended recipe)	
	Per Litre
2M Tris (base)	242.28 g
	Per Litre
2 M Tris – HCL	315.2 g

Add an equal amount of both solutions for dilution to 1 ×. Before adding ddH_2O to total final amount, measure pH and adjust using 1 × Tris – HCL until pH is 8.9

5 × Cathode Buffer (pH 8.25)	
	Per Litre
Tris (base)	60.6 g
Tricine	89.6 g
SDS	5 g

Do not adjust pH. When diluted to 1×, the pH should be about 8.25

1 × Cathode (Schägger's; pH 8.25)	
0.1 M Tris	Do not correct pH
0.1 M Tricine	
0.1% SDS	

Gel Buffer (pH 8.45)	
	Per 100 ml
3 M Tris	36.34 g
0.3% SDS	0.3 g

Dissolve chemicals in about 70 – 80 ml water and adjust pH to 8.45 using HCL. Add up to 100 ml and check pH again.

Bis – Acrylamide solution (49.5% T, 3% C)	
	Per 100 ml
Acrylamide	48 g
Bis	1.5 g

Add up to 100 ml water and dissolve. Filter solution using a no. 4, or finer, filter paper. Store in 4°C, wrapped in foil.

	4%	10%	20%
Bis – Acrylamide solution	1 ml	6.1 ml	12.12 ml
Gel Buffer	3.1 ml	10 ml	10 ml
Glycerol	-	4 ml	4 ml
ddH2O	8.36 ml	9.86 ml	3.84 ml
TEMED	10 µl	10 µl	10 µl
10% Ammonium persulphate	30 µl	30 µl	30 µl

Allow the 10% & 20% resolving layer to solidify, at most, overnight before casting the 4% stacking layer.

Solutions for peptide extraction, Zip – Tip and MALDI – TOF analysis

Chemicals used for making these solutions must always be of the highest grade in purity and quality (HPLC grade or "pro analysis"). All buffers and solutions are to be made with nanopure (MilliQ) water. All solutions can be stored at ambient temperature unless stated otherwise.

100 mM Ammonium bicarbonate (pH 8.8)	
	Per 100 ml
$(NH_4)HCO_3$	

For 50mM Ammonium bicarbonate, simply dissolve 50:50 with water.

0.2% TFA	
	1 ml
TFA	2 µL

Caution: TFA vapour is poisonous & noxiously pungent. Use in fume cupboard!

Trypsin (2.5 µg final concentration)

For a 25 µg vial, dissolve trypsin in 50 µl of MQ distilled water.

Aliquot 5 µl per Eppendorf tube and SpeedVac to lyophilize the trypsin solution.

Store at 4 °C and use within 3 – 6 months.

Eluting Solution

100% ACN / 0.2% TFA in 70:30 ratios

This solution is used for eluting peptides immediately after Zip Tip / reconstituting lyophilized peptides for spotting MALDI plate. This helps to avoid over– saturation and clumping of matrix crystals on the plate. Basic formula: 1 µl Elution Solution + peptide + 1 µlmatrix solution.

Other solutions normally used:

100% ACN

100% ACN / 0.2% TFA in 50:50 (& various) ratio

MALDI Matrix Solutions	
CHCA in ACN / 0.2% TFA (50:50)	= *For up to 3000 Da*
	Per 1 ml
CHCA♠	10 – 20 mg

CHCA in IFW (Isopropanol : Formic : water, 2:1:3)	= *For up to 4000 Da*
	Per 1 ml
CHCA♠	10 – 20 mg
MQ ddH_2O	450 µl
Isopropanol	300 µl
Formic Acid	150 µl
Sinapinic Acid in 100% ACN / 0.2% TFA (50:50)	= ***For larger peptides***
	Per 1 ml
Sinapinic Acid♠	10 – 15 mg

♠ Keep to the middle of the weight range in order to avoid over – saturation and clumping of crystals on the MALDI plate.

Sonicate crystals in the solution for 5 min before vortexing for a minimum of 30 min.

Prior to use, spin down crystals at 10,000 RPM for 5 min. Use only the supernatant

Staining & destaining solutions (including methods)

All buffers and solutions are to be made with nanopure (MilliQ) water. All solutions can be stored at ambient temperature unless stated otherwise. Percentages of chemicals are stated as the final concentration in the staining / destaining solutions.

Coomassie Blue R – 250 stain	
	Per L
0.025% Coomassie R – 250 [w/v]	250 mg
40% Methanol [v/v]	400 ml
7% Acetic acid [v/v]	70 ml
53% water [v/v]	530 ml

This stain can be reused until staining power wears out. Preferably stain gels overnight.

Destain solution for R - 250
40% Methanol [v/v]
7% Acetic acid [v/v]
53% water [v/v]

Colloidal Coomassie Blue G – 250 stain	
	Per L
0.1% Coomassie G – 250 [w/v]	1 g
34% Methanol [v/v]	340 ml
17% Ammonium sulphate [w/v]	170 g
2% Phosphoric acid [v/v]	20 ml

Make fresh when needed. Fix the gel in 50% Ethanol / 2% Phosphoric acid overnight. The next day, wash the gel in 3 changes of cold water (30 min intervals) and stain the gels in G – 250 for approximately 4 days maximum.

Sypro® Ruby stain
Fix Solution [v/v] 50% Methanol, 7% Acetic acid
Fix Solution for IEF gels [v/v] 40% Methanol, 10% Trichloroacetic acid
Wash Solution [v/v] 10% Methanol, 7% Acetic acid

Fix gel in Fix Solution for 3 hours. Ensure container has been thoroughly rinsed with ethanol and MQ water before staining so as to avoid elevated background levels.

Wash in 3 washes, 10 min each time, in ultrapure water.

Stain overnight in Sypro Ruby gel stain.

Transfer gel to clean container.

Wash in Wash Solution for 30 min.

Rinse gels in ultrapure water twice (5 min each time) before scanning.