Follicular growth and atresia in the mare

Hanne Gervi Pedersen

Doctor of Philosophy - The University of Edinburgh - 2000

Declaration

I hereby declare that this thesis has been prepared by myself and has not been submitted for any other degree elsewhere. The work presented herein is my own with the exception of testosterone and progesterone assays, which were performed by Sheila Thomson and preparation of sections for histology, which was done by John Binnie. All work of other authors is duly acknowledged.

Hanne Gervi Pedersen

Table of Contents

Acknowledgements

I would like to thank first of all my supervisors, Prof. Elaine Watson and Dr. Evelyn Telfer, for making it possible for me to complete this thesis. Their continuous help, advice and encouragement have been invaluable and much appreciated. My oocyte guru, Katrin Hinrichs, USA, has functioned as a shadow supervisor, providing good criticism of my theories.

Thank you to the many people who helped out in various ways: Sheila Thomson and John Binnie for a lot of help in the lab; Michael Jakobsen, Orestis Papasouliotis and Dennis Fielding for advice on the statistics; Michael the Genius for creating mathematical programs for the follicle maps; the anaesthetists (Eddie, Pamela, Oliver, Kathleen and Karen) for getting up early in the morning for the ovariectomies; Dave, Mo and June for taking good care of the mares; Hinke Ten Hulscher, Tove Bjorksten and Niamh Collins for being excellent assistants over the busy summers.

My stay at Bush was made very enjoyable by the flow of people through the "Tower of Babel" house – Constanze Fintl, Patricia Touw, George Caldow, Antonio Miguel Hinojosa, Blanca Berrocal, Susanne Weber and many others.

A special thank you to Mark Dagleish, who provided me with advice on just about everything in life.

I especially wish to thank the best family in the universe, Annelise, Boye and Per, for always being there for me and for being immensely patient and accepting and encouraging my pursuit of dreams wherever in the world I have made them come true.

Finally, thank you to Michael for sailing through oceans of euphoria, dark depression, elation and hysteria without breaking any masts. Thanks for the long sessions of sorting out my thesis and the ropes on the mountains.

Publications arising from the thesis

Refereed paper

Pedersen HG, Watson ED and Telfer EE (2000) Apoptosis in granulosa cells and the relation to cumulus expansion and oocyte chromatin configuration in ovarian follicles. Journal of Reproduction and Fertility, Suppl 2000; 55: (In Press).

Conference presentations

Pedersen HG, Watson ED and Telfer EE (1998) Apoptosis in granulosa cells and the relation to cumulus expansion and oocyte chromatin configuration in ovarian follicles. Proceedings of 7th International Symposium on Equine Reproduction, Pretoria, abstract p. 121.

Pedersen HG, Watson ED and Telfer EE (1998) Apoptosis in granulosa cells and the relation to cumulus expansion and oocyte chromatin configuration in ovarian follicles. Journal of Reproduction and Fertility, abstract series, 21, 5.

Pedersen HG, Telfer EE and Watson ED (1999) Follicular granulosa cell apoptosis on day 6, day 11, day 15 and during oestrus in the mare. Journal of Reproduction and Fertility, abstract series, 24, 13.

Pedersen HG, Telfer EE and Watson ED (2000) Effect of temperature and holding time on equine oocyte chromatin configuration and cumulus morphology. Proceedings of Equine Embryo Transfer, EET V, Helsinki, 2000, abstract p. 17.

Pedersen HG, Telfer EE and Watson ED (2000) Holding temperature and time affect equine oocyte chromatin configuration and cumulus morphology. Journal of Reproduction and Fertility, abstract series, 25, 4.

Conference poster

Pedersen HG, Telfer EE and Watson ED (2000) Follicular granulosa cell apoptosis through the equine oestrous cycle. 14th International Congress on Animal Reproduction, Stockholm, abstract 1:55.

Abstract

The growth and atresia in equine follicles has received little attention although the mare is a unique model because of the size of the animal, the size of the ovarian follicles and the unusual ovarian anatomy. The size of the animal allows ultrasound scanning to monitor follicle dynamics in vivo and the size of each follicle allows for several parameters to be studied without having to pool follicles together and thereby potentially introducing errors. Several studies in the equine species have focused on follicle dynamics, others on atresia and follicular fluid hormone levels and yet others on the oocyte, but none have combined all of these areas. The aims of this thesis were to establish whether granulosa cells of regressing equine follicles degenerate via an apoptotic mechanism and whether the presence of apoptotic cell death in granulosa cells was correlated with other indices of follicle health such as steroid hormone levels and oocyte quality.

At first each parameter was investigated in detail at a microscopic level: oocyte chromatin configuration, cumulus morphology, follicle wall atresia, granulosa cell apoptosis and steroid content in follicular fluid. Then followed a whole animal approach with monitoring of follicular growth via ultrasonography. In the end this information was put together when studying ovaries recovered at different stages of the cycle in order to reveal when selection took place and how this was reflected in the parameters of the individual follicle.

The temperature at which the ovaries were held did not significantly affect ($P > 0.05$) chromatin configuration in oocytes ($n = 73$), but the length of time at which the ovaries were held, did significantly change chromatin configurations ($n = 222$) after 6 hours ($P < 0.001$). Health status of the follicle appeared to affect chromatin configuration ($n = 118$), but this failed to reach significance ($P = 0.08$). Oocyte chromatin configuration tended to change from even distribution within the germinal vesicle, through different degrees of condensation, to metaphase stages with increasing apoptosis or atresia of the follicle. The puzzling configuration condensed chromatin may be part of the normal development but may also be an end-stage chromatin configuration as it tended to be most frequent in atretic follicles and increased in frequency after long storage in the ovary. The relationship between cumulus morphology and chromatin configuration ($n = 149$) was evident: compact cumulus morphology was associated with immature oocyte chromatin configurations and fully

expanded cumuli were correlated with metaphase chromatin ($P < 0.001$). Each sequential step of cumulus expansion represented a further step towards meiotic maturation.

Evaluation of follicle health based on granulosa cell morphology immediately upon retrieval, follicular wall atresia and apoptosis showed that each of the methods to detect atresia and apoptosis had advantages and limitations and that it was necessary to use the methods in conjunction because they supplemented each other. Histological atresia and ethidium bromide detection of apoptosis ($n = 20$) was significantly correlated ($P < 0.001$), and the stereomicroscopic evaluation of freshly isolated granulosa cells were significantly correlated ($P < 0.001$) with the histological findings ($n = 33$). Apoptotic granulosa cells and theca cells were detected histologically, a phenomenon which has not previously been described in equine follicles.

In a study with 293 follicles from 23 mares, it was found that apoptosis was not a definite measure of death, because growing follicles, as determined by ultrasonography, could be apoptotic and have no other signs of impending death of the follicle, although apoptosis generally was associated with atresia of the follicle ($P < 0.001$). There was a population of small, atretic follicles with expanded cumuli and oocytes with chromatin progressed towards meiosis, and a population of large, healthy follicles, with young oocyte configurations and no cumulus expansion, and finally a population of follicles larger than 30 mm, which were healthy, but had started to move towards maturation in vivo, i.e. getting ready for ovulation. Size of the follicle appeared to be more important than stage of cycle for predicting chromatin configuration, cumulus morphology and atresia and apoptosis, but size of follicle was correlated with stage of cycle. This led to the theory that despite the larger follicles having stopped growing according to the follicle maps made from ultrasound scans, regression may not initially be related to detectable apoptosis, and that overt apoptosis may be a later event. The fact that most atretic follicles were small suggested that follicles regressed and slowly became overtly atretic and contained low levels of oestradiol and degenerate oocytes surrounded by expanded cumuli.

When used in combination with plasma hormone levels, daily mapping of follicles in 7 ponies and 5 mares throughout the cycle made it possible to detect days of changes in follicular growth both of the individual large follicle and smaller follicles in groups in relation to hormone changes. The growth of the largest follicle to a point where it was capable of producing sufficient oestradiol, resulted in decreasing levels of FSH. The

decreasing levels of FSH caused the smaller follicles to regress. The increased oestradiol concentrations promoted increased LH production as well as partaking in luteolysis, with resulting further increased levels of LH due to lack of negative feed back from progesterone. When the inhibiting effect of oestradiol disappeared just before ovulation, FSH started to increase, resulting in growth of a new wave of follicles, emerging on day 6 ($P < 0.01$). Divergence in growth rates between the dominant follicle and the largest subordinate follicle happened on day 9 and this became significant on day 12 of the cycle ($P < 0.05$). At this point FSH levels were high, LH was low and progesterone was still produced. The cause of deviation in this study remains unknown. The subordinate follicles grew until day 17 – 18, at which point they started to regress. The follicle maps were a reliable method of determining growth and regression, when keeping in mind that there are influences from regressing, atretic follicles during periods of general growth and that monitoring of the individual small follicle is not possible when there are many follicles.

In conclusion this thesis demonstrated that oocyte chromatin configuration, cumulus morphology, follicular fluid steroid concentration and granulosa cell health are parameters, which change with follicle health, and that ultrasound scanning is an important and necessary tool to use in conjunction with the above measurements when studying follicle growth, selection and regression. Death of follicles happens gradually, and selection for dominance is a gradual process, where one follicle gains advantage over the competitors, resulting in dominance at an early stage during the cycle.

List of abbreviations

CC: condensed chromatin

CP: compact cumulus cells

DAPI: 4',6-diamidine-2'-phenylindole dihydrochloride

DNA: deoxyribonucleic acid

E2: oestradiol

EDTA: ethylene diamine tetraacetic acid

EX: expanded cumulus cells

FN: fluorescent nucleus

F ex: fully expanded cumulus cells

FSH: follicle stimulating hormone

GnRH: gonadotrophin releasing hormone

GVBD: germinal vesicle breakdown

H&E: haematoxylin and eosin

LCC: loosely condensed chromatin

LH: luteinising hormone

M199: medium 199

M ex: moderately expanded cumulus cells

P4: progesterone

SFN: spotty fluorescent nucleus

Sl ex: slightly expanded cumulus cells

T: testosterone

UV: ultra violet

V ex: very expanded cumulus cells

1 Literature review

1.1 The oestrous cycle of the mare

In a paper from 1926, W.A. Aitken (Aitken, 1926) pointed out how little research had been done and how little was known about the mare's oestrous cycle. At that time as now, people drew on knowledge from more well-studied species like the cow and sow, mentioning that this is often misleading because of the mare's unique reproductive features. Aitken palpated several mares per rectum over 8-13 months as well as examining material retrieved at ovariectomy and post mortem. He found that the mare's oestrous cycle lasts for 20-25 days with the mare being in oestrus for 7 days, and with great differences between individual mares. With the later use of ultrasound scanners and hormone assays, it is known that the cycle lasts for on average 19.2 – 27.5 days depending on type of horse and time of year (Ginther, 1974; Sirois *et al.*, 1989). The cycle consists of the follicular phase with the development of usually one preovulatory follicle and increased oestradiol secretion, and the luteal phase with the development of the corpus luteum and progesterone production. The length of oestrus is on average 7 – 8 days and the length of dioestrus is 15 – 16 days (Back *et al.*, 1974; Ginther, 1992b). Ovulation occurred within the last two days of the mares showing oestrous behaviour in 69% of animals (Ginther, 1992b). The day of ovulation is defined as day 0.

The mare is seasonally polyoestral, with a phase of anoestrus lasting from November until February, when spring transition starts. Spring transition lasts for 1-2 months with multiple follicles growing and regressing, but none ovulating. From March - May in the Northern Hemisphere, depending on latitude, mares will cycle regularly until October-November. If the mares are bred and become pregnant, the pregnancy lasts for on average 335-342 days (McKinnon *et al.*, 1993). Approximately 7 days after foaling, the mares will come into oestrus and ovulate, and in theory the mare can have a foal per year.

1.1.1 Pony mares versus horse mares

In a study by Driancourt *et al.* (1982b), no difference between pony and horse mares was found with regard to mean number of primordial follicles, mean number of growing follicles, the distribution into various size classes, or in atresia in these follicles. Pony mares' cycles are on average 25 days versus 22.7 days in horse mares. The length of the breeding season is

shorter in ponies with real anoestrus more often seen in ponies than in horses. Occurrence of multiple ovulations is more frequent in horse mares than in pony mares (Ginther, 1974).

1.2 Hormones regulating the oestrous cycle

1.2.1 Gonadotrophin Releasing Hormone

1.2.1.1 Release and regulation of GnRH

Circulating levels of GnRH are not detectable in the mare, but cannulation of the pituitary vein has allowed study of GnRH output (Irvine *et al.*, 1987). Equine GnRH is released in a pulsatile as well as in a continuous manner from the axons in the hypothalamus and is carried via the hypothalamic-pituitary portal system to the anterior pituitary gland where it binds to receptors, which triggers the release of LH and FSH (Irvine *et al.*, 1993 and 1995),. The mechanisms of release of GnRH involve interactions between neurotransmitters and neuroendocrine tissue (Naor *et al.*, 1981). The effect of GnRH on the uterus and ovary of horses has not been studied, but in rats GnRH has a direct effect on uterus and the ovaries (Rippel *et al.*, 1976).

The GnRH pulse frequency is higher near ovulation. Pulse frequency was one pulse per 2 hours early in the LH surge and one pulse per half-hour at the time of ovulation. During the periovulatory period, secretion of GnRH, LH and FSH occurred continuously with synchronous pulses superimposed on the tonic background (Alexander *et al.*, 1987b). About 90% of GnRH pulses in the mare are followed by gonadotrophin pulses and it appears that synchrony of pulses among the hormones occurs throughout the oestrous cycle (Alexander *et al.*, 1987b). In many species including the mare, FSH and LH are secreted differentially at various points in the ovulatory cycle. The FSH and LH pulses are synchronous, but the amplitude varies (Irvine *et al.*, 1998). Modulation of pituitary responsiveness to GnRH is caused by ovarian hormones and is partly the explanation for the differential regulation of the gonadotrophins. In the mare the differential secretion of FSH and LH is marked early in the periovulatory period, with FSH concentrations declining to their lowest point in the cycle whilst LH concentrations rise to form the ovulatory surge, and at mid-dioestrus, when only FSH increases (Evans *et al.*, 1975). Rapid GnRH pulses favour LH synthesis and secretion, whereas slow GnRH pulses favour FSH synthesis and secretion (Irvine *et al.*, 1998).

Ovarian hormones (inhibin, oestrogens, progesterone) have a negative feedback effect on GnRH, either directly on the hypothalamus or via other centres in the brain, or by regulating the release of gonadotrophins from the pituitary gland. The negative feed back on the release of gonadotrophins does not necessarily have a negative effect on the synthesis of gonadotrophins (Thompson *et al.*, 1983; Garza *et al.*, 1986a). These can be stored and released in a surge when GnRH is released. In ewes, an oestradiol-induced LH surge was associated with a decrease in GnRH mRNA expression, which occurred in advance of the onset of the GnRH surge. This suggested that neural mechanisms controlling GnRH biosynthesis may be distinct from those regulating the GnRH secretion (Harris *et al.*, 1998).

1.2.2 Luteinising Hormone

Equine LH is polymorphic with isoforms with different sialic acid content, which affects their half-life and bioactivity. The half-lives of LH isoforms range from 2-5 hours. The circulating forms appear to vary during the ovulatory cycle (Irvine *et al.*, 1997).

1.2.2.1 Function of LH:

LH is involved in the regulation of follicular growth, steroid secretion and luteinisation of the follicle. In the cow the early stages of follicle development (follicles less than 4 mm) are not dependent on gonadotrophins, but LH pulses are necessary for follicle development beyond 9 mm in diameter (Gong *et al.*, 1996). LH affects the secretion of steroids by the preovulatory follicle and the corpus luteum, because LH sustains steroidogenic enzyme activity. In some species oestradiol induces LH receptor formation on the granulosa cells after which the follicle develops to the preovulatory stage (Gore-Langton *et al.*, 1994). Under the influence of LH, the theca cells produce dehydroepiandrosterone and androstenedione. Androstenedione diffuses into the granulosa cells and is aromatised to oestradiol-17B in horses (Sirois *et al.*, 1991). Oestrogen secretion by the follicles increases during the second half of the follicular phase when the frequency of LH pulses increase and FSH secretion declines in humans (Hillier, 1994). In the pig, LH enhanced relaxin secretion by granulosa cells from large preovulatory follicles in vitro (Loeken *et al.*, 1983).

The preovulatory rise in LH causes ovulation, formation of corpus luteum and the onset of luteal progesterone production. In pigs, LH and FSH stimulated granulosa cell progesterone secretion and induced morphological changes associated with luteinisation in vitro (Thanki *et al.*, 1976). The corpus luteum is dependent on LH for progesterone production in the

macaque, (Fraser *et al.*, 1985), human (Mais *et al.*, 1986) and cow (Peters *et al.*, 1994). Mares that had antiserum against LH and FSH during oestrus, failed to ovulate and if given during dioestrus, the corpus luteum decreased in weight (Pineda *et al.*, 1972a and 1972b). In the mare, antral follicles contain LH receptors on the theca interna and granulosa cells and FSH receptors in the granulosa cell membrane (Fay *et al.*, 1987). In mares mRNA for LH receptors were found initially only in the theca cell layer of follicles larger than 8 mm, but from day 12, the largest follicles' granulosa cell layer also expressed LH mRNA, and this expression increased in the preovulatory follicle (Lawler *et al.*, 1998).

1.2.2.2 Concentrations of LH during the cycle

The ovulatory mechanism in the mare is different from other species because there is no abrupt surge of LH from the pituitary gland. The LH surge in cattle lasts 8-12 hours whereas the mare's surge lasts for almost a week. LH pulse amplitude declines between day 4 and day 10 (Irvine *et al.*, 1998). Concentrations increase progressively after luteolysis, reach a maximum 1 - 3 days after ovulation and then diminish to dioestrous levels over the following 4 - 6 days (Whitmore *et al.*, 1973; Irvine *et al.*, 1994). Pulses of LH occur regularly between day 4 and day 12 with a mean frequency of 1.6 pulses day (Irvine *et al.*, 1998). The slow postovulatory fall is partly caused by continued secretion of LH, but the long circulatory half-life of eLH of 2-5 hours may also contribute (Irvine *et al.*, 1997). In the ewe the half-life is 30 - 40 minutes (Robertson *et al.*, 1991). Another reason for the long LH surge could be that immunoreactive forms of LH appear at different times during oestrus. Different isoforms of LH have been isolated at different times during the cycle in the mare (Adams *et al.*, 1986; Pantke *et al.*, 1991). The bioactive forms reach a maximum before ovulation as in other species, but both bioactive and immunoactive forms are present after the peak (Alexander *et al.*, 1982). An indication of LH being bioactive during the whole surge is the occurrence of dioestrous ovulations with the second ovulation occurring 2-6 days after the first ovulation (Ginther, 1992b).

1.2.2.3 Regulation of LH

Oestradiol stimulated and progesterone decreased secretion of LH in ovariectomised mares (Garcia & Ginther, 1978; Thompson, Jr. 1991). Administration of dihydrotestosterone to ovariectomised mares did not suppress LH concentrations (Garza *et al.*, 1985), but testosterone propionate did (Garza *et al.*, 1989). In intact mares, oestradiol treatment caused increased LH levels after regression of the corpus luteum (Burns & Douglas, 1981), but

progesterone and oestradiol treatment in combination also caused decreasing concentrations of LH (Garcia & Ginther, 1978; Evans *et al.*, 1982). LH concentrations and biological efficiency was stimulated by administration of oestrogen to ovariectomised horses, whereas FSH concentrations were suppressed (Garza, Jr. 1986b; Sharp *et al.*, 1991). Oestradiol and GnRH interact to create isoforms of LH with biological effect in the mare (Pantke *et al.*, 1991). LH pulse frequency is directly related to blood concentrations of progesterone in horses (Evans *et al.*, 1975; Irvine *et al.*, 1997). The negative feedback effect of progesterone on LH appears to involve a central site of action because gonadotrophin and GnRH pulses are slowed by progesterone (Fitzgerald *et al.*, 1993a; Irvine *et al.*, 1997). Oestradiol seems to work on the hypothalamus rather than the pituitary, as pituitary stalk-sectioned, ovariectomised mares did not have increased levels of either LH or FSH after administration of oestradiol (Porter *et al.*, 1997).

1.2.2.4 Release of LH

The pulsatile release of LH in the mare is caused by the pulsatile release of GnRH from the hypothalamus (Irvine *et al.*, 1987). Pulses of LH in systemic circulation were detectable only during the luteal phase and during the periovulatory phase with the LH surge, the pulses were only detected in pituitary venous blood. Frequency varied from one pulse per two hours early in the LH surge to almost one pulse per half-hour at the time of ovulation (Alexander *et al.*, 1987b). LH pulse amplitude declines as pulse frequency rises. The LH profile during the cycle was similar whether determined by sampling once daily or sampling every 4 hours (Irvine *et al.*, 1998).

1.2.3 Follicle Stimulating Hormone

In the mare, there are several bioactive and immunoactive forms of follicle stimulating hormone (FSH) (Alexander *et al.*, 1987a). In heifers, FSH isoform patterns did not change during the cyclic changes in FSH, although this was the case in hamsters, rats and humans (Cooke *et al.*, 1997). The half-life of FSH was 30 minutes in the ewe (Robertson *et al.*, 1991) but there is no information on the half-life of FSH in mares.

1.2.3.1 Function of FSH

Both FSH and LH are necessary for regulation of follicular development and differentiation from the earliest preantral to preovulatory stages (Wang & Greenwald, 1993) but follicles of

hypophysectomised rats can grow to antral stages despite the absence of gonadotrophins (Hirshfield, 1985). FSH stimulates proliferation of granulosa cells, follicle growth and induces the LH-responsive mechanisms that sustain steroid secretion. Granulosa cells are the only cells that have FSH receptors. FSH is involved in the regulation of P450arom, P450scc, LH-receptor production, IGF, IGFBP, inhibin, activin and follistatin (Hillier, 1994). In cattle, follicular growth from 4 mm up to 9 mm was found to be dependent on FSH after suppression of FSH with a GnRH agonist (Gong *et al.*, 1996).

1.2.3.2 Concentrations of FSH during the cycle

Mean concentrations of FSH were low during oestrus, increased during dioestrus and then decreased approximately 8 days before ovulation. FSH secretion was inhibited during the growth of the dominant follicle, and increased after ovulation (Evans *et al.*, 1975; Bergfelt *et al.*, 1993). The interval between FSH peaks was longer for day 0 than for days 7-9 and day 15 (Evans, 1990). Between days 4 and 12, synchronous pulses of FSH and LH occurred, but pulse amplitude varied independently (Irvine *et al.*, 1998). The number of FSH pulses was on average 1.9 pulses per day (Irvine *et al.*, 1998). Mean concentrations were higher on day 4-5 and day 7-10 than in oestrus, but not all mares studied had this pattern (Irvine *et al.*, 1998). Dioestrus pulses of FSH were slow and of high amplitude (Irvine *et al.*, 1998). A period of high amplitude pulses were detected 10 days before ovulation, which is around the time of the emergence of the ovulatory follicle (Irvine *et al.*, 1998). Evans and Irvine reported the occurrence of 2 or more FSH surges during the cycle. Two broad surges of FSH at 10-12 day intervals were described: one during late oestrus to early dioestrus and the other during late dioestrus with a peak 10-13 days before ovulation (Evans *et al.*, 1975). The profiles of circulating levels of FSH during the cycle apparently exhibited different patterns with 1-3 peaks, often due to surges detected during sampling only once daily. According to (Irvine *et al.*, 1998) this is an insufficient sampling regimen because of the marked pulsatility of FSH during dioestrus. Pituitary content of FSH did not differ due to season, contrary to LH which, during anoestrus is only 15% of the content during the breeding season (Garza, Jr. 1986a).

1.2.3.3 Regulation of FSH

In the mare, FSH is regulated by feedback from ovarian hormones. Inhibin causes decreased FSH synthesis and secretion (Garza, Jr. 1986b; Bergfelt *et al.*, 1991). Exogenous oestradiol and testosterone cause depression in FSH concentrations (Garza, Jr. 1989; Thompson, Jr.

1991). Progesterone decreases GnRH pulse frequency and thereby favours FSH synthesis and release (Thompson, Jr. 1991; Irvine *et al.*, 1997).

1.2.3.4 Release of FSH

In the mare, pulses of FSH and LH often occur together and are sometimes tightly coupled, especially during dioestrus (Thompson, Jr. 1987; Irvine *et al.*, 1993; Irvine *et al.*, 1998). In a study by Evans (1990), duration of FSH peaks was 5.9 minutes and occurred every 15 minutes as detected by sampling every 3 minutes. When pulses are infrequent as in anoestrus, FSH pulses are large and LH pulses relatively small. Synchronous high amplitude FSH and LH pulses occur 0-4 times daily during anoestrus and 1-4 times daily during dioestrus (Irvine, 1995). This pattern of stimulation, which is characteristic of the transitional and luteal phases, causes follicular development but not ovulation. As pulse frequency accelerates due to e.g. a decrease in progesterone, FSH pulse amplitude decreases whereas LH increases. The LH to FSH ratio rises to a maximum before ovulation, when GnRH pulses occur approximately half-hourly (Irvine, 1995). FSH may be more loosely regulated by GnRH than LH, as immunisation against GnRH appeared to reduce FSH concentrations to a lesser degree than LH (Garza *et al.*, 1986a).

1.3 Ovarian morphology

The equine germ cells are located centrally in the ovary in contrast to other species. When the follicle grows, it reaches the ovarian surface but ovulates through the ovulation fossa, ventromedially in the ovary (Aitken, 1926). In order to allow the follicle to expand and for a part of the follicle to migrate to the ovulation fossa, extracellular matrix remodelling is necessary. Matrix metalloproteinases (MMP-1, MMP-9) and tissue inhibitors of MMPs (TIMP-1, TIMP-2, and TIMP-3) have been found to be synthesised by equine stroma cells (Song *et al.*, 1999) and is thought to play a role in the tissue remodelling.

The antral follicle consists of a fluid filled cavity surrounded by layers of granulosa cells in which the oocyte with the cumulus oophorus is located. Surrounding this is the basal lamina and the theca interna and externa. The theca interna is active in androgen, relaxin and prostaglandin production (Hirshfield, 1991). Theca externa is smooth muscle-like and contains muscle actins. In healthy follicles these cells are elongated, but during atresia they contract (Hirshfield, 1991).

1.4 The follicle

1.4.1 Follicle growth

In 1926, Aitken wrote: "It would be interesting to know just how the follicles, which rupture at ovulation, are selected". In 2000 we are still not sure.

The mechanisms of folliculogenesis in domestic animals are not fully understood. There are approximately 35,000 primordial follicles in the adult mare ovary, of which 100 are growing (Driancourt *et al.*, 1982b). The mare follicle develops an antrum and theca cells when about 300 μm in diameter (Kenney *et al.*, 1979). Antral follicular development occurs in waves. Only 1 or 2 of the follicles in a wave will become dominant, grow to the preovulatory size and ovulate. The dominant follicle present at luteolysis will, through oestradiol production, induce the preovulatory rise in LH (Tortonese *et al.*, 1990).

1.4.1.1 Follicle recruitment

As in other species, it is not known how the dormant follicles are selected to grow from the pool of primordial, resting follicles in mares. Follicular development is partly regulated by gonadotrophins with the later follicle stages being acutely dependent on gonadotrophins. Tonic FSH and LH stimulation is necessary for the follicles to reach an immature antral stage (5 mm in humans, 4 mm in cattle) (Hillier, 1994; Gong *et al.*, 1995 and 1996). Recruitment of these follicles for further growth requires additional stimulation by FSH and the rise in FSH stimulates proliferation and functional differentiation of the granulosa cells. Preantral murine follicles cultured in vitro increased in size in response to FSH, but the administration of activin A blocked the effect of FSH, suggesting that activin A, secreted by secondary follicles, causes preantral follicles to become dormant at the preantral stage (Mizunuma *et al.*, 1999). FSH alone will support bovine follicles until 9 mm, where LH becomes necessary for further growth (Gong *et al.*, 1996). The size at which the ovine follicles become dependent on gonadotrophins is 2 mm (Dufour *et al.*, 1979; McNeilly *et al.*, 1991). In the mouse, the preantral stages are gonadotrophin dependent (Hirshfield, 1991; Mizunuma *et al.*, 1999). In the mare, the dominant follicle appears after exposure to 2-8 days of rhythmic, high amplitude FSH pulses (Irvine *et al.*, 1998). If FSH support is removed, antral follicles will become atretic in sheep (Driancourt *et al.*, 1979 and 1987).

1.4.1.2 Follicle selection

Once a group of follicles has been recruited, selection of the dominant follicle takes place, possibly by the future dominant follicle inducing atresia in the competing follicles. There are several theories as to how dominance is established. Dominance may be obtained in part by the largest follicle interfering with the gonadotrophin supply to smaller follicles of the cohort, perhaps by producing inhibin and oestradiol, which via negative feed back on the hypophysis will reduce FSH secretion. The FSH levels will be insufficient to support the subordinate follicles (Baird *et al.*, 1991). In heifers, the declining FSH beginning after day 2 of oestrus induced growth and enhanced the oestradiol-producing capacity of the dominant follicle and atresia of subordinate follicles. Administration of FSH delayed selection of the dominant follicle and atresia in the subordinate follicles (Mihm *et al.*, 1997). The dominant follicle grows despite the decreasing levels of FSH, because at it is supported by LH (Fortune, 1994). LH receptors develop on the granulosa cells and gives the follicle an advantage over the smaller follicles when FSH concentrations are low (Driancourt, 1991b). In the mare, increasing amounts of LH receptor mRNA develop as the follicle gets closer to ovulation (Lawler *et al.*, 1998).

The dominant follicles may also inhibit the growth of the smaller follicles via secretion of paracrine factors. Follicular fluid growth factors or peptides are likely to affect follicle function or to modify gonadotrophin action. These factors may include inhibin, activin or Insulin-like Growth Factors (IGFs). In cattle, administration of follicular fluid after ovulation resulted in a delay in development of a follicular wave (Turzillo *et al.*, 1990). Charcoal extracted follicular fluid (no progesterone, oestradiol or inhibin) administered to mares altered FSH concentrations. The steroid-free follicular fluid induced atresia in follicles larger than 11 mm and initiated a new follicle wave (Plata-Madrid *et al.*, 1992). In sheep and rats, antiserum against granulosa cell inhibitory factor (GCIF), which is present in follicular fluid, resulted in increased number of large follicles and ovulation rate in both species (Hynes *et al.*, 1999). In sheep, IGF-I stimulated follicle development and androgen and oestradiol secretion. Transforming Growth Factor alpha (TGFα) resulted in atresia in large follicles and decrease in ovarian hormone secretion. Intra-arterial infusion of inhibin A resulted in an acute depression in ovarian steroid secretion. This depression was also associated with an acute depression in circulating FSH concentrations. These data suggest that factors can modulate the action of gonadotrophins on follicular cells to augment (IGF-I, inhibin A) or inhibit (TGF alpha/EGF) granulosa and thecal cell differentiation (Baird & Campbell, 1998). Regulators of ovarian somatic cells can be divided into mitogenic factors and differentiation-

induction factors, based on proliferation and differentiation that are fundamental to folliculogenesis and ovarian functions. Epidermal growth factor (EGF) is a mitogen, and transforming growth factor beta is a differentiation-induction factor, in modulating gonadotrophin action in the ovary, particularly in the preantral follicles (Roy *et al.*, 1996).

1.4.2 Follicular atresia

Depending on species, 70-99% of follicles in the ovary will undergo atresia (Byskov, 1979). Follicles can become atretic at any point during development from primordial germ cells to preovulatory follicle (Hsueh *et al.*, 1994). A histological study of the developing germinal epithelium in the foetal horse ovary showed widespread atresia of oocytes during the meiotic phase, between days 73 and 150 of pregnancy and peaking around day 100. The first groups of oocytes to enter this phase underwent mass degeneration and eventually disappeared. Few oocytes developed to primordial follicles at that stage, but by day 150 primordial follicles were common (Deanesly, 1975).

Once the primordial follicle pool is established, depletion of most of the remaining oocytes occurs indirectly as a result of atretic degeneration of follicles not selected for ovulation. In most mammals the majority of follicles undergo atresia during the late preantral to early antral stage (shown by in situ end labelling of DNA in ovarian sections) when continued growth is dependent upon gonadotrophin (Tilly, 1996). Atresia regulates the size of the follicle cohort for ovulation. The proportion of healthy and atretic follicles varies among species, but remains constant within the species (Hsueh *et al.*, 1994). Most mare follicles less than 1 mm were not atretic although atresia could be seen in follicles of 350 μm (Driancourt *et al.*, 1982a). No atresia was seen in preantral follicles in mares. In the cow and ewe, apoptotic death of granulosa cells as well as mitosis may occur within the same follicle, suggesting that atresia of a follicle is determined by a dynamic equilibrium between cell division, differentiation and death (Jolly *et al.*, 1994b and 1997a).

1.4.2.1 Apoptosis

Atresia is the result of cells undergoing apoptosis. The degeneration of granulosa cells as atresia advances has the characteristics of apoptotic cell death. Apoptosis is a form of physiological cell death in contrast to necrosis, which is "accidental" (pathological) cell death, generally caused by a wide variety of harmful conditions (Kerr *et al.*, 1972; Schwartzman *et al.*, 1993; Darzynkiewicz *et al.*, 1998). Apoptosis is apparently the primary

mechanism by which cell loss is mediated during follicle degeneration in all species studied (rats (Nahum *et al.*, 1996; Boone *et al.*, 1997a), sheep (Jolly *et al.*, 1997a), cattle (Jolly *et al.*, 1994a; Blondin *et al.*, 1996) and pigs (Hughes, Jr. 1991; Tilly *et al.*, 1991; Guthrie *et al.*, 1994; Pesce *et al.*, 1994)). Apoptotic cell death is regulated by a defined genetic pathway and so this process is referred to as being programmed (Schwartz *et al.*, 1993; Schwartzman *et al.*, 1993; Hale *et al.*, 1996).

1.4.2.2 Factors stimulating or preventing atresia

During follicle development, gonadotrophins and oestrogens, together with local ovarian growth factors (Insulin like Growth Factor I (IGF-I) (Hynes *et al.*, 1996), Epidermal Growth Factor (EGF) (Tilly *et al.*, 1992; Hynes *et al.*, 1996) and oestrogens appear to have anti-atretogenic as well as mitogenic activity (Kenney *et al.*, 1979). In contrast, Tilly *et al* (1992) found IGF-I to be ineffective to prevent spontaneous onset of DNA fragmentation in culture of rat granulosa cells. Circulating levels of gonadotrophins, FSH in particular, determines if an antral follicle will grow or undergo atresia. Hormone withdrawal triggers cell death and tissue remodelling and initiates a fresh cycle (Gosden *et al.*, 1997b). FSH reduces apoptosis in granulosa cells in cows (Blondin *et al.*, 1996). In rats, FSH and LH suppress apoptosis and prevent atresia, possibly through growth factors. Transforming Growth Factor (TGF-alpha) (Flaws *et al.*, 1995), basic Fibroblast Growth Factor (FGF) (Nuttinck *et al.*, 1996) and interleukin-1 beta (Chun *et al.*, 1995) activate different intracellular pathways to suppress apoptosis (Tilly *et al.*, 1992; Hsueh *et al.*, 1994). In contrast, Tumour Necrosis Factor (TNF-alpha) (Kaipia *et al.*, 1996), Fas ligand (Quirk *et al.*, 1995; Kim *et al.*, 1998) and androgens are atretogenic factors. These diverse hormonal signals influence death genes (including genes such as BCL-2 and ICE families) to regulate apoptosis (Kaipia *et al.*, 1997).

1.4.2.3 Effectors of apoptosis

Gene families CED-3 and ICE encode for proteases, which regulate the action of the endonucleases that cleave DNA. Caspases are intracellular, cytoplasmic cysteine proteases, which are cell death regulators. They may initiate or execute apoptosis induced by various stimuli. The caspases are present in most cells in an inactive pro-enzyme form, which can be activated by cleavage (Tilly, 1996). An endonuclease responsible for the apoptotic DNA degradation has been identified as a caspase-activated deoxyribonuclease (CAD) (Boone *et al.*, 1997a; Enari *et al.*, 1998).

Bcl-2 is a proto-oncogene, which encodes for an intracellular protein, which prevents apoptosis by inhibiting a central step in the apoptotic pathway (Tilly, 1996). Bcl-2 over-expression in transgenic mice reduced the number of follicles undergoing apoptosis and increased litter sizes (Hsu *et al.*, 1996) and in BCL-2 knock-out mice, there were fewer oocytes and primordial follicles in the postnatal ovary (Ratts *et al.*, 1995). Other genes either enhance (BAX, Bcl-X_{short}, BAD) or repress (Bcl-X_{long}) apoptosis (Billig *et al.*, 1996; Tilly, 1996). P53 tumour suppressor protein induces apoptosis, maybe through enhancing transcriptional activity of the BAX gene, while suppressing the bcl-2 gene (Tilly, 1996).

1.4.2.4 Morphology of apoptosis

Biochemically, apoptosis is characterised by loss of DNA integrity following endonuclease-mediated fragmentation (Wyllie, 1980a). The internucleosomal cleavage of DNA results in high molecular weight fragments (oligonucleosomes, 180 to 200-bp). Morphologically, apoptosis is characterised by loss of cell volume (cytoplasmic condensation), nuclear pyknosis resulting from margination of the chromatin and redistribution against the nuclear envelope (Kerr *et al.*, 1972). The residual bodies from apoptosis are phagocytosed. In rats it has been shown that neighbouring granulosa cells engulf the apoptotic cells (Boone *et al.*, 1997b). Cells undergoing apoptosis may form apoptotic bodies and completely disappear within 24 hours (Kerr *et al.*, 1972).

1.4.2.5 Methods of determining atresia

Distinguishing between healthy and atretic follicles has been done in a variety of ways. Follicles have been classified as healthy or atretic based on surface opacity (Grimes *et al.*, 1987), translucency and vascularisation of the follicle (Moor *et al.*, 1978), on follicular fluid hormone content (Kenney *et al.*, 1979; Jolly *et al.*, 1994b; Nahum *et al.*, 1996; Guthrie *et al.*, 1994 and 1996) as well as by histologic means. New techniques are emerging at a fast pace.

1.4.2.5.1 Histological evaluation of atresia

Equine follicles have been characterised by histology (Kenney *et al.*, 1979; Driancourt, 1979, 1982a and 1982b). Sections of follicular wall stained with H&E allows estimation of numbers of pyknotic nuclei as well as mitotic index in the granulosa cells by histology. Histologically, it may not be possible to detect apoptosis in its first stages, as histological detection of apoptosis involves evaluation of nuclear chromatin condensation, which may

occur in the final stages of apoptosis. Because atresia is characterised in only a small section of the follicular wall and because pyknosis is seen to some extent in dominant, viable follicles (Ireland *et al.*, 1982) the onset of atresia is difficult to estimate. In the mare the duration of the atretic process from viable follicle to pronounced atresia has not been established. To reach a very advanced stage of atresia where the granulosa cells have gone and only fibroblasts are present takes 4 days in mice (Byskov, 1974).

1.4.2.5.2 Biochemical detection of apoptosis

The internucleosomal cleavage of DNA in oligonucleosomes results in "DNA ladders" when visualised by agarose gel electrophoresis (Wyllie, 1980a; Tilly *et al.*, 1991). By contrast, necrosis is characterised by random fragmentation of DNA by multiple endonucleases, which produces a continuous spectrum of DNA fragment sizes and shows up as even staining in the lane after agarose gel electrophoresis (Afanas'ev *et al.*, 1986; Gold *et al.*, 1994).

Histochemical and biochemical alterations in granulosa cells may precede morphological changes in atretic granulosa cells. Whether DNA laddering in agarose gels is recognisable before or after histological changes in the follicle is questionable. In rats, DAPI (4',6-diamidine-2'-phenylindole dihydrochloride) staining of the granulosa cell nuclei detected apoptotic nuclei at the same time point as DNA fragmentation was detected, but a major increase in oligonucleosome formation happened 24 hours earlier than a major increase in apoptotic nuclei as detected morphologically in hypophysectomised rats (Nahum *et al.*, 1996). Apoptotic death of granulosa cells in the cow and ewe may also occur in healthy follicles during the luteal phase and/or occur early in the atresia process before other morphological or biochemical signs of degeneration are evident (Jolly *et al.*, 1994b and 1997b). The workers correlated DNA fragmentation with morphological atresia, follicular fluid hormone content and aromatase activity and concluded that granulosa cells may die by apoptosis before there is an appreciable decrease in the capacity of the granulosa cell layer to respond to gonadotrophins or to produce oestradiol.

1.4.2.5.3 Other methods of evaluating atresia

Electron microscopy of granulosa cells shows the ultrastructural changes that occur during apoptosis (Kerr *et al.*, 1972). Flow cytometry has been used to detect apoptosis in porcine and bovine follicles. This method assesses the percentage of cells found at each stage of the

cell cycle and detects cells with less DNA (less fluorescence after excitation), presumably resulting from apoptosis (Guthrie *et al.*, 1994; Blondin *et al.*, 1996; Susin *et al.*, 1999). Detection of translocation of phosphatidylserine to the surface of cell membranes with Annexin V staining of fresh granulosa cells specifically detects apoptotic cells (Van Blerkom *et al.*, 1998). In addition to detection of apoptosis, cell proliferation detectors can be used to determine whether a follicle is growing or undergoing atresia. For follicular fluid hormone content and steroidogenic enzymes, see the following paragraphs.

1.5 Follicular fluid

In connection with formation of the follicular antrum, follicular fluid is produced. The follicular fluid contains a variety of steroid hormones, peptides, proteoglycans and heparin sulphate (Greenwald *et al.*, 1994). The main precursor in the steroidogenic pathway of the steroids produced in the follicular fluid is cholesterol. Cholesterol is converted to pregnenolone, and then further to either progesterone or via 17αhydroxypregnenolone to dehydroepiandrosterone and androstenedione. Androstenedione can be converted to oestrone or testosterone, which both can form oestradiol. Theca interna and the interstitial cells primarily function in de novo production of androgens. Granulosa cells are the main sites of oestrogen synthesis and also de novo synthesis of progesterone in mares and other species studied (Sirois *et al.*, 1991; Tucker *et al.*, 1991; Conley *et al.*, 1997).

1.5.1 Follicular fluid hormones and atresia

Atretic follicles often have a degenerating oocyte, low levels of oestradiol and progesterone but elevated levels of androstenedione and testosterone (Greenwald *et al.*, 1994). Follicles have been classified as healthy or atretic based on reduced follicular fluid oestrogen. Concentrations of oestradiol in follicular fluid were higher than both progesterone and androgens in non-atretic follicles in cows (Ireland *et al.*, 1983). Severely atretic bovine follicles contained granulosa cells with a low capacity to metabolize testosterone to oestradiol and the lowest concentrations of androstenedione and testosterone, but had the highest concentrations of progesterone (McNatty *et al.*, 1985). Studies in the horse indicate lower levels of progesterone with the presence of atresia (Kenney *et al.*, 1979). Other reports indicate that atresia is correlated with increased progesterone production (rats (Nahum *et al.*, 1996), pig (Guthrie *et al.*, 1994 and 1996), cow (Jolly *et al.*, 1994b)). The basis for the shift in the oestrogen to progesterone ratio appears to be species specific and has been attributed to both a decrease in substrate for granulosa cell aromatisation and a loss of aromatase

activity. The ability of atretic follicles to continue production of androgens and progesterone may be related to the retention of LH-specific binding and increased progesterone production in response to LH or may be a sign of accumulation due to decreased utilisation of substrate for oestradiol production.

The decline in oestrogen is paralleled by a decrease in aromatase and a decrease in FSH and LH receptor mRNA. In cattle, healthy follicles expressed higher amounts of mRNA for gonadotrophin receptors and steroidogenic enzymes than did atretic follicles. Loss of aromatase activity is an important characteristic of atresia in porcine (Garrett *et al.*, 1997), ovine and bovine follicles (Guthrie *et al.*, 1994). Loss of aromatase expression in vivo may be one of the earliest functions lost during apoptosis of granulosa cells, decreasing before signs of internucleosomal DNA cleavage can be detected and is followed by decreased cell proliferation and decreased expression of P450c17α but unaffected 3βHSD expression in pigs (Garrett *et al.*, 1996 and 1997). Alterations in steroidogenic activity as measured by changes in concentrations of steroids in follicular fluid, does not appear to occur prior to atresia in cattle (Grimes *et al.*, 1987).

1.6 The oocyte

A major obstacle for characterising equine oocytes is the lack of success in fertilising oocytes and development of the fertilised oocyte to a blastocyst and beyond. To characterise oocytes biochemically and morphologically, as well as obtaining criteria for the quality of the oocytes, but also to sustain embryonic development, it is necessary to overcome these problems. In 1990, the only foal that has been produced by in vitro maturation and in vitro fertilisation was born (Palmer *et al.*, 1991). Several pregnancies have been obtained after intracytoplasmic sperm injection of matured oocytes, but only 2 live foals have been born (Squires, 1996; Cochran *et al.*, 1998).

1.6.1 The granulosa cell - oocyte relationship

The oocyte cannot develop in isolation from the granulosa cells. From the primordial stage, the oocytes are surrounded by granulosa cells, which produce growth factors and hormones and protects the oocyte physically. The oocyte on the other hand may be controlling the granulosa cell development and function rather than just being in the follicle (Eppig *et al.*, 1997). Oocytes are known to secrete paracrine growth factors, which are necessary for cumulus expansion and induction of hyaluronic acid synthesis. Bovine oocytes produced a

factor, which enabled oocytectomised murine cumulus cells to expand (Ralph *et al.*, 1995). The presence of oocytes in culture of murine preantral follicles reduced apoptosis in the granulosa cells (Telfer *et al.*, 1999). By removing the oocyte from the cumulus complex, it was evident that the oocyte stimulated granulosa cell mitosis by secreting a factor (Vanderhyden *et al.*, 1992). Also steroid hormone production by granulosa cells appeared to be modulated by oocytes throughout folliculogenesis (Vanderhyden *et al.*, 1998).

Recent studies have demonstrated the importance of Growth differentiation factor-9 (GDF-9). GDF-9 is a transforming growth factor-β (TGF-β) family member, which is expressed in the oocytes in mouse ovaries (McGrath *et al.*, 1995) and human ovaries (Aaltonen *et al.*, 1999). GDF-9 is necessary for normal folliculogenesis as female mice deficient for the GDF-9 gene are infertile due to an arrest of follicular growth at the primary follicle stage (Dong *et al.*, 1996). As well as functioning as a growth and differentiation factor during early folliculogenesis, GDF-9 is also an oocyte-secreted paracrine factor, which regulates granulosa cell enzymes involved in cumulus expansion (Elvin *et al.*, 1999). As of yet, no studies on GDF-9 in horses was performed.

1.6.2 Meiosis

1.6.2.1 First stages of meiotic division

Oogenesis starts in the equine foetus with primordial germ cell formation (Deanesly, 1975 and 1977). The primordial germ cells develop into oogonia, which undergo mitotic divisions. In the horse mitosis of the oogonia starts from day 50 of pregnancy until day 150 - 160. Meiosis is seen from around day 70 of pregnancy and continues beyond day 150. Only few germ cell divisions are seen after day 180 of pregnancy. The oogonia are transformed into primary oocytes via leptotene, zygotene, pachytene, and diplotene stages of the first meiotic prophase, where development is arrested. In the diplotene stage of prophase, the chromosomes decondense (Tsafriri *et al.*, 1994). The meiotic arrest occurs at the time that granulosa cell precursors start to form the primordial follicle. At this point, the oocyte chromosomes have duplicated but the nuclear membrane (germinal vesicle) is still intact. The oocytes will stay arrested at the first meiotic prophase during the growth of the follicle and oocyte, which can take place anytime from the birth of the animal until senescence (Deanesly, 1975 and 1977). Not until the follicle is close to ovulation will the oocyte undergo meiosis.

1.6.2.2 Meiotic arrest

The factors involved in maintenance of meiotic arrest and in the resumption of meiosis are not well known in the horse oocyte. The growing follicle keeps the oocyte in meiotic arrest, and once removed from the follicle, the oocyte will mature without gonadotrophin stimulation (Pincus *et al.*, 1935). Follicle atresia is associated with meiotic competence of the oocyte and equine oocytes within atretic follicles may undergo maturation (Hinrichs, 1991a and 1997). This suggests that follicular components actively suppress maturation when the follicle is not dying (Pincus *et al.*, 1935). Oocytes cultured in the presence of either follicular fluid or granulosa cells will mature, but presence of follicular wall, sheets of granulosa cells or intact follicles can suppress maturation in oocytes from the mare (Hinrichs *et al.*, 1995). In the mouse, follicular fluid suppress maturation, apparently through modulation of cAMP levels within the oocyte, but this does not appear to be the case in sheep (Eppig *et al.*, 1983; Crosby *et al.*, 1985).

1.6.2.2.1 Chromatin configurations in the meiotic prophase

The chromatin configurations in the equine oocyte arrested in the meiotic prophase are not fully elucidated. Some authors reported germinal vesicles with diplotene (Torner *et al.*, 1995) chromatin, whereas others reported several different configurations within the germinal vesicle (Hinrichs *et al.*, 1993a; Grondahl *et al.*, 1995a). It is not known precisely at which stage the germinal vesicle disappears, but presumably it happens between diakinesis and metaphase I (Suzuki *et al.*, 1989).

1.6.2.3 Resumption of meiosis

Oocytes in growing follicles gradually gain the capacity to resume meiosis. An increase in LH concentrations 1-2 days before ovulation (Whitmore *et al.*, 1973; Irvine *et al.*, 1994) initiates the final oocyte maturation (Moor *et al.*, 1981; Channing *et al.*, 1982; Tsafriri *et al.*, 1984). During meiotic maturation, cytoplasmic and nuclear changes occur, which prepare the oocytes for fertilisation.

1.6.2.3.1 Factors involved in meiosis activation

The timing of onset of meiosis corresponds with a marked decline in follicular fluid oestrogen and with cumulus expansion (Ainsworth *et al.*, 1980). In sheep, there is little evidence of the induction of meiosis through hormone stimulated steroidogenesis, but it is

clear that initial high levels of oestrogen are necessary for the cytoplasmic maturation required for subsequent embryonic development (Moor *et al.*, 1978). Mouse and rabbit oocytes appear to produce a steroid-regulating factor that inhibits progesterone and enhances oestradiol production by regulating the activity of 3β-HSD or progesterone metabolism during follicular development (Gosden *et al.*, 1997a; Vanderhyden *et al.*, 1998). This was demonstrated by oocytectomy, which induced premature luteinisation and progesterone secretion.

The LH surge is crucial for meiosis activation. Gonadotrophins and various growth factors can induce resumption of meiosis in oocytes in vitro. Meiosis activating sterols (MAS) secreted by the cumulus cells induce resumption of meiosis in cultured cumulus-enclosed and naked mouse oocytes (Byskov *et al.*, 1995). FSH, but not LH or hCG, induced the cumulus cells to produce and secrete MAS (Byskov *et al.*, 1997). Changes during meiotic maturation occur simultaneously with an increase in the activity of several kinases. The maturation-promoting factor (MPF or histone H1 kinase) is a cell cycle regulator in mitosis and meiosis. The factor consists of cyclin B and the kinase cdk1 ($p34^{cdc2}$). MPF maintains cells in a metaphase state, but at fertilisation or activation, the activity of MPF is disturbed (Fulka, Jr. 1992; Naito *et al.*, 1995). MPF takes part in regulation of changes in the phosphorylation patterns of cellular proteins (Goudet *et al.*, 1998a). These proteins are involved in nuclear membrane formation, chromatin condensation and microtubular reorganisation. MPF is measured by phosphorylation of histone H1. In equine oocytes, MPF was lower in the germinal vesicle stage oocytes, than in metaphase I and II oocytes (Goudet *et al.*, 1998a). In bovine oocytes, there were low levels of MPF activity during germinal vesicle stage during the first 8 hours after maturation. This was followed by a peak of activity during MI (12-14 hours of maturation) and an abrupt reduction in activity at 16-18 hours (anaphase and telophase), followed by an increase and plateau at 20-24 hours (MII) (Fissore *et al.*, 1996; Wu *et al.*, 1997). Mitogen-activated protein (MAP) kinase is also activated around germinal vesicle breakdown in the bovine (Fissore *et al.*, 1996). Amongst maturation activating factors, the epidermal growth factor (EGF) seems to play an essential role in several species (pig (Grupen *et al.*, 1997; Abeydeera *et al.*, 1998), human (Goud *et al.*, 1998), cow (Lorenzo *et al.*, 1995; Rieger *et al.*, 1998)). Other factors such as ovarian peptides (inhibin, activin) and other growth factors may also be involved in maturation.

1.6.2.3.2 Nuclear changes during meiotic maturation

The nuclear changes involve progression from the diffuse diplotene stage of the first meiotic

prophase to metaphase II of meiosis. What happens between the meiotic prophase and metaphase I is not clear. The meiotic maturation is characterised by dissolution of the germinal vesicle membrane (Germinal Vesicle Break Down, GVBD), condensation of chromatin through diakinesis, metaphase I, anaphase I and telophase I, emission of the first polar body and arrest of meiosis with the chromosomes aligned on the metaphase II spindle (Wassarman *et al.*, 1976b; Wassarman *et al.*, 1994). The equine oocyte is ovulated in metaphase II (King *et al.*, 1987). Meiosis is completed when fertilisation occurs and the second polar body is formed (Wassarman *et al.*, 1994). Resumption of meiosis can occur spontaneously in culture without necessarily achieving competence for embryonic development (Cran, 1985). Under some circumstances MII arrested mouse oocytes can become parthenogenetically activated, for example after exposure to ethanol, protein synthesis inhibitors or calcium ionophore (Clarke *et al.*, 1983; Kubiak, 1989; McConnell *et al.*, 1995). Oocytes at earlier stages can not be activated by exposure to calcium ionophore (McConnell *et al.*, 1995).

1.6.2.3.3 Meiotic spindle

The equine meiotic spindle was described by (Goudet *et al.*, 1997). As part of the normal maturation, the spindle assembles in a specific way in order for the chromosomes to align. The meiotic spindle in oocytes matured in vitro was longer and wider than in vivo matured oocytes' spindles, indicating a change in the concentration of cytoplasmic proteins, e.g. tubulin, during IVM, which could be a sign of imperfect maturation (Goudet *et al.*, 1997). Meiotic spindle disorganisation was seen in human oocytes with normal chromosome morphology (Pickering *et al.*, 1990). As oocyte quality usually is evaluated by staining the chromatin only, other important parameters of oocyte health may be overlooked.

1.6.2.3.4 Organisation of cytoplasmic organelles during final maturation

Assessment of maturation of the oocyte has included nuclear as well as cytoplasmic maturation state, as both are thought to be important for fertilisation and subsequent embryo development. In the mouse, studies indicate that the potential for the oocyte to move to interphase from MII is dependent on cytoplasmic maturation rather than nuclear maturation (McConnell *et al.*, 1995). During oogenesis cytoplasmic organelles and molecules accumulate in order to make fertilisation and cleavage possible. The cytoplasmic maturation changes include breakdown of the intermediate junctions between the cumulus cell projections and the oolemma. The perivitelline space is enlarged and the Golgi apparatus

forms a large number of cortical granules beneath the oolemma. The cortical granules are lysosome-like structures that fuse with the oolemma at fertilisation in order to alter functional properties of the zona pellucida. In the small oocyte, many organelles are arranged around the nucleus, but as the oocyte matures, there is centripetal organelle migration (Grondahl *et al.*, 1995a; Gosden *et al.*, 1997a; Avery *et al.*, 1998). In the mare, the cytoplasm takes longer to mature (36 hours) than does the nucleus (15 hours) and this could explain the low in vitro fertilisation rates (Grondahl *et al.*, 1995a). The cytoplasmic maturation appears to be more important than the nuclear maturation for mouse oocytes to respond to calcium ionophore activation (McConnell *et al.*, 1995).

1.6.2.3.5 Biochemical changes during oocyte maturation

During maturation, changes take place in protein synthesis that may be essential for cytoplasmic maturation (Moor *et al.*, 1977; Crosby *et al.*, 1981). Resumption and completion of meiosis involves synthesis and phosphorylation of proteins (Wassarman *et al.*, 1976a). The necessary time needed for protein synthesis during meiotic maturation of equine oocytes having either compact or expanded cumuli was studied with the use of cycloheximide. Cycloheximide inhibits peptidyltransferase, and thereby suppresses oocyte maturation (germinal vesicle break down) by blocking synthesis of stage specific proteins. Nuclear maturation of horse oocytes was reversibly suppressed by incubation with cycloheximide. Oocytes with different initial cumulus type, differed in the time required for protein synthesis essential for maturation. Expanded cumulus oocytes required less maturation time than compact cumulus oocytes to go through germinal vesicle break down and to reach metaphase II (Alm *et al.*, 1996).

1.6.2.4 Oocyte and follicle size

The size of the follicle and oocyte is an essential parameter for meiotic maturation (Sorensen *et al.*, 1976). The ability to resume meiosis is acquired at a specific stage of oocyte growth in the juvenile mouse, but the ability to complete meiosis is acquired subsequently (Sorensen *et al.*, 1976). The mouse oocyte grows from 15 to 80 μm over a period of 2-3 weeks. The human oocyte takes several months to grow from 35 μm to the final size of 120 μm (Gosden *et al.*, 1997a). The final size of the sheep oocyte (110 μm) appears to be reached when the follicle is approximately 0.6 mm (Avery *et al.*, 1998). The murine oocyte finishes growing and obtains meiotic capacity when the antrum starts to form. Porcine (Motlik *et al.*, 1984; Motlik *et al.*, 1986), and bovine (Arlotto *et al.*, 1996) oocytes keep growing during the

growth of the antral follicle, and the larger the follicles are the better in vitro maturation rates are obtained. Increasing diameter of the follicle increased maturation rate of the equine (Goudet *et al.*, 1997 and 1998b), suggesting that larger follicles are better prepared for maturation. Time for the bovine oocyte to extrude polar bodies was lower for larger follicles than for smaller follicles' oocytes (Arlotto *et al.*, 1996).

1.6.2.5 Oocyte maturation and stage of cycle

The hormonal environment at the end of the follicular phase and during pregnancy provided better conditions for acquisition of meiotic competence than the environment during the luteal phase in mares (Goudet, 1998b). In the follicular phase the growth of a dominant follicle until the preovulatory stage increased the percentage of competent oocytes in subordinate follicles (Goudet, 1998b). No effect was found on bovine oocyte in vitro maturation capacity when taking oestrous cycle stage into account (Arlotto *et al.*, 1996). Injection of crude equine gonadotrophin before the collection of oocytes in the luteal phase increased competence for in vitro maturation in follicles larger than 10 mm (Goudet, 1998b), suggesting that the oocytes benefited from increased concentrations of gonadotrophins.

1.6.3 Oocyte atresia

The fate of most oocytes is to die in follicles that are under-stimulated by FSH. These follicles become atretic and the granulosa cells become apoptotic. Common characteristics of atresia in the oocyte of many species are meiosis-like alterations. This includes germinal vesicle breakdown, alignment of the chromosomes in metaphase and possibly expulsion of a polar body. In women synchronous degeneration of oocyte and granulosa cells occurs. In the mouse foetus, the degeneration of oogonia and oocytes occurs via apoptosis (Tilly, 1996) and there is evidence that ovulated murine oocytes also undergo apoptosis after 24 hours of incubation with or without doxorubicin, which induces fragmentation in oocytes (Perez *et al.*, 1999).

1.6.3.1.1 Nucleolus

The nucleus contains the nucleolus. In cattle the last stages in nucleolar differentiation includes condensation and fragmentation of the one or two initial nucleoli into a few smaller and compact nucleoli of 2-3 μm (Crozet, 1989). At the time of germinal vesicle break down,

the nucleolus disappears. The formation of a rim of chromatin around the nucleolus in large oocytes is coinciding with readiness to resume meiosis (Gosden *et al.*, 1997a).

The nucleolus is believed to be the site of RNA synthesis in all cells. During oogenesis, gene transcription must be very active to provide the accumulation of maternal RNAs necessary for early embryonic development. The RNA is produced and stored for the purposes of oogenesis and the early stages of post-fertilisation development, where the RNA is used (Gosden *et al.*, 1997a). There is an indication of a correlation between the regulation of transcription and chromatin organisation (Felsenfeld, 1992). Generally, highly compacted chromatin is associated with low transcription activity, and transcription is low during meiotic maturation (Gosden *et al.*, 1997a; Zuccotti *et al.*, 1998). In the mouse, the RNA synthesis is elevated during oocyte growth, but decreases at the time of follicular antrum formation (Crozet, 1989). The formation of a chromatin ring around the nucleolus appears to be a prerequisite for germinal vesicle break down (Zuccotti *et al.*, 1998). Presumably, the configuration with no chromatin ring around the nucleolus precedes the form with the surrounded nucleolus in time. The chromatin surrounding the nucleolus leads to the formation of chromosomes of the first meiotic metaphase (Zuccotti *et al.*, 1998). This means that the oocytes with no surrounded nucleolus is a more immature stage which will mature by going through the surrounded stage and then move on to the germinal vesicle break down stages (Zuccotti *et al.*, 1998).

1.7 Cumulus oophorus

The equine cumulus oophorus is attached to the follicle wall with a broad base (Bruck *et al.*, 1999). The location of the equine cumulus oocyte complex within the follicle is random in contrast to cattle and llama, where the follicular attachment of the oocyte is more frequently found in the hemisphere closest to the expected site of ovulation (Delcampo *et al.*, 1995a; Bruck *et al.*, 1999),. During maturation in vivo, the cumulus cells surrounding the oocyte expand.

1.7.1 Factors involved in cumulus expansion

Meiotically competent murine oocytes secrete an expansion-enabling factor, but the cumulus cells do not expand until after the LH surge (Vanderhyden *et al.*, 1998). Other reports mention that the oocytes secrete a factor that allows cumulus cells to undergo expansion in response to FSH (Buccione *et al.*, 1990; Salustri *et al.*, 1990; Tirone *et al.*, 1997; Nagyova *et*

al., 1999). Just before ovulation gonadotrophins stimulate cumulus cells to produce and secrete hyaluronic acid that disperses the cumulus cells and embeds them in a mucus-like matrix (Dekel *et al.*, 1978; Eppig, 1979; Salustri *et al.*, 1989). Cumulus expansion results in an up to 40 times increase in the volume of the cumulus mass (Chen *et al.*, 1990). The expanded cumulus extracellular matrix is stabilised through the binding of hyaluronan by serum glycoproteins (Hess *et al.*, 1999). Oocyte maturation changes include breakdown of the intermediate junctions between the cumulus cell projections and the oolemma, which is part of the cumulus expansion (Grondahl *et al.*, 1995a). Mouse granulosa cells, which were deprived of their oocyte, did not respond to FSH or cyclic AMP by producing hyaluronic acid, and thus did not have cumulus expansion (Buccione *et al.*, 1990). The pig oocytes are different in that their absence did not affect the cumulus cells' production of hyaluronic acid in vitro (Nagyova *et al.*, 1999) and bovine cumulus complexes expanded with or without the presence of the oocyte (Ralph *et al.*, 1995). IGF-I appears to have a role in cumulus expansion (Nagyova *et al.*, 1999). In relation to timing of cumulus expansion, (Bezard *et al.*, 1997) showed that 0%, 17%, 83% and 100% of equine oocytes had expanded cumulus 0, 6, 12 and 35 hours after induction of ovulation with gonadotrophin administration in vivo. After in vitro culture, most oocytes have expanded cumulus as part of the maturation. Expanded cumulus oocytes are more likely to be recovered from atretic follicles. In the mare, studies indicate that expanded cumulus oocytes mature to MII at higher rates and more rapidly than compact cumulus oocytes (Zhang *et al.*, 1989; Hinrichs *et al.*, 1997).

Introduction and aims

Reproductive efficiency in the horse is still poor compared to other species. Because the horse is considered a pleasure animal rather than livestock, there has not been the same demand as in cows and pigs to optimise production of horses, and there is hardly any economical gain from producing horses. The only money-producing interest in horses is the racing industry. Unfortunately this industry is governed by a conservative belief that horse reproduction should be as natural as possible, i.e. only natural cover of the thoroughbred mare is allowed. The reason for this is primarily traditional but with referral to keeping the genetic material as wide as possible. Assisted reproduction is permitted outside of the racing community, but the economic backing in any other area of the equine industry is poor. These circumstances have resulted in research in equine reproduction falling far behind other domestic species. Due to the horse's unique size of ovary and follicles, it is an excellent species to use for studying follicle dynamics, and to collect samples from for studying several parameters at a time.

Several studies in the equine species have focused on follicle dynamics, others on hormone levels and yet others on oocyte maturation, but none have combined these areas, i.e. investigated several hormones during the cycle at the same time as studying the follicular growth and regression. Only a few have researched the relationship between the follicle and its oocyte. Apoptosis of the equine follicle is a completely untouched area, and the study of atresia in follicles has virtually not progressed after publications in 1979 (Kenney *et al.*, 1979), 1991 (Hinrichs, 1991b) and 1997 (Hinrichs *et al.*, 1997).

The overall aim of this study was to determine when selection of the dominant follicle occurs and how this is reflected in the dominant and the subordinate follicles in horses. To this end there were a series of experiments, which addressed three main goals:

1. to map the pattern of chromatin configurations changes in equine oocytes, which have not been cultured (chapter 3). As material was obtained from slaughterhouses, it was also necessary to find out whether holding temperature and time altered chromatin configuration and cumulus morphology post mortem.

2. to investigate whether equine follicles undergo apoptosis (chapter 4). Methods of detecting apoptosis were investigated as well as effect of holding temperature and time on granulosa cell health post mortem.

3. to map patterns of follicular growth, selection and regression as seen by ultrasonography in relation to hormone levels in the animal (chapter 5).

Once these three goals were obtained, all of the information gathered in the previous chapters was brought together in order to establish whether findings on ultrasonography correlate with biochemical and histological findings in both oocytes, cumulus, granulosa cells and follicular fluid hormone content (chapter 6).

This thesis was undertaken in the expectation that we would obtain a better understanding of the events surrounding growth, selection and regression of follicles with the hope to further elucidate how to control follicular growth, atresia and ovulation in the mare.

2 Materials and methods

2.1 Animals

2.1.1 Experimental animals

All experimental animal procedures were carried out under the Animal (Scientific Procedures) Act, 1986 at Easter Bush Veterinary Centre. They were either kept grouped in a large outdoor pen with partial cover or individually stabled indoors. The animals were fed haylage with additional hard feed. The animals weighed between 198 - 584 kg, and were aged between 2-20 years.

2.1.2 Slaughter house animals

Samples from slaughtered animals were collected at abattoirs in Bristol, UK and Massachusetts, USA. The animals ranged from 2-30 years of age and weighed an estimated 200-600 kg. The majority were horses rather than ponies.

2.2 Examination of the reproductive tract

Mares were scanned daily from day 0 until ovulation or ovariectomy. The cervix, uterine body and horns and ovaries were palpated to examine tone and size. A 5 MHz linear array transrectal probe was inserted into the rectum and the vagina, cervix, uterus and ovaries were visualised on an ultrasound scanner (Aloka Co. Ltd., Tokyo, Japan). The vagina was examined for presence of fluid, the cervix for density, the uterus for presence of fluid and oedema and the ovaries for follicular activity and presence of corpora lutea. Care was taken to distinguish other non-echogenic areas from follicles. Cross-sections of blood vessels could be mistaken for small follicles and cystic vestiges of embryonic structures associated with the oviducts could mimic follicles, although they could be identified just outside the ovary. Maps were drawn at the time of scanning of each ovary's structures. The diameter of the follicles was measured on the screen, and presence or absence of an echogenic rim along the inside of follicle was noted, as well as any signs of luteinisation such as trabeculae in the lumen. Diameters of non-spherical follicles were obtained by averaging length and width.

2.3 Blood sampling

Blood samples (15 ml per sample) were collected by jugular venipuncture into heparinised, evacuated glass tubes and kept on ice until centrifugation (2000 *g* for 15 minutes at 4 °C). The plasma was dispensed into 1.5 ml eppendorfs and stored at –20 °C until assayed.

Frequent blood sampling was done through an indwelling intravenous catheter. A 10 x 6 cm area over the vena jugularis was clipped and surgically prepared with chlorhexidine scrub and sprayed with chlorhexidine solution. Local analgesia was obtained with 1 ml lignocaine and a catheter was then inserted and sutured to the skin. The catheter was flushed after use with sterile physiological saline containing heparin. The catheter was removed immediately after the last blood sample was taken. Blood samples from slaughtered animals were collected at the time of bleeding the carcass (opening of vena jugularis).

2.4 Collection of ovaries

2.4.1 Ovariectomy

Ovaries were obtained from cycling mares after standing ovariectomy using the method described by (Colbern *et al.*, 1987). The mares were sedated and analgesed with acepromazine (0.02 mg/kg i.v.; C-Vet Ltd., Bury St. Edmunds, UK), flunixin meglumine (1.1 mg/kg i.v.; Finadyne, Schering-Plough Animal Health, Welwyn City Garden, UK), romifidine (0.05 mg/kg i.v.; Sedivet, Boehringer Ingelheim Ltd., Bracknell, UK), xylazine (0.2-0.4 mg/kg i.v.; Virbaxyl 10%), morphine (0.5 mg/kg). After washing the vulva, anus and surrounding area with iodine scrub, a small incision was made in the mucosa of the vagina at the 4 o'clock position, 3-5 cm from the cervix in a safe distance from the internal iliac artery. Blunt, digital dissection was used to penetrate the peritoneum to enter the abdominal cavity. A lignocaine soaked swab was held around the ovarian pedicle for 1 minute to anaesthetise the area where the ecraseur would cut. An ecraseur (JorVet, Germany) was used to crush the ovarian artery and the ligaments. The mares were given prophylactic tetanus antitoxin (6,000 i.u.) s.c. and received procaine penicillin (20,000 i.u./kg) i.m. s.i.d. for 5 days. Phenylbutazone (4 mg/kg) s.i.d. was administered either orally or intravenously as needed.

2.4.2 Slaughter house ovaries

Ovaries were collected from abattoirs after slaughter of the animals. The ovaries were exteriorised within 30 minutes of slaughter.

2.5 Ovary processing

The exteriorised ovaries were kept at 20 - 30 °C or 35 - 37 °C in M199 with Hanks salts and 25 mM Hepes (Gibco BRL, Life Technologies LTD, Paisley, UK) for 0.5 - 24 hours. The holding temperature and time is indicated for each study in the respective chapters. All visible follicles on the surface were processed followed by the follicles found after slicing the ovary thinly. Each follicle was measured with a ruler before aspiration of all follicular fluid (see Figure 2.1 and Figure 2.2). The follicular fluid was stored at –20 °C until assaying for steroid hormones. The follicle was cut open and the inner wall was scraped with a bone curette to release both granulosa cells and the cumulus oophorus with the oocyte. The granulosa cells were flushed off with M199 with Hank's salts. The granulosa cells were pelleted by centrifugation at 2,000 *g* for 30 seconds at room temperature, snap frozen and stored at -70 °C until DNA extraction. After examination of the cumulus oophorus using a stereomicroscope, the oocyte was denuded by pipetting with smaller and smaller bore pipettes (0.2 - 0.6 mm in diameter) either with or without a solution of 0.25% trypsin/EDTA (Gibco BRL, Life Technologies LTD, Paisley, UK). The oocytes were kept in phosphate buffered saline (PBS) or Medium 199 with Hanks salts and 25 mM Hepes (Gibco BRL, Life Technologies LTD, Paisley, UK) during denuding.

2.6 DNA extraction

Granulosa cells were lysed with 0.5% sodium dodecyl sulphate, 0.1M sodium chloride, 0.05M tris (pH 8.0), 2.4 mM EDTA and digested with proteinase K (100 mg/ml) at 57 °C for 6 hours. Protein precipitation was initiated with potassium acetate (8M) (75 microlitres) and chloroform (500 microlitres) either at 5 °C for 1 hour or at -20 °C overnight. After centrifugation for 8 minutes at 10,000 *g* at 4 °C, further extraction and precipitation was performed with isopropanol (500 microlitres) for 2 hours at -70 °C and finally ethanol (80%) (1 millilitre). After removal of the ethanol, the samples were air dried and resuspended in deionised, filter sterilised H_2O and stored at -20 °C. DNA concentration in the samples was quantified spectrophotometrically (Beckmann Spectrophotometer, D4® 650, USA) by reading the absorbance at 260 nm and 280 nm.

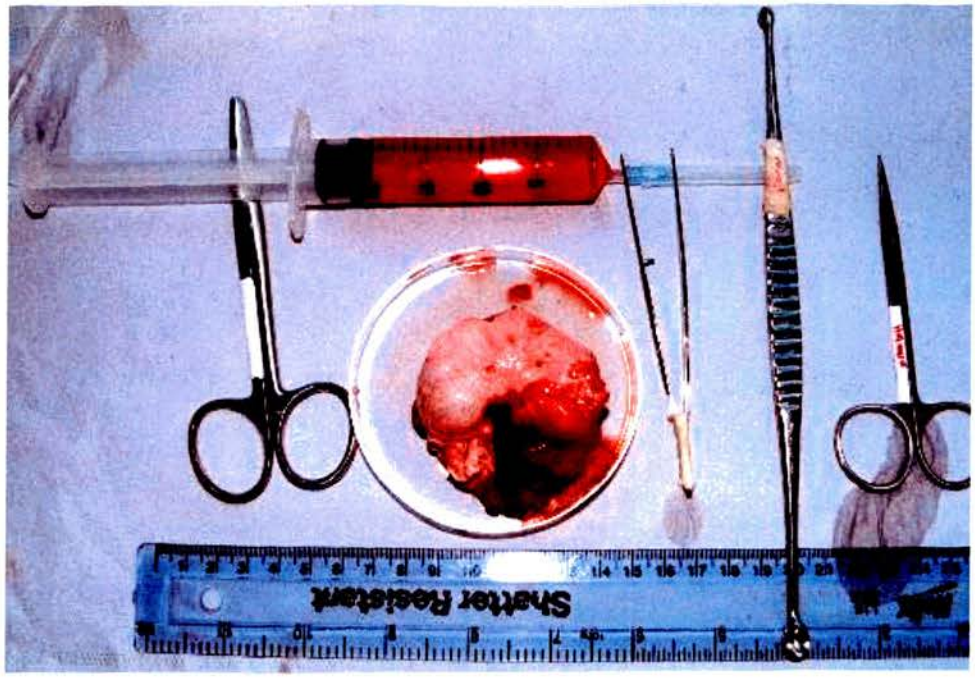


Figure 2.1. Equine ovary with dissection equipment.

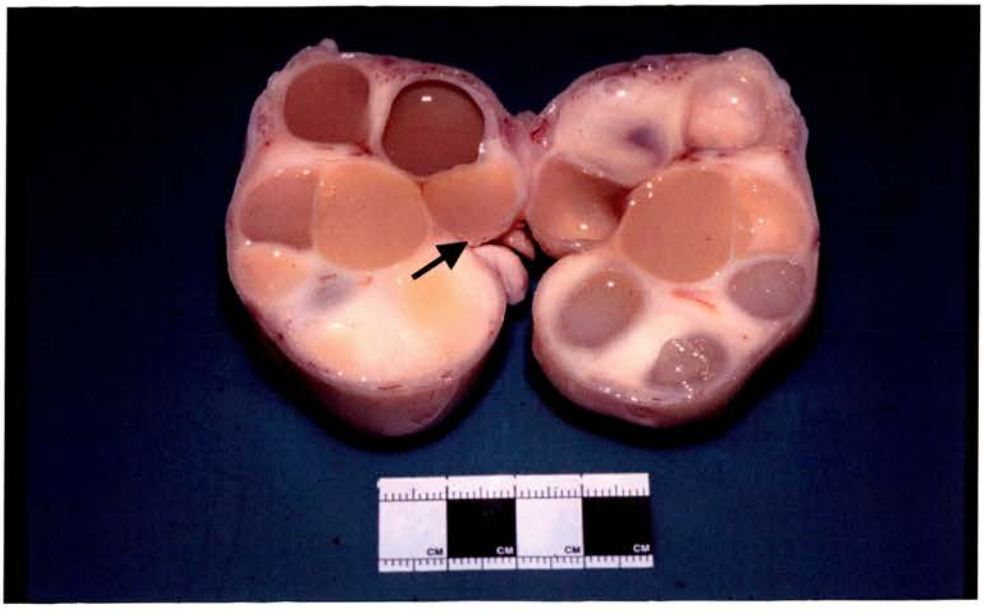


Figure 2.2. Follicles in equine ovary. This ovary was fixed before it was cut open. Arrow: ovulation fossa.

2.7 Agarose gel electrophoresis

In samples with 10 μg or more DNA, the DNA fractions were separated by agarose gel electrophoresis, using 10 μg DNA from one follicle per lane in a 1.5% agarose gel. The DNA was stained with ethidium bromide incorporated in the gel (1 mg/ml) and visualised with ultraviolet light using a transilluminator. Ethidium bromide is a fluorescent dye, which binds to DNA. DNA from freshly isolated murine thymocytes was used as negative (non-apoptotic) controls and DNA from murine thymocytes incubated for 24 hours with dexamethasone (100 μg dexamethasone/ml culture medium) (Sigma-Aldrich Company Ltd., Poole, UK) was used as a positive (apoptotic) control (Wyllie, 1980a; Cohen *et al.*, 1984).

2.8 DNA 3'-end labelling

Samples with 2 - 10 μg DNA were analysed through the use of DNA 3'-end labelling. DNA samples were labelled at the 3'-end with [^{32}P]dideoxy-ATP (50 uCi) (2 microlitres) (Amersham Life Science Ltd, Little Chalfont, UK) by incubation for 60 minutes at 37 °C with Terminal Transferase, 1 microlitre (5 units) (Sigma-Aldrich Company, Poole, UK), reaction buffer (10 microlitres reaction buffer consisting of 1 M Na (1.6 g), 0.125 M Tris HCl (0.197 g), BSA (12.5 g) at pH 6.6) and cobalt chloride (5 microlitres of a 10 mM solution (Sigma-Aldrich Company, Poole, UK). The incubation was terminated with EDTA (5 μl of a 0.25 M solution), the samples were centrifuged on spin columns (Boehringer Mannheim, Lewes, UK) for 3 minutes at 1000 *g* for purification of the radio-labelled DNA. The labelled DNA was separated by electrophoresis as above. The gel was vacuum dried, sealed in plastic wrap and exposed to Kodak X-ray film for 0.5 – 12 hours at room temperature.

2.9 Histology of the follicular wall

2.9.1 Preparation of sections for histology

Samples were fixed in buffered formalin for 24 hours and stored in 70% alcohol until processing. Samples were dehydrated by 90% alcohol, 95% alcohol and absolute alcohol followed by cedar wood oil. The samples were transferred to toluene and finally embedded in paraffin wax. Sections (5-7 μm) were cut using a microtome, mounted on gelatin-coated slides and dried overnight in an oven before staining with haematoxylin and eosin. The sections were evaluated at x20, x40 and x100. The sections were stained with Harris

haematoxylin for 1 minute, washed in water for 5 minutes, and washed with Scott's Tap Water Substitute for 30 seconds. Counter staining was done with Putt's eosin for 30 seconds, washed in water for 2 minutes, dehydrated, cleared and mounted in DPX mountant.

2.9.1.1 Histological evaluation of apoptosis

In the earliest stage where apoptosis is recognised, most of the chromatin is aggregated in large compact granular masses that border the nuclear membrane and the cytoplasm is starting to condense. In the next phase, the nucleus fragments and the cell buds to produce membrane-bounded apoptotic bodies of varying sizes and structure, which are phagocytosed by nearby cells and degraded within lysosomes. Where cellular budding has been relatively restricted during the formation of apoptotic bodies, they tend to be only a little smaller than the cells of origin. Where cellular budding has been extensive, the apoptotic bodies vary widely in size. Cell remnants are contained within a membrane after budding and separating from the cell surface, leaving apoptotic bodies of varying sizes. Only the larger apoptotic bodies are seen with a light microscope. It may be difficult to determine whether individual bodies have been phagocytosed or are still extracellular. Inflammation is characteristically absent (Wyllie *et al.,* 1980b; Kerr *et al.,* 1994).

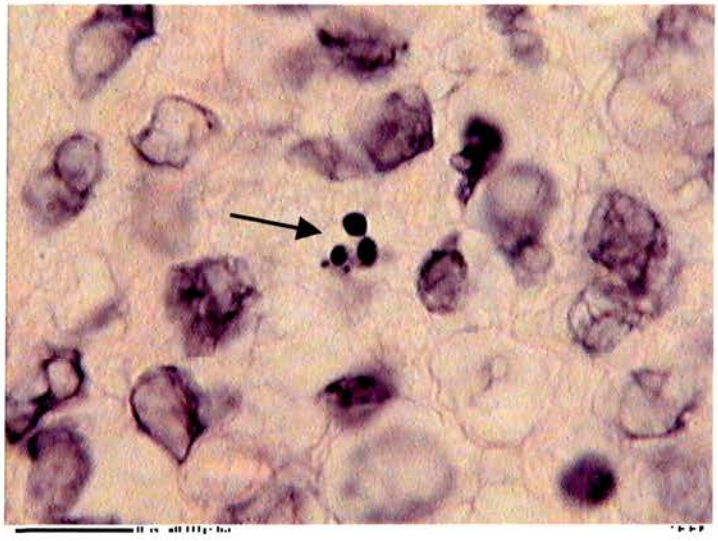


Figure 2.3. Apoptotic theca cell. Arrow: apoptotic nuclear fragments. H&E stain, X100 objective, bar = 10 μm,.

2.10 Evaluation of follicular wall colour

As part of the evaluation of the gross morphology of the follicle, the colour of the inside of the follicular wall was noted. The granulosa cells and basal membrane are transparent, but the theca cells are vascularised in varying degrees, which gives the follicular wall colouring. The colours were White, Pink, Yellow, Rose, Dark Red.

2.11 Granulosa cell morphology at the time of retrieval

Granulosa cells were evaluated with a stereo microscope at the time of retrieval before snap freezing. A grading system was developed.

Granulosa cell grade 0: There were many granulosa cells, and they were in tight connection with each other in large light brown sheets.

Granulosa cell grade 0.5: There were many granulosa cells, and in tight connection with each other but the sheets appeared a little thinner than normal.

Granulosa cell grade 1: There were many granulosa cells, and they were in tight connection with each other in large light brown sheets, but a few small sheets with expanded cells in a yellow gel-like substance (hyaluronic acid) were seen.

Granulosa cell grade 1.5: There were many granulosa cells, and approximately 50% were normal granulosa cells sheets and 50% were expanded sheets with areas of gel with few cells therein.

Granulosa cell grade 2: There were few granulosa cells, some of which were normal but most were expanded. There was a lot of gel-like substance.

Granulosa cell grade 3: Hardly any cells were present and there was either presence or absence of gel.

2.12 Cumulus oophorus evaluation

Cumulus oophorus morphology was classified either as compact, slightly expanded, moderately expanded, very expanded, fully expanded, or denuded using the criteria of (Hinrichs *et al.*, 1993b). The compact cumulus gradually develops into the expanded cumulus. Hyaluronic acid causes the granulosa cells to lose the tight connections with each other. The compact cumulus has tightly connected granulosa cells over and around the oocyte and is smaller than the expanded cumulus. The slightly expanded cumulus has a few granulosa cells protruding from the surface and a slight yellowish colouring in the matrix surrounding the oocyte. The moderately expanded cumulus has more granulosa cells loosely

connected particularly in the periphery, making it have an appearance like a fried egg. The very expanded cumulus has even more granulosa cells expanded but there is still an obvious cumulus. The fully expanded cumulus has granulosa cells expanded all around the oocyte with copious matrix visible between cells, giving it the appearance of a sun. The matrix in between is yellow and gel-like. When the retrieved oocyte lacked part of or all of the cumulus it was classified as denuded of cumulus. A truly denuded oocyte had no cumulus left, possibly due to ageing of the cumulus oocyte complex. The partial denuding of oocytes was possibly caused by the manipulation of the granulosa cells during recovery. See Figure 2.4.

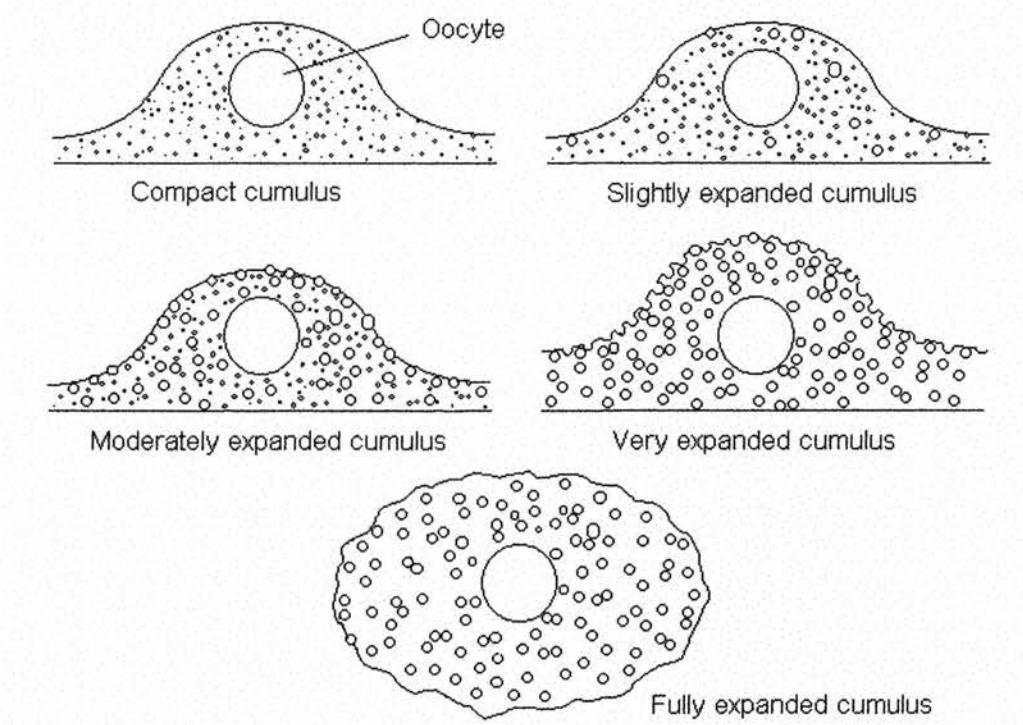


Figure 2.4. Cumulus morphologies.

2.12.1 Oocyte fixation

In chapter 3, different fixatives were used for the oocytes.

2.12.1.1 Paraformaldehyde

To make 4% paraformaldehyde, 66 ml of distilled water and 4 g paraformaldehyde was heated to 60 °C with a stirrer in a fume hood. The solution was cleared (paraformaldehyde dissolved) with 0.1N NaOH added drop by drop. Twenty ml 0.5M Na-phosphate buffer at pH 7.0 was added to give a final 0.1M. Distilled water was added to make a total volume of 100 ml. NaOH was added to adjust pH to 7.0.

2.12.1.2 Buffered formalin

Ten percent formalin was buffered with 9 g NaCl per litre.

2.12.1.3 Acetic acid and methanol

The oocytes were fixed in a solution of 30% glacial acetic acid and 70% methanol with Hoechst added.

2.12.1.4 Chromatin staining

After or at the time of fixation the oocytes were stained with 2.5 µg/ml Hoechst stain 33258 (bisBenzimide) (Sigma-Aldrich Company Ltd, Poole, England). To 25 mg Hoechst 33258 was added 7.5 ml glycerol and 2.5 ml sterile PBS to make a stock solution. This was kept frozen. The final solution was made up of 100 µl stock solution in 75 ml glycerol and 25 ml PBS to a final concentration of 2.5 µg/ml. This solution was kept at –20 °C. 10-14 µl of solution was used per oocyte. The glycerol helped support the oocyte so the cover slip would not squash it, and furthermore it prevented the drying out of the oocyte. The cover slip was sealed onto the slide by nail varnish. The fixed, stained and sealed oocytes can be kept at –20 °C for at least 4 years with no fading of the stain. The chromatin configuration was evaluated under a fluorescence microscope (G365 exciter filter for blue dye) at a magnification of x20, x40 and x100 (oil).

2.13 Oocyte chromatin evaluation

The oogonia are transformed into primary oocytes via leptotene, zygotene, pachytene, and diplotene stages of the first meiotic prophase, where development is arrested. In the diplotene stage of prophase, the chromosomes decondense. At this point, the oocyte chromosomes have duplicated but the nuclear membrane (germinal vesicle) is still intact.

The oocytes will stay suspended at the first meiotic prophase until the follicle and oocyte is selected to grow (Suzuki *et al.*, 1989; Tsafriri *et al.*, 1994). When the oocyte gains meiotic competence, the chromatin goes through phases of organisation. The chromatin configuration changes between the decondensation in the diplotene stage until recondensation into diakinesis and metaphase stages are not well characterised.

The oocytes were either grouped as prophase or metaphase, but with subdivisions. In prophase, oocytes with a large, evenly stained germinal vesicle were classified fluorescent nucleus (FN). If there were spots of condensed chromatin, the term spotty fluorescent nucleus (SFN) was applied. Further condensation of the chromatin with an unevenly stained, regular or irregular large to medium sized fluorescent nucleus was termed of loosely condensed chromatin (LCC). This configuration sometimes had strands of chromatin which were almost lamp brush-like. Diakinesis was a configuration with further condensation and individual chromosomes became visible. At this stage, the germinal vesicle is reported to disappear. The Hoechst stain only occasionally allowed for detection of the outline of the germinal vesicle, depending on the background of the ooplasm. With the germinal vesicle break down, the metaphase stages appear. These consist of prometaphase I, metaphase I, anaphase, prometaphase II and metaphase II. A configuration where the chromatin was condensed to a small, dense mass of chromatin within the germinal vesicle was labelled condensed chromatin. Oocytes were classified as having abnormal or degenerated configurations if clumps or strands of chromatin were scattered at random within the oocytes. Finally, no chromatin described oocytes with no fluorescence present. The chromatin configurations are a continuum rather than distinct steps, and thus there may be overlaps between the different groups. See Figure 2.5.

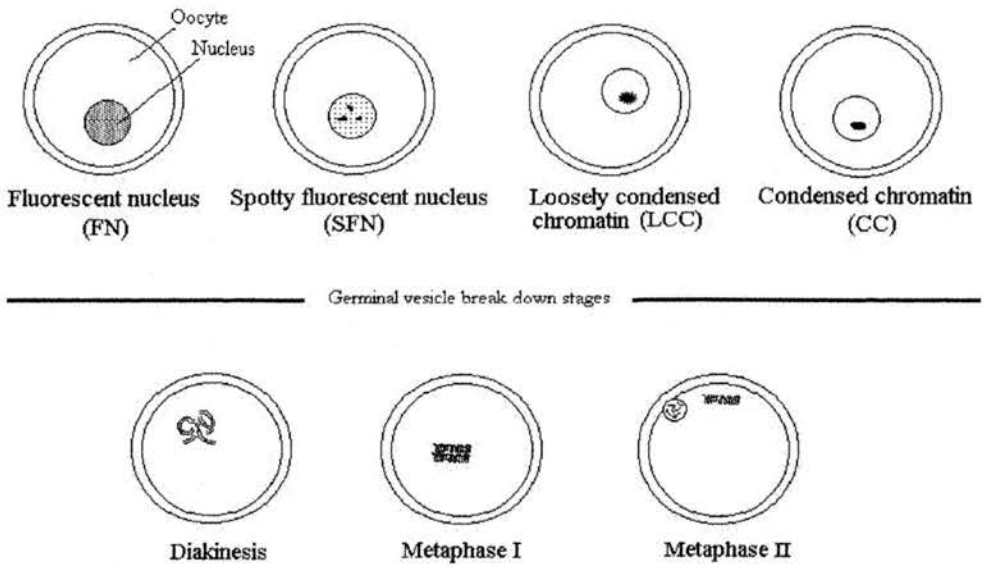


Figure 2.5. Chromatin configurations.

2.14 Hormone assays

Hormone concentrations were measured in plasma and follicular fluid. With the exception of plasma oestradiol, concentrations of hormones were measured by radioimmunoassay directly in plasma without extraction using techniques previously validated for LH (Watson *et al.*, 1995), progesterone (Corrie *et al.*, 1981) and FSH (Roser *et al.*, 1991). For each hormone, displacement curves, which were produced by serial dilutions of plasma and spiking of samples containing low concentrations of the respective hormone, were parallel to the standard curves. Quality controls were plasma collected from mares at different stages of the cycle or hormone spiked assay buffer. All tubes were counted on an LKB-Wallac Clinigamma 1272. The results were calculated by the computer using a LOG Spline Curve Fit.

2.14.1 Follicle Stimulating Hormone RIA assay

In the FSH assay a rabbit anti-human FSH antibody was used (NIDDK-anti-hFSH-6) at a final dilution of 1:10,000. The equine FSH (e265B) standards were prepared by Dr H Papkoff and kindly supplied by Dr J Roser, University of California. Assay sensitivity was

0.5 ng/ml for FSH with intra- and inter-assay coefficients of variation of 6.3 and 10.2%. The average recovery of a known amount of hormone added to equine plasma was 94% for FSH.

2.14.2 Luteinising Hormone RIA assay

The equine LH (e263B) standards were prepared by Dr H Papkoff and supplied by Dr J Roser, University of California. The antiserum for the LH assay was donated by Dr. DL Thompson, Louisiana State University, USA, and was raised in rabbits against equine chorionic gonadotrophin. Ovine LH for radioiodination was obtained from NHPP, NIDDK, HICHHD and USDA, Rockville, MD, USA. Assay sensitivity were 0.6 ng/ml for LH with intra- and inter-assay coefficients of variation of 13.8 and 14%. The average recovery of a known amount of hormone added to equine plasma was 92% for LH.

2.14.3 Oestradiol RIA assay

Concentrations of oestradiol-17β were determined in diethyl ether-extracted plasma samples and directly in follicular fluid samples. Sheep anti-oestradiol-17β was supplied by Prof. S. Hillier, Reproductive Medicine Laboratory, MRC Reproductive Biology Unit, Edinburgh. Assay sensitivity for oestradiol was 8 pg/ml with intra- and inter-assay coefficients of variation of 4.6 and 7.8%. The average recovery of a known amount of hormone added to equine plasma was 91% for oestradiol. All cross-reactivities were less than 0.2%, except for oestrone sulphate which was 8%.

2.14.4 Progesterone RIA assay

Progesterone concentrations were determined directly in plasma using the method of (Corrie *et al.*, 1981) modified by Law *et al.*, (1992). Anti-progesterone antiserum was provided by the Scottish Antibody Production Unit, Carluke. Main cross-reactivities of the antiserum were with 5-pregnan-3,20 dione, 11-deoxycorticosterone and 17-hydroxyprogesterone (9.5%, 6.2% and 3.4% respectively). Progesterone standards were prepared in ovariectomised mare plasma. The sensitivity of the assay was 0.5 ng/ml, and the intra- and inter-assay coefficients of variation were 9.0 and 12.6% respectively.

2.14.5 Testosterone ELISA assay

For the testosterone assay, a polyclonal rabbit antibody raised against testosterone-3-CMO was used (Helena Biosciences, Sunderland, Tyne & Wear). Main cross-reactivity of the antibody was with dihydrotestosterone (12 %). The sensitivity of the assay was 0.08 ng/ml, intra-assay coefficient was 3.7% and the inter-assay coefficient was 11.3%. A displacement curve produced by spiking of samples containing low concentrations of testosterone was parallel to the standard curve. Regression coefficient emerging after spiking of follicular fluid with a known amount of testosterone compared with the recovery was $R^2 = 0.99$, ($P < 0.001$), ($y = 1.15x + 14.9$). The average recovery of a known amount of testosterone added to equine follicular fluid was 95%.

2.14.6 Reagents

Assay buffer

PBSG: Phosphate buffered saline with 0.1% gelatine (0.05M, pH 7.2).

$NaH_2PO_42H_20$: 2.3 g

$Na_2HPO_42H_20$: 6.3 g

Na Cl: 9.0 g

Gelatine: 1.0 g

Thiomersal: 0.1 g

Made up to 1 litre with filtered water (Purite Ltd., Chimnor Road, Thame, UK) and stored at 4 °C.

Dextran coated charcoal (DCC)

Activated charcoal: 12.5 g

Dextran T70: 1.25 g

Made up to 1 litre in assay buffer and stored at 4 °C.

Reagents were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, UK.

2.15 Statistical analysis

In chapters 3, 4 and 6 mainly frequencies were analysed and a Chi-square test was appropriate. In chapter 5 different tests were used, which are described in materials and methods of that particular chapter.

Data for investigation of an association between frequency of parameters were analysed with a chi-square test. When the data was arranged in one row (e.g. frequency of apoptosis over 4 days), a one way classification was used, and a chi-square analysis performed. To analyse frequencies of observations between categories (apoptosis and health between day 6 and day 11) the data was put into a contingency table, using a two-way classification, and a chi-square contingency analysis was performed. In cases where data was presented in a 2 by 2 table, Yates' correction for continuity was applied to avoid the calculated value of the test statistic being too high. Where there were more than 2 rows and columns, the data was put into an r x c contingency table. To find the expected frequency for a cell in a table, the total of a column was multiplied with the total of a row and divided by the product of the grand total. Degrees of freedom was calculated as number of columns minus one, multiplied number of rows minus one. Once the calculated value and the degrees of freedom were obtained, a chi-square table was consulted in order to find the level of significance (Fowler *et al.*, 1998).

Conditions for test for a 2 x 2 contingency table: total observations should be at least 40. When total observations were between 20-40, the chi-square test was used if calculated frequencies were more than 5. If any cell's expected frequency was less than 5, a Fisher test was used. The Fisher exact probability test is a non-parametric test for use when sample sizes were small. Data was presented in a 2 x 2 contingency table. Tocher's modification may or may not have been used (Fowler *et al.*, 1998). When degree of freedom was larger than 1, the Chi-square test required that no more than 20% of cells had an expected frequency of less than 5, and that no cell had an expected frequency less than 1. If the data did not fulfil these requirements, columns were added together to obtain larger values (Siegel, 1956).

The null hypothesis of the statistical test assumed that there was no difference. A P-value of 0.05 or less was considered significant.

3 Oocyte chromatin configuration and cumulus oophorus morphology

3.1 Introduction

Growth of the follicle and the simultaneous oocyte growth and acquisition of meiotic competence is a prerequisite for fertilisation. Oocyte maturation and cumulus expansion are processes that involve reorganisation of the cumulus cells and the oocyte chromatin. Chromatin configuration in the germinal vesicle stage in rodent oocytes has been studied in detail (Albertini, 1984; Mattson *et al.*, 1990; Wickramasinghe *et al.*, 1991), but so far only one report specifically tried to map the changing chromatin configurations in immature equine oocytes before in vitro maturation (Hinrichs *et al.*, 1993a). Investigators primarily study in vitro maturation and thus are concerned about the stages after germinal vesicle breakdown rather than the more immature stages. The oocytes therefore are often only classified as germinal vesicle intact or after germinal vesicle breakdown. This does not take into account the effect of the different stages of meiotic competence of the germinal vesicle intact oocyte. Several investigators work with equine oocytes, but use different methods and terminology to assess the oocytes and cumulus (Shabpareh *et al.*, 1993; Torner *et al.*, 1995; Goudet *et al.*, 1997; Hinrichs *et al.*, 1997). In order to better compare findings between groups, it is necessary to standardise the terms for cumulus morphology and nuclear configuration and to evaluate effects of fixatives and holding time, which vary with different workers.

A thorough study into the connection between cumulus morphology and chromatin configuration is needed. When estimating success of maturation of oocytes, investigators initially classify the cumulus as compact or expanded, because it is not possible to evaluate chromatin configuration before maturation without removing the cumulus. Ideally, the same oocyte should be stained before and after maturation, but at the moment staining of the oocyte involves either fixation and sectioning or denuding and staining. The UV light used when illuminating the stained chromatin is likely to be detrimental to the oocyte, and therefore it may not be capable of maturing after having been exposed to UV light.

The oogonia are transformed into primary oocytes via leptotene, zygotene, pachytene, and diplotene stages of the first meiotic prophase, where development is arrested. In the diplotene stage of prophase, the chromosomes decondense. At this point, the oocyte

chromosomes have duplicated but the nuclear membrane (germinal vesicle) is still intact. The oocytes will stay suspended at the first meiotic prophase until the follicle and oocyte is selected to grow (Suzuki *et al.*, 1989; Tsafriri *et al.*, 1994). As the oocyte prepares for meiotic division, the chromatin goes through phases of organisation. Meiotic maturation is characterised by dissolution of the germinal vesicle membrane (germinal vesicle break down), condensation of chromatin through metaphase I, anaphase I and telophase I, emission of the first polar body and arrest of meiosis with the chromosomes aligned on the metaphase II spindle (Suzuki *et al.*, 1989; Wassarman *et al.*, 1994). The chromatin configurations in the meiotic prophase in the horse are not fully elucidated. Some authors have reported several different configurations (both diffuse but also condensed chromatin) within the germinal vesicle (Fulka, Jr. 1981; Hinrichs *et al.*, 1993a; Grondahl *et al.*, 1995a; Torner *et al.*, 1995). Follicles that become atretic may contain oocytes, which show configurations usually related to acquisition of meiotic competence, and in late atresia meiosis-like changes appear. This includes germinal vesicle break down, alignment of the chromosomes in metaphase and expulsion of a polar body. In women synchronous degeneration of oocyte and granulosa cells occurred. In sheep, oocytes from atretic follicles underwent parthenogenesis and developed into blastocysts (Greenwald *et al.*, 1994). In horses, atretic follicles contained more oocytes with chromatin associated with resumption of meiosis. Actual meiosis, including metaphase II, was seen only in oocytes from the most atretic follicles (Hinrichs *et al.*, 1997). Resumption of meiosis was also shown to occur when oocytes were removed from the inhibiting effect of the follicle (Pincus *et al.*, 1935), pointing at the possibility of meiosis-subduing factors disappearing in atretic follicles.

Few studies have investigated the relationship in vivo between cumulus expansion and chromatin configuration in the mare. The highest proportion of prophase oocytes was found in compact cumulus oocytes (Torner *et al.*, 1995; Hinrichs *et al.*, 1997 and 2000). In atretic follicles, cumulus expansion was more frequent than in non-atretic follicles (Hinrichs *et al.*, 1997). Cumulus expansion was not related to the normal preovulatory expansion of the cumulus in these follicles, but was a sequel to atresia. In the mare, some studies indicated that oocytes with expanded cumulus cells at the time of retrieval from the follicle matured to metaphase II at higher rates and more rapidly than oocytes with compact cumulus cells (Zhang *et al.*, 1989; Hinrichs *et al.*, 1997), but other studies could not confirm these findings (Goudet *et al.*, 1997).

In this chapter an attempt was made to standardise techniques used for processing oocytes. The aim was to find out which storage conditions affected, and could potentially be detrimental to the oocyte, as this was of importance for the following chapters, where optimal conditions were needed in order to avoid artefactual results, which could lead to wrong conclusions. Based on my findings a hypothesis was developed as to how the chromatin changed in the prophase in the oocyte within the follicle, as this is an area of research, which is still lacking in information. Many different chromatin configurations exist, and little knowledge of what they signify is available. The effect of cumulus expansion on the chromatin configuration was determined in order to establish the relationship between the two parameters.

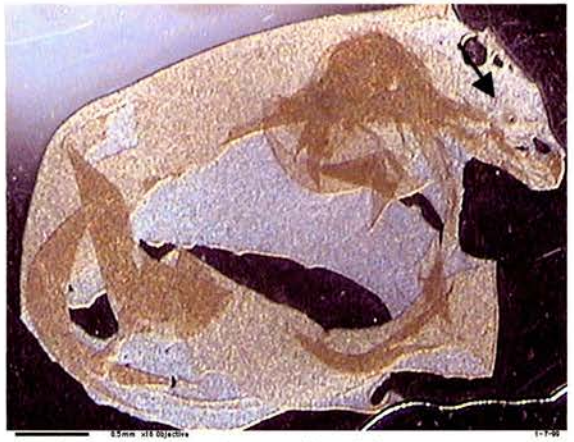
3.2 Materials and methods

The present study was a retrospective study and hence the numbers of oocytes and cumuli in each group varied greatly. Ovaries were obtained from horses and ponies after slaughter or after ovariectomy. The animals weighed between 198-600 kg, and were aged between 2-30 years. The exteriorised ovaries were kept at 20 - 30 °C or 35-37 °C in M199 with Hanks salts and 25 mM Hepes for 0.5 - 24 hours. Holding temperature and time is indicated for each experiment. Processed follicles ranged from 2 – 60 mm in diameter. The follicle was cut open and the inner wall was scraped with a bone curette to release both granulosa cells and the cumulus oophorus with the oocyte. The granulosa cells were flushed off with M199 with Hank's salts. After examination of the cumulus oophorus using a stereomicroscope, the oocyte was denuded by pipetting with smaller and smaller bore pipettes (0.2 - 0.6 mm in diameter) either with or without a solution of 0.25% trypsin/EDTA. The oocytes were kept in polybuffered sulphate or Medium 199 with Hanks salts and 25 mM Hepes during denuding.

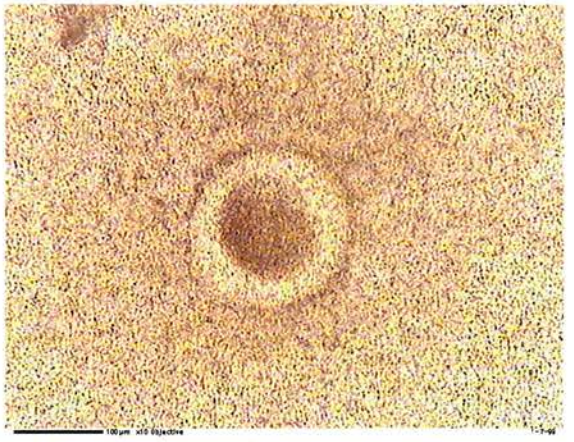
Cumulus oophorus morphology was classified either as compact, slightly expanded, moderately expanded, very expanded, fully expanded, or denuded using the criteria of (Hinrichs *et al.*, 1993b). See chapter 2 for further detail and colour atlas page 45.

After or at the time of fixation the oocytes were stained with 2.5 μg/ml Hoechst stain 33258 (bisBenzimide) apart from experiment 3. The chromatin configuration was evaluated under a fluorescence microscope (G365 exciter filter for blue dye) at a magnification of x20, x40 and x100 (oil).

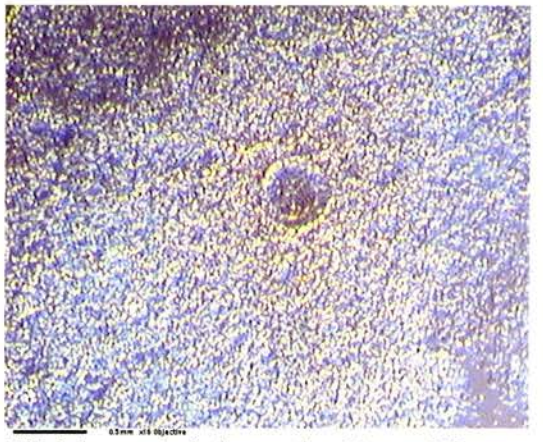
The oocytes in the present study were either grouped as prophase or metaphase, but with subdivisions (see chapter 2 for further detail and colour atlas pages 46 - 50).



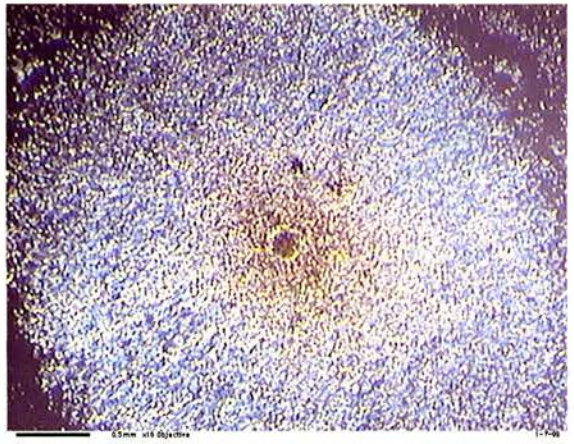
Sheet of granulosa cells with compact cumulus oocyte (arrow). Bar = 2 mm.



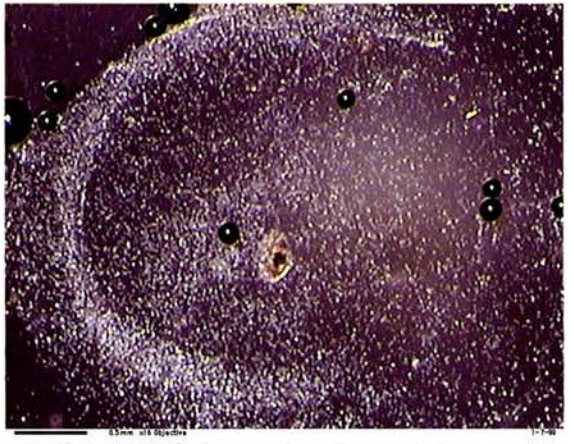
Compact cumulus. Bar = 100 μm.



Slightly expanded cumulus. Bar = 0.5 mm.

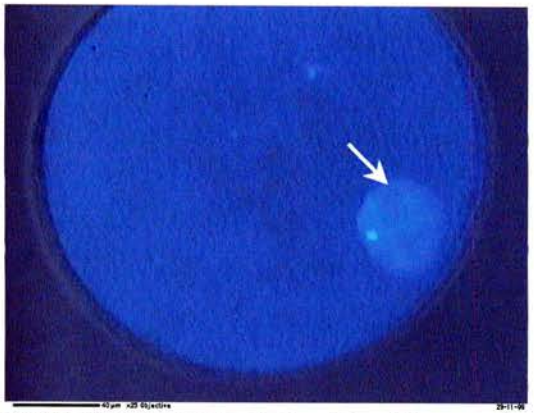


Moderately expanded cumulus. Bar = 0.5 mm.

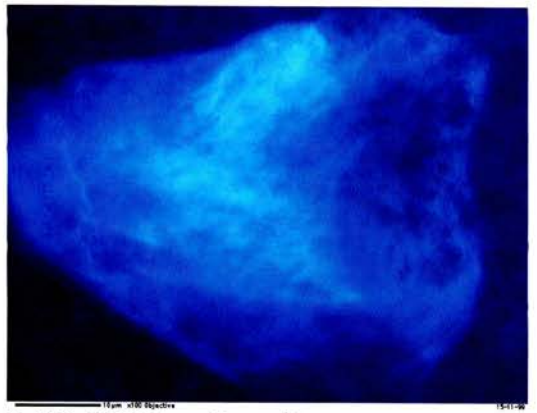


Fully expanded cumulus. Bar = 0.5 mm.

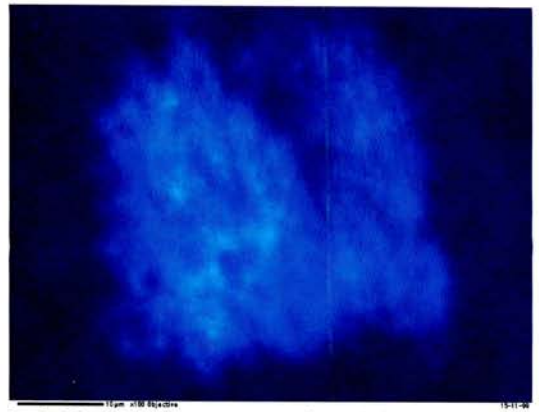
Fully expanded cumulus. Bar = 100 μm.



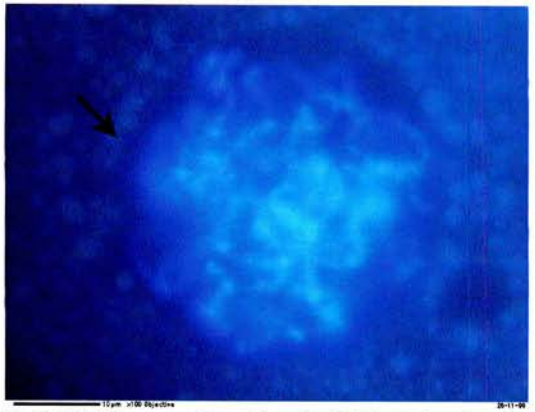
1 : FN. Fluorescent germinal vesicle with one spot. Bar = 40 μm.



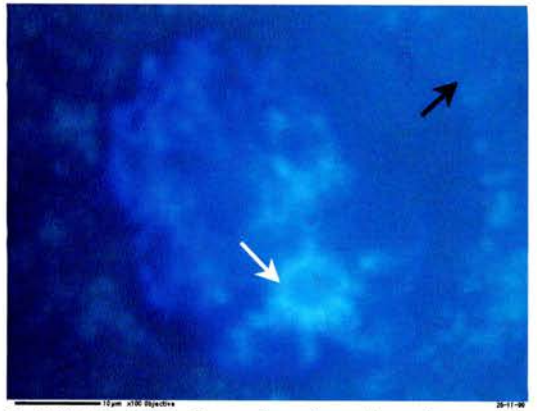
2 : FN - LCC, large. Bar = 10 μm.



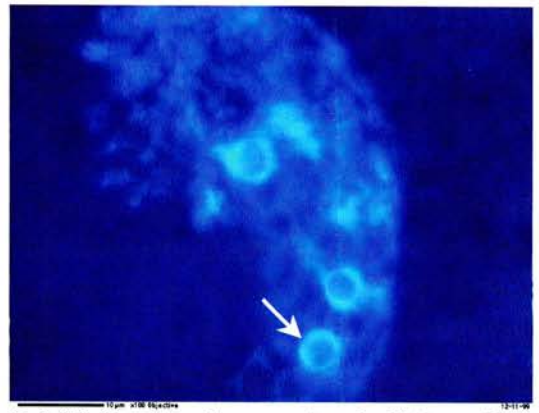
3 : LCC, large, but more condensed than no. 3. Bar = 10 μm.



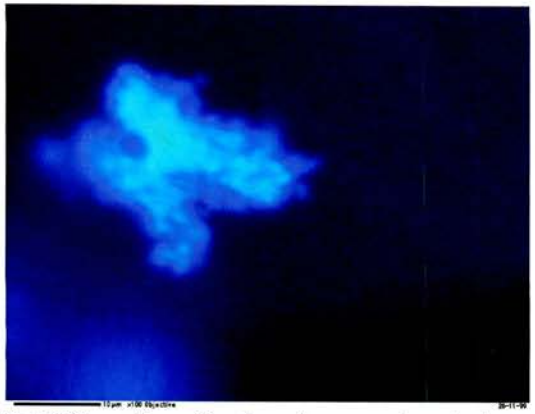
4 : LCC. More condensation, individual strands of chromatin are visible. Note outline of the germinal vesicle. Bar = 10 μm.



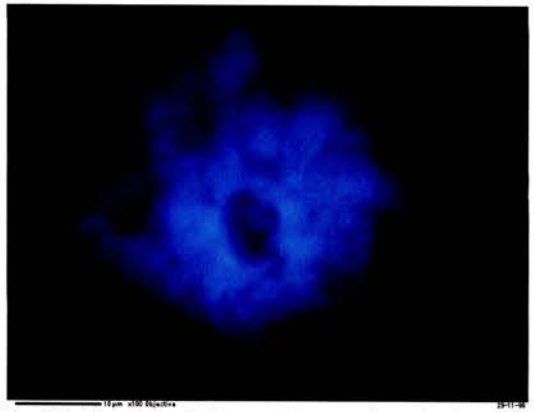
5 : LCC. Individual strands and condensation around nucleoli. Note outline of germinal vesicle. Bar = 10 μm.



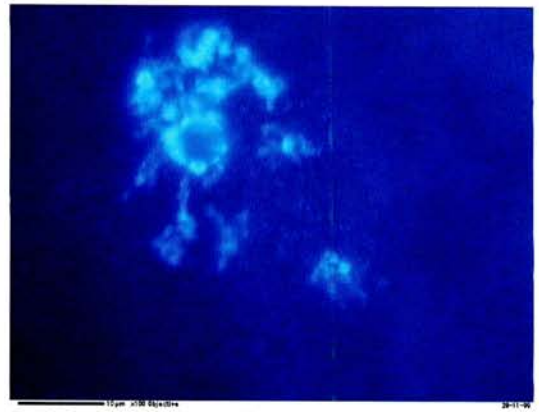
6 : LCC. Condensation around nucleoli. Bar = 10 μm.



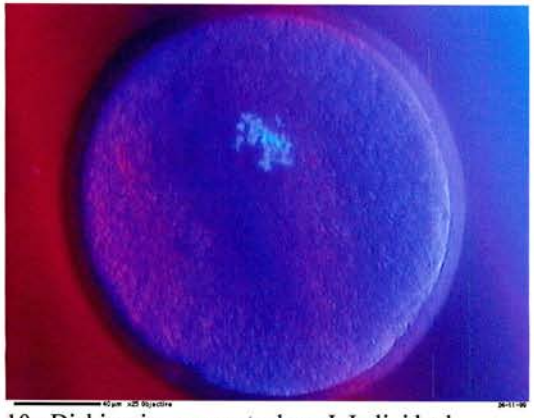
7 : LCC, medium. Condensation around nucleolus. Bar = 10 μm.



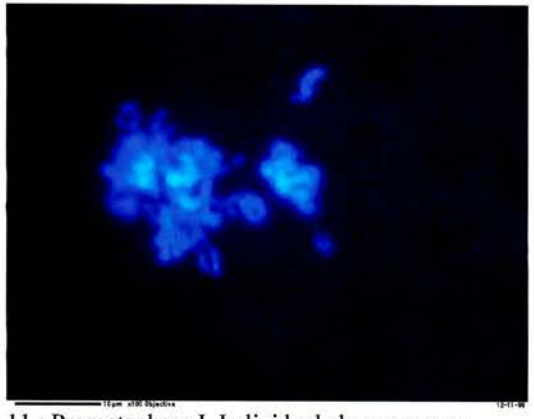
8 : CC. Condensed chromatin around nucleolus. Bar = 10 μm.



9 : CC – diakinesis. Individual chromatin strands condensing and becoming visible. Bar = 10 μm.

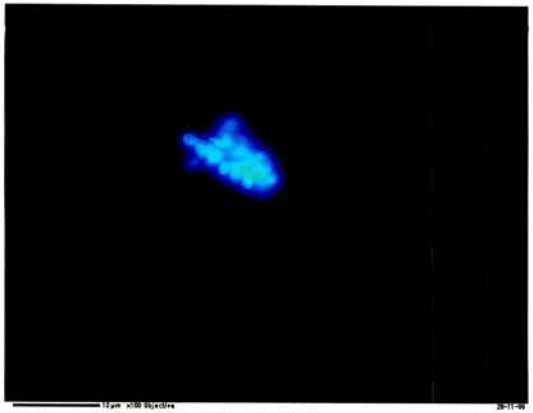


10 : Diakinesis – prometaphase I. Individual chromosomes. Nucleolus no longer visible. Bar = 40 μm.

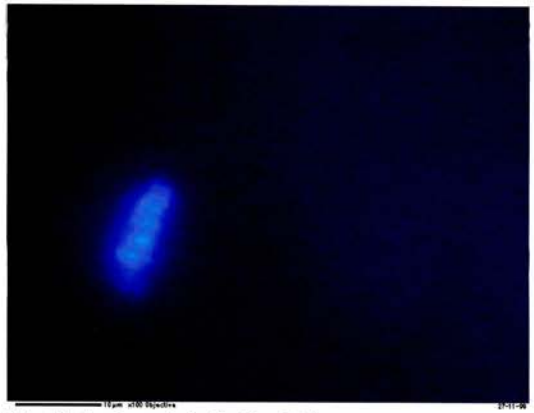


11 : Prometaphase I. Individual chromosomes becoming visible. Bar = 10 μm.

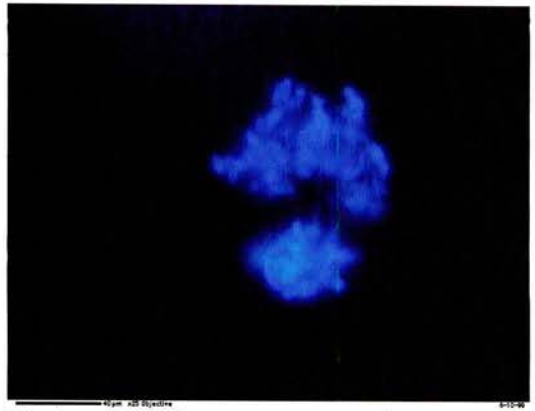
12 : Prometaphase I. Bar = 40 μm.



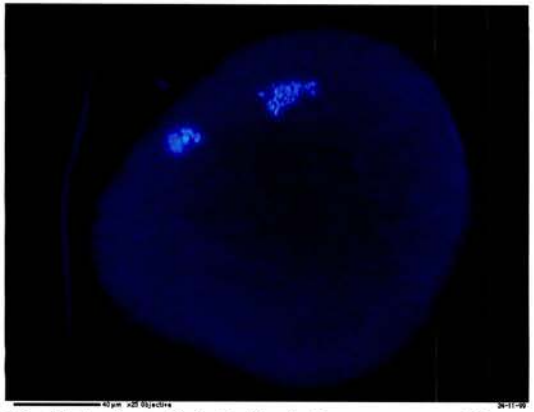
13 : Metaphase I. Individual chromosomes lining up. Bar = 10 μm.



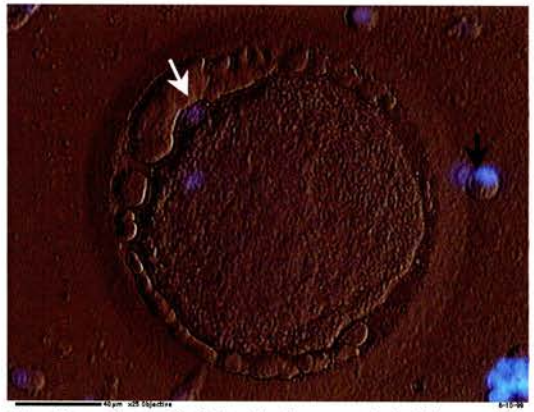
14 : Metaphase I. Individual chromosomes on a line. Bar = 10 μm.



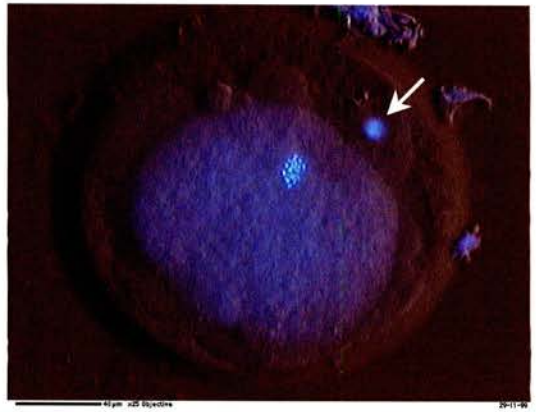
15 : Anaphase. Chromosomes separating. Bar = 10 μm.



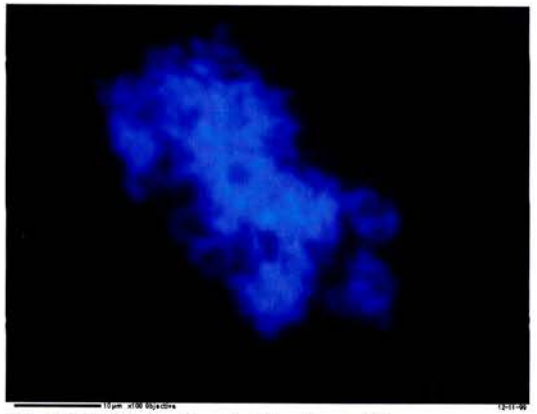
16 : Metaphase II. Individual chromosomes still visible. Polar body forming. Bar = 40 μm.



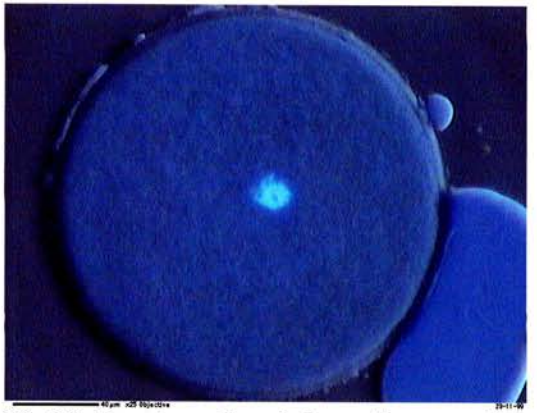
17 : Metaphase II. Polar body extruded. Chromatin from granulosa cell outside the oocyte. Bar = 40 μm.



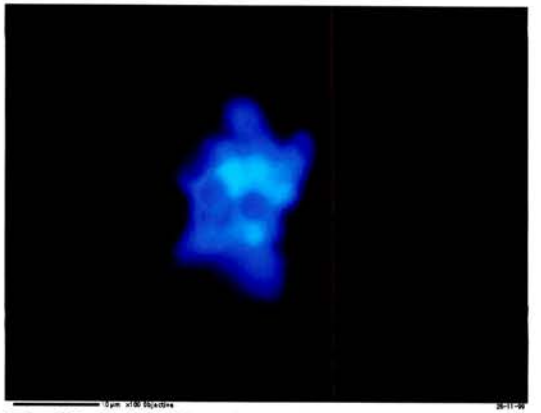
18 : Metaphase II. Individual chromosomes in the ooplasma and polar body extruded (out of focus). Bar = 40 μm.



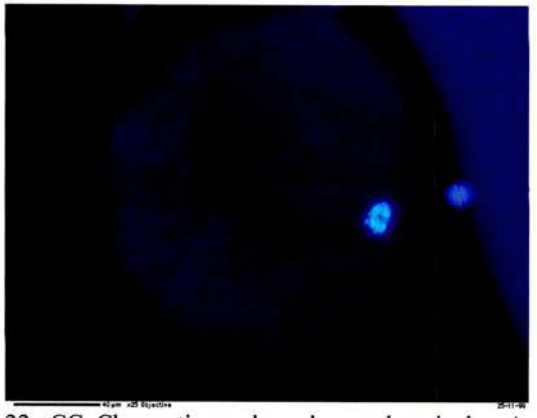
19 : LCC. Nucleolus visible. Bar = 10 μm.



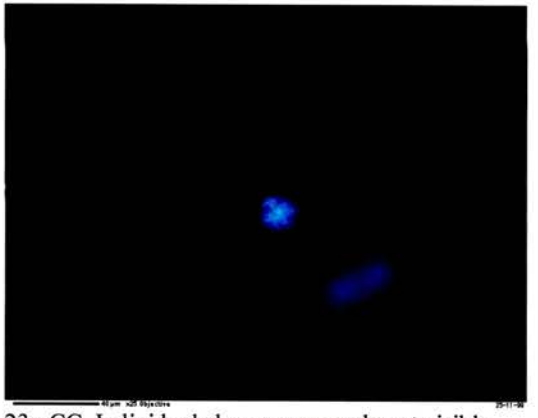
20 : CC. Densely condensed chromatin surrounding nucleolus. Bar = 40 μm.



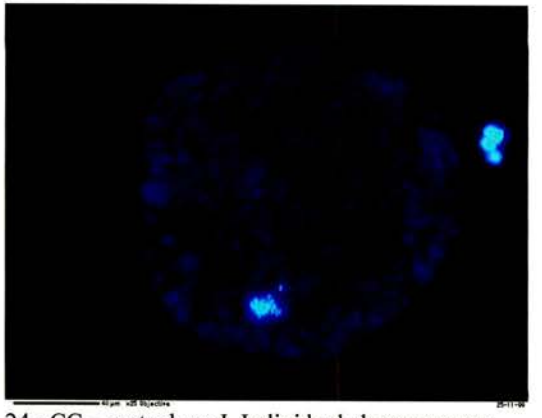
21 : CC. Condensing chromatin surrounding nucleolus. Bar = 10 μm.



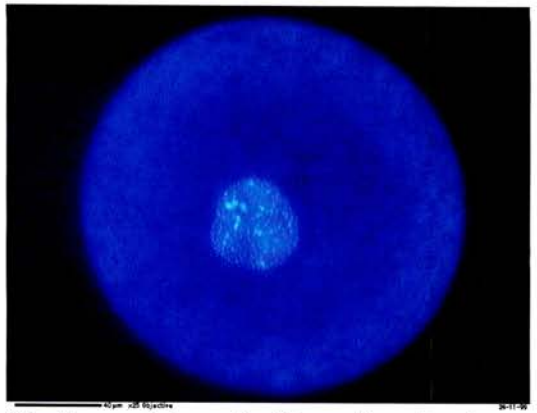
22 : CC. Chromatin condensed around nucleolus. A granulosa cell is visible. Bar = 40 μm.



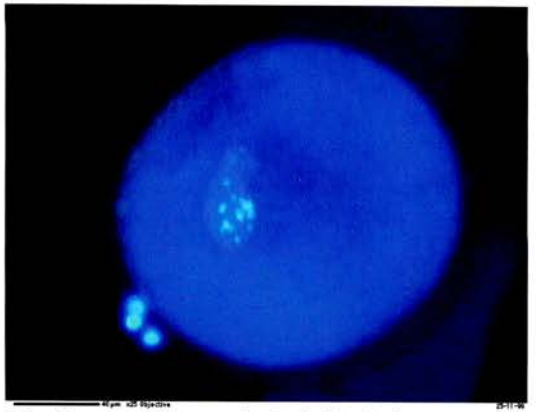
23 : CC. Individual chromosomes almost visible. Bar = 40 μm.



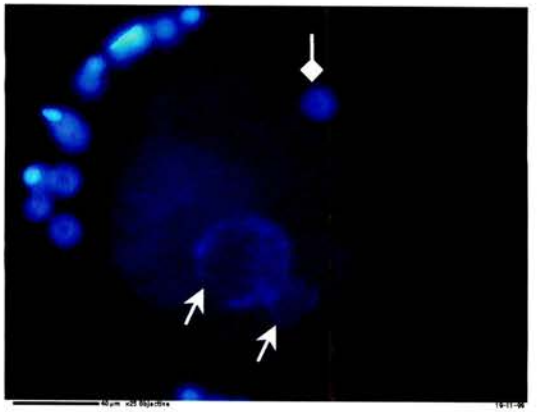
24 : CC – metaphase I. Individual chromosomes becoming visible. Bar = 40 μm.



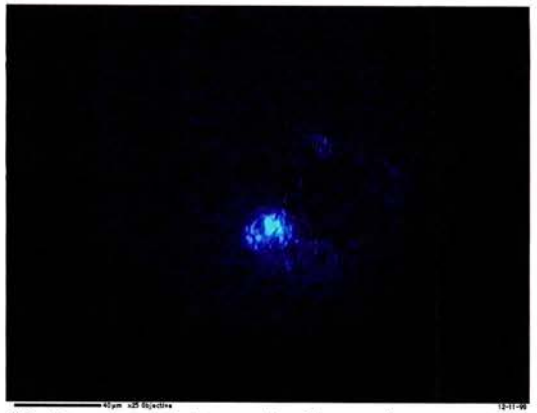
25 : Abnormal chromatin. Chromatin scattered within the germinal vesicle. Bar = 40 μm.



26 : Abnormal chromatin. Individual chromosomes visible within the germinal vesicle. Bar = 40 μm.

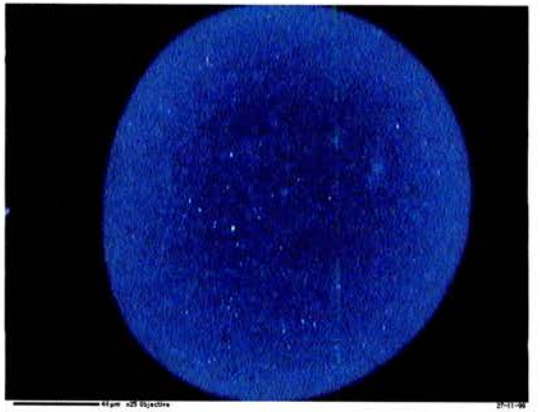


27 : Parthenogenesis. Two pronuclei and a polar body. Bar = 40 μm.



28 : Degenerated chromatin. Chromatin scattered within the ooplasma. Bar = 40 μm.

29 : Degenerated chromatin. Chromatin scattered within the oocyte. Bar = 40 μm.



30 : No chromatin. No chromatin visible within the oocyte. Bar = 40 μm.

This study consisted of 5 small experiments and an examination of the data on cumulus morphology in relation to chromatin configuration. As the study was a retrospective study, numbers of oocytes or cumuli in each group vary. To avoid interference from different treatments at one particular parameter, an arbitrary baseline was chosen to be follicles, which had been stored for less than 3 hours and whose oocytes had been fixed in buffered formalin. At first the effect of temperature was investigated on both chromatin and cumulus, then based on these results follicles were selected for studying the effect of time, avoiding any confounding effects of temperature. Follicles were selected based on the findings in the two previous experiments to examine the effect of fixative on oocyte chromatin. Finally, based on all the previous experiments' findings of which conditions may induce artefactual results, the chromatin and cumulus relation was investigated.

Slaughterhouse material was more abundant than ovaries from ovariectomised mares, and therefore slaughterhouse ovaries were stored for longer periods and at different temperatures as well as the same time periods and temperatures as the surgically removed ovaries. Surgically removed ovaries were not stored at temperatures less than 30 °C and storage time was kept at a minimum, but processing time stretching over several hours was inevitable with ovaries with many follicles. Time from retrieval of the ovary until processing of each specific follicle was recorded. Fixation methods varied equally between slaughterhouse and surgically removed ovaries. Slaughterhouse ovaries were obtained in March – June, and surgically removed ovaries were recovered in May – October.

3.2.1 Experiment 1: The effect of temperature on chromatin configuration

A total of 73 oocytes were examined. Oocytes from ovaries stored at 20 - 30 °C (n = 14) or 35 - 37 °C (n = 59) were used. To avoid confounding effects of time, only oocytes that had been sitting for less than 3 hours in the follicles were selected. To avoid any effect of the fixation method only oocytes that were fixed in 1 ml of buffered formalin were used. Chromatin configuration was evaluated in order to assess whether temperature had any effect on the chromatin configuration.

3.2.2 Experiment 2: The effect of time on chromatin configuration

Oocytes (n = 222) were left in the follicles at 20 – 37 °C for varying lengths of time ranging from 30 minutes to 12 hours after exteriorisation of the ovary. Oocytes were grouped as 0-1

(n = 23), >1-2 (n = 35), >2-3 (n = 38), >3-4 (n = 34), >4-6 (n = 44), >6-8 (n = 34), and >8-12 (n = 14) hours. To avoid any effect of the fixation method, only oocytes that were fixed in 1 ml of buffered formalin were used. Chromatin configuration was evaluated to detect any differences in chromatin configuration in relation to storage time.

3.2.3 Experiment 3: The effect of fixation method on chromatin configuration

Oocytes (n = 345) from follicles kept between 20-37 °C for less than 6 hours were fixed in either

a. 1 ml 4% paraformaldehyde (n = 30)
b. 1 ml 10% buffered formalin (n = 165)
c. 15 µl 10% buffered formalin with 2.5 µg/ml Hoechst directly on a glass microscope slide (n = 23)
d. 15 µl 10% buffered formalin with glycerol (3:1) and 2.5 µg/ml Hoechst directly on a glass microscope slide (n = 93)
e. 15 µl acetic acid and methanol (1:3) with 2.5 µg/ml Hoechst directly on a glass microscope slide (n= 16)
f. 15 µl follicular fluid with 2.5 µg/ml Hoechst directly on a microscopy slide (no fixative) (n = 18).

The oocytes fixed in a large volume with no stain in the fixative, were subsequently stained with 2.5 µg/ml Hoechst in PBS and glycerol (1:3). The different treatments were used in large blocks of time and thus in several animals and at periods of time stretching over the whole season. Treatment c and e was performed within the same animals, alternating between the two methods for every second oocyte. Treatment e was only performed in March and in September. Chromatin configuration was evaluated to assess the effect of fixative on the oocyte.

3.2.4 Experiment 4: The effect of temperature on cumulus morphology

Cumulus oocyte complexes (n = 74) were recovered from follicles within 2 hours of exteriorisation. The follicles had been kept at 20 - 30 °C (n = 34) and 35 - 37 °C (n = 40). Morphology of the unfixed cumulus was evaluated to determine the effect of temperature.

3.2.5 Experiment 5: The effect of time on cumulus morphology

Cumuli (n = 298) from follicles kept at 35 - 37 °C for 30 minutes to 10 hours were examined morphologically to detect effects of time on cumulus expansion.

3.2.6 The relationship between cumulus morphology and chromatin configuration

Cumulus oocyte complexes (n = 149) were retrieved from follicles which had been stored for 0.5 - 6 hours at 35 - 37 °C. Cumulus morphology and chromatin configuration were evaluated to detect any relationship between the two. The oocytes were fixed in buffered formalin or paraformaldehyde.

3.3 Statistical analysis

The effects of the changing parameters (temperature, time, fixation) on oocyte chromatin configuration and cumulus morphology were analysed by a Chi-square test or a Fisher test in case of small sample numbers (Fowler *et al.*, 1998). To analyse frequencies of observations between categories (e.g. chromatin configuration after different lengths of storage) the data was put into a contingency table, using a two-way classification, and a chi-square contingency analysis was performed. In cases where data was presented in a 2 by 2 table, Yates' correction for continuity was applied to avoid the calculated value of the test statistic being too high. The Fisher exact probability test is a non-parametric test, which was used when sample sizes were small. Data was presented in a 2 x 2 contingency table. The null hypothesis of the statistical test assumed that there was no difference. A P-value of 0.05 or less was considered significant. See chapter 2 for further detail.

3.4 Results

3.4.1 Experiment 1: The effect of temperature on chromatin configuration

Holding of ovaries at either 20-30 °C or 35-37 °C did not significantly affect ($P > 0.05$) chromatin configuration in oocytes, which were fixed in buffered formalin within 3 hours of the ovary leaving the animal. See Table 3-1.

Table 3-1. Chromatin in oocytes from ovaries held at different temperatures

Chromatin (%)	n	FN - SFN	LCC - diakinesis	CC
20-30 °C	14	0.0	64.3	35.7
35-37 °C	59	1.7	78.0	20.3

There was no significant difference in chromatin distribution between oocytes held at different temperatures.

3.4.2 Experiment 2: The effect of time on chromatin configuration

Oocytes were grouped as storage times 0-1, >1-2, >2-3, >3-4, >4-6, >6-8, and >8-12 hours. There was no difference ($P > 0.1$) in oocyte chromatin configuration within the first 6 hours. The configurations started to change from >4 hours, but were not significantly affected until after 6 hours. There was a difference between 0-6 and >6-12 hours ($P < 0.001$). There was no effect of time between 6-8 and >8-12 hours ($P > 0.1$). The major changes between 0-6 hours and >6-12 hours were detected in the FN configuration ($P < 0.01$), which increased with time whereas LCC decreased ($P < 0.05$). Condensed chromatin, metaphase stages and no chromatin oocytes were unaffected by time, but significantly more abnormal configurations were seen as time passed ($P < 0.01$). The relationship between chromatin configuration and time suggested that the LCC configurations changed into abnormal configurations and FN due to holding time. See Figure 3-1.

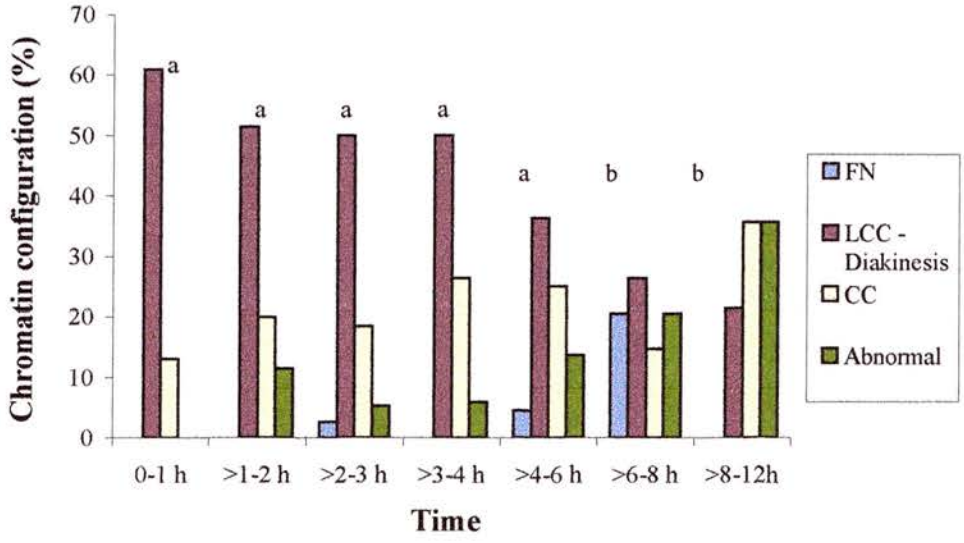


Figure 3-1. The effect of time on chromatin configuration. There was a significant change between 0-6 and >6-12 hours. Numbers of oocytes: 0-1 h (n = 23), >1-2 h (n = 35), >2-3 h (n = 38), >3-4 h (n = 34), >4-6 h (n = 44), >6-8 h (n = 34), >8-12h (n = 14). Letters a and b denote difference ($P < 0.001$).

3.4.3 Experiment 3: The effect of fixation method on chromatin configuration

Because oocyte chromatin configurations changed after storage in the follicle for 6 hours, only oocytes that were retrieved earlier than 6 hours were used. There was a significant difference ($P < 0.001$) in frequencies of LCC and diakinesis and condensed chromatin configurations between the different fixation methods. Fixing oocytes in paraformaldehyde versus buffered formalin made no difference ($P > 0.1$). There was a tendency towards paraformaldehyde having a higher proportion of oocytes in the FN and LCC configurations resulting in fewer condensed chromatin oocytes. There was no difference ($P > 0.1$) between fixing oocytes in the small volumes of buffered formalin with Hoechst and buffered formalin with glycerol and Hoechst, but there was a difference between fixing the oocytes in 1 ml buffered formalin and 15 in µl buffered formalin with Hoechst ($P < 0.01$). See Figure 3-2.

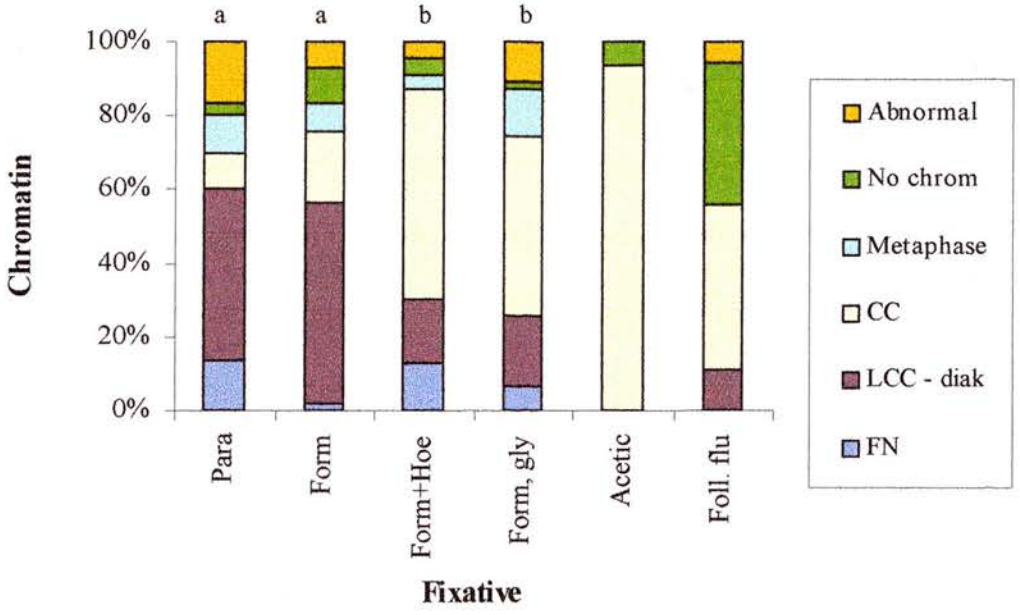


Figure 3-2. The effect of fixation method on chromatin configuration. There was a significant difference between fixing oocytes in large volumes of fixative compared to small volumes. There was no significant difference between fixing in paraformaldehyde and formalin. The acetic acid and follicular fluid groups did not contain enough oocytes to allow inclusion in the statistical test, but frequencies are shown. Number of oocytes in each group: Paraformaldehyde (n = 30), Formalin (n = 165), Formalin + Hoechst (n = 23), Formalin + glycerol + Hoechst (n = 93), Acetic acid + Hoechst (n = 16), Fol. fluid + Hoechst (n = 18). Abbreviations: para, paraformaldehyde, 1 ml; form, buffered formalin, 1 ml; form+hoe, buffered formalin and Hoechst, 15 µl; form+gly, buffered formalin and glycerol with Hoechst, 15 µl; acetic, acetic acid with Hoechst, 15 µl; foll flu, follicular fluid with Hoechst, 15 µl. Letters a and b denote difference ($P < 0.01$).

3.4.4 Experiment 4: The effect of temperature on cumulus morphology

Storing the ovaries for less than 2 hours at 20 - 30 °C compared to 35 - 37 °C affected cumulus morphology ($P < 0.001$). Cumulus cells held at temperatures lower than 30 °C were more expanded and more oocytes had lost part of the cumulus than those held at 35 - 37 °C (Figure 3-3).

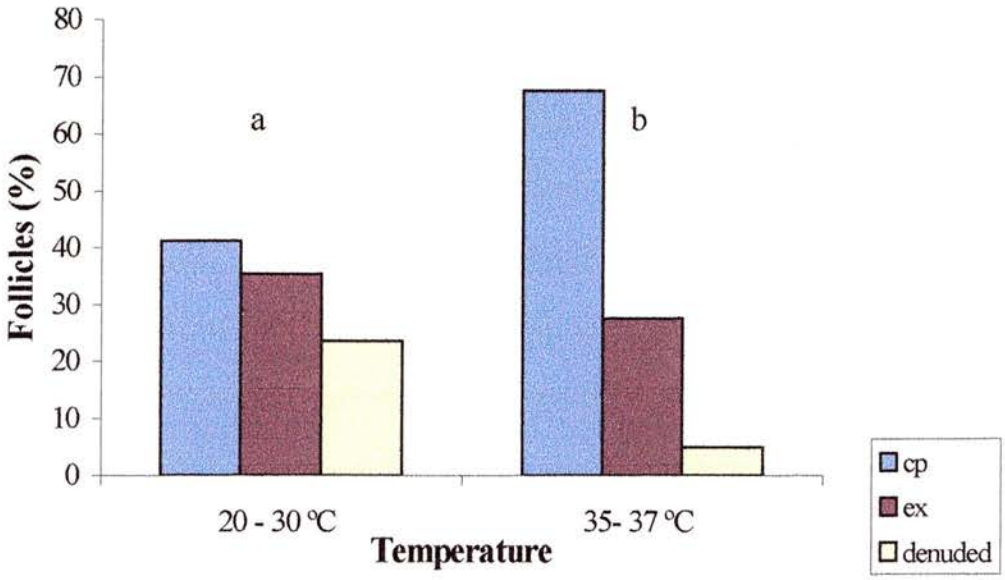


Figure 3-3 Effect of temperature on cumulus morphology. Cumulus cells held at temperatures lower than 30 °C were more expanded than those held at 35 - 37 °C. Numbers of cumuli in each group: 20 – 30 °C (n = 34), 35 – 37°C (n = 40). Letters a and b denote difference ($P < 0.001$).

3.4.5 Experiment 5: The effect of time on cumulus morphology

Because temperature had an effect on cumulus morphology, only cumuli kept at 35 - 37°C were studied. There was a difference ($P < 0.05$) between the cumulus at 0-2 and >2-4 hours, suggesting that cumulus expansion started almost immediately. There was no difference ($P > 0.1$) between the cumulus at >2-4, >4-6 and >6-10 hours. From 0-2 hours onwards there were decreasing numbers of compact cumuli, and increasing numbers of expanded cumuli, with the most expansion in the >6-10 hour group (Figure 3-14).

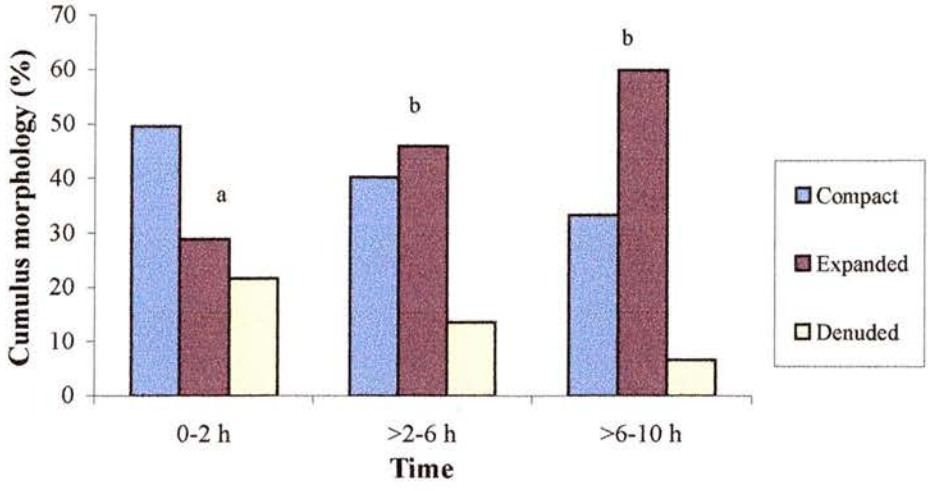


Figure 3-4. The effect of time on cumulus morphology. There was a significant increase in cumulus expansion as time went on. Number of cumuli: 0-2 h (n = 111), >2-6 h (n = 169), >6-10 h (n = 15). Letters a and b denote difference ($P < 0.05$).

3.4.6 The relationship between cumulus morphology and chromatin configuration

There was a change ($P < 0.001$) in distribution of chromatin configuration with changing cumulus morphology. Compact cumulus was the only group, which contained oocytes with a fluorescent nucleus. With increasing cumulus expansion, the chromatin configurations indicated a maturation of the oocyte because there were increasing frequencies of diakinesis and metaphase stage oocytes. Of fully expanded cumuli, 41.7% contained a metaphase stage oocyte and 25% contained a diakinesis oocyte. Condensed chromatin appeared to increase with cumulus expansion. In the fully expanded cumulus, CC frequency decreased, perhaps because the chromatin configuration had moved on to either diakinesis or metaphase stages (Figure 3-5).

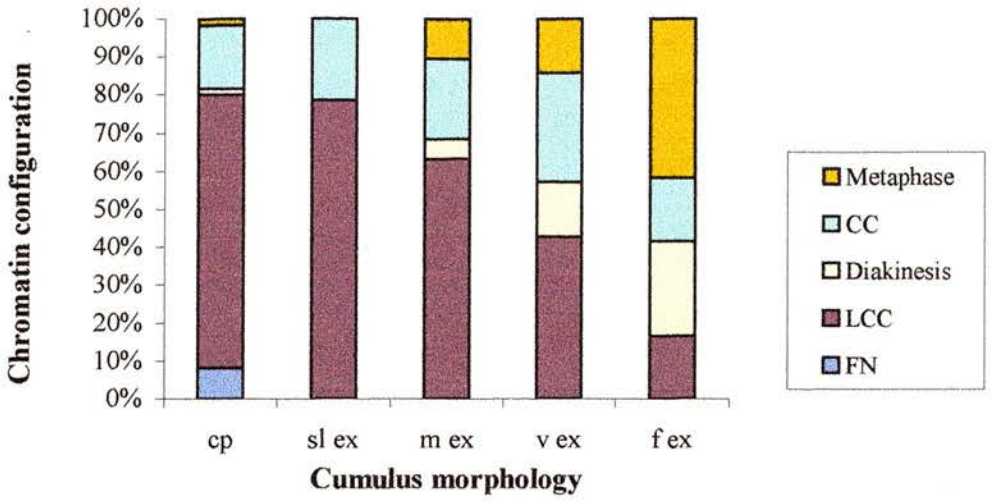


Figure 3-5. Chromatin configuration in relation to cumulus morphology. An overall Chi-square test revealed a significant change in chromatin with changing cumulus ($P < 0.001$). Numbers of oocytes: Compact cumulus (n = 60), Slightly expanded (n = 14), Moderately exp. (n = 19), Very expanded (n = 7), Fully expanded (n = 12)

3.5 Discussion

The present study indicated that the temperature at which the ovaries were held did not influence oocyte chromatin, but did influence cumulus morphology. There was a change in cumulus morphology with temperature, resulting in more denuded and expanded cumuli with falling temperature. This was opposite to what would be expected, as lower temperatures would decrease metabolism and any influence from enzymes, which may be involved in the production of hyaluronic acid from the cumulus cells. Perhaps factors other than enzymes were involved in the cumuli becoming denuded. A factor, which could introduce a bias in this particular study, was the fact that the cumuli held at the lower temperatures were recovered from slaughterhouse material, which was collected earlier in the season, than the surgically removed ovaries.

There was more condensed chromatin in the 20-30 °C group, but this was not statistically significant. The apparent difference in the effect of temperature on cumulus and chromatin may have been caused by slower nuclear reaction to a suboptimal environment than the time taken for cumulus expansion. Pig oocytes at the germinal vesicle stage did not survive cooling to 15 °C or below (Didion *et al.*, 1990) and sheep oocytes were sensitive to cooling to 20 °C at various stages of meiosis with cooling inducing chromosomal abnormalities (Moor *et al.*, 1985).

In the present study, chromatin configuration changed gradually with time and was significantly changed after 6 hours of holding time. There were more FN and abnormal configurations and fewer LCC and diakinesis with time. Surprisingly, the increase in FN indicated that this was not an immature stage. However, this was not in agreement with other data from the present study: FN was only found within compact cumuli. Also, FN was only increased between >6 – 8 hours but not in the >8 – 12 hour group, although the low numbers of oocytes could bias the results of this group. Hinrichs observed that oocytes left sitting in medium in a petri dish at room temperature for 1 – 4 hours would significantly change chromatin configuration to more CC and FN configurations, suggesting that the LCC configuration of the juvenile, incompetent oocytes would disperse to FN and the oocytes competent to mature would condense from LCC to the CC configuration in the petri dish (personal communication). The changes caused by sitting at room temperature for 1 – 4 hours before in vitro maturation did not affect the maturation rates. These findings were reflected in the present study's decreased LCC and increased FN, and to a certain extent CC, but only after storage for longer than 1 – 4 hours. The majority of oocytes at >8-12 hours contained condensed chromatin and abnormal configurations, whereas this was the case in few of oocytes at 0-1 hours, clearly showing that time had an effect. The effect of storage time on maturation rates was not examined in this study, but has been investigated by other workers with differing conclusions.

In a study in horses, storage time of >6 - 8 hours was not found to affect maturation rates compared to oocytes stored in ovaries for 1.5 – 4 hours (Guignot *et al.*, 1999). Another study reported no effect on maturation rates after storage time for up to 15 hours compared to 3 – 9 hours in horses (Delcampo *et al.*, 1995b). The same conclusion was reached after comparisons were made between storing the equine oocytes at 5 – 6, 6 – 7 and 7 – 8 hours, and furthermore, no difference was found in cumulus morphology either (Shabpareh *et al.*, 1993). Frequency of bovine oocytes in the germinal vesicle stage was unaffected by storage for up to 6 hours (Richard *et al.*, 1996). By contrast, storing ovine oocytes for 4, 8 and 24 hours at 5°C, 22°C, or 37°C, resulted in a negative effect on the oocytes' capacity for reaching metaphase II. Storing the oocytes at 22°C affected the oocytes less than at 5°C and 37°C (Moodie *et al.*, 1989) but another study found that exposing bovine germinal vesicle oocytes to temperatures below 23 °C reduced their viability as determined by membrane integrity (Zeron *et al.*, 1999). The developmental capacity of murine oocytes to the 2-cell stage embryo in vitro following storage in the ovaries for up 6 hours was no different than

control oocytes, but storage for 9 – 12 hours did affect the developmental capacity negatively (Schroeder *et al.*, 1991).

The apparent lack in some studies of effect of time and temperature on nuclear maturation but lowered cleavage rates may be explained by other factors, e.g. cytoplasm, zona pellucida or cell membranes, being more sensitive to temperature and time than chromatin configuration (Azambuja *et al.*, 1998; Zeron *et al.*, 1999). Human oocyte spindles disassembled and were accompanied by chromosomal dispersion in oocytes after 30 minutes at room temperature (Pickering *et al.*, 1990). In vitro matured bovine oocytes left for 30 minutes at room temperature resulted in abnormal spindles in the majority of the oocytes, whereas chromatin configuration was abnormal in only few of the oocytes (Aman *et al.*, 1994). The detrimental effect was more marked when the oocytes were cooled to 4 °C. In summary, reports differ on conclusions as to whether time affects oocyte maturation or not, but most seem to agree on maturation rates not being affected as determined by chromatin configuration, but factors other than chromatin may be more sensitive and development after fertilisation may be affected. The studies on equine oocytes need better control groups, because in two of the studies, the groups serving as controls ranged from storing the ovaries for 3-5 hours to 8-9 hours (Shabpareh *et al.*, 1993; Delcampo *et al.*, 1995b).

There appeared to be an effect of fixative volume on chromatin configuration. Fixing the oocytes directly on the slide with a lesser volume of fixative seemed to result in a greater number of oocytes with condensed chromatin. It is not known whether it was the effect of the Hoechst stain or the contact with the slide or lesser volume of fixative that caused the difference. The fixative volume to oocyte volume was more than 1000 times, so it seems unlikely that there was inadequate fixation. The graph with fixatives and the follicular fluid control (Figure 3-2) demonstrated the possible influence of fixative. Fixation with a small volume of acetic acid-methanol with Hoechst had almost no other configurations than condensed chromatin, and none of the supposedly immature configurations. This method of fixation may have damaged the oocyte. Adding of a drop of acetic acid-methanol with Hoechst to the oocyte lying in a drop of M199 caused the oocyte to swirl around until the two types of fluid had mixed. Perhaps this induced damage to the oocyte, inducing chromatin condensation. Sitting in follicular fluid appeared to cause more condensed chromatin configurations and a lot of these oocytes had no chromatin staining up. These two latter groups contained fewer oocytes than the other fixative groups due to the retrospective nature of the study, and further studies into this subject are needed to get a more detailed

picture. Other workers have used acetic acid as fixative without any apparent problem (Shabpareh *et al.*, 1993; Delcampo *et al.*, 1995b), and the difference to the present study seems to be the volume used. To see if the Hoechst stain caused the change in chromatin configuration, oocytes could be fixed in the same manner, but without the Hoechst stain added to the fixative. Buffered formalin and paraformaldehyde appeared to give the most variation in nuclear configurations including the presumed immature configurations, so fixing in these fixatives seemed to be the method of choice. Paraformaldehyde appeared to yield the greatest variation in chromatin configuration, although the difference between the 2 fixatives was not significant.

In the present study, there was a correlation between changing cumulus morphology and a change in distribution of chromatin configuration. Compact cumulus was the only group, which contained oocytes with a fluorescent nucleus. With increasing cumulus expansion, the chromatin configurations indicated a progression of meiosis in the oocyte because there were increasing frequencies of diakinesis and metaphase oocytes. Some oocytes showed pronucleus formation. Pronucleus formation can be induced by contact with ethanol (Shaw *et al.*, 1989), but apparently also results from advanced follicular atresia as seen in the present study, where the pronucleus oocytes were surrounded by expanded cumuli. Of fully expanded cumuli, more than 40% contained a metaphase oocyte. Hinrichs *et al* (1997) reported that more expanded than compact cumulus oocytes had condensed chromatin (61% vs. 32%) and that after culture, more expanded than compact cumulus oocytes had matured to metaphase (74% vs. 30%). This was in agreement with our study, suggesting that oocytes with expanded cumulus cells had initiated meiotic maturation, which was completed during culture.

Oocytes which were classified as denuded, had either no cumulus (true denuded) or had an incomplete cumulus. The incomplete cumulus made it impossible to classify the cumulus oocyte complex (COC) into one of the cumulus morphology classes, because the COC was not sitting on a reasonably sized sheet of granulosa cells. The partial cumulus oocytes looked like a compact cumulus, which had been damaged or separated from a sheet of granulosa cells during the scraping of the follicle. These oocytes looked like and were as easy to denude as the compact cumulus oocytes. I hypothesise that the denuded oocytes were a mixed population of true denuded oocytes, which had lost their cumulus naturally, and a population of compact cumulus oocytes, which had been accidentally partially denuded. To examine this assumption, 15 denuded oocytes were examined for chromatin configuration in

relation to granulosa cell health. The distribution of these oocytes was 33.3% in the early chromatin configurations (FN, LCC) in healthy follicles, and 13.3% were in atretic follicles. Only 6.7% of late and degenerated chromatin configurations (condensed chromatin, metaphase stages, no chromatin and abnormal) were from healthy follicles and 46.7% were from atretic follicles. This supports the theory that denuded oocytes consisted of a mixed population of naturally denuded oocytes from atretic follicles and with maturing chromatin, and damaged oocytes from healthy follicles and with young chromatin configurations most likely being originally compact cumulus oocytes. Denuded oocytes decreased with time alongside the compact cumulus when cumulus morphology was assessed in relation to holding time of the follicle. The denuded oocytes were excluded from analysis because the data was very mixed and showed no trends.

This study raised a question as to whether the condensed chromatin is not part of the normal development of the chromatin to metaphase II, but perhaps a degenerative configuration or related to atresia. In follicles stored at 5 °C for 30–33 hours, mainly condensed chromatin was found, indicating that this configuration may have resulted from degeneration. Also the oocytes, which were kept in follicular fluid with no fixative, had condensed chromatin configuration. The way to investigate these findings would be to incubate these oocytes after storage. If any of them progressed to metaphase II, condensed chromatin would be shown to be a normal feature of preparation for meiosis.

Opposing the theory that condensed chromatin is a sign of atresia was the finding that in the mouse highly compacted chromatin was a normal feature during the germinal vesicle stage (Mattson *et al.*, 1990). Condensed chromatin was associated with low transcription activity, and transcription was low during meiotic maturation (Gosden *et al.*, 1997a; Zuccotti *et al.*, 1998). In the mouse, oocytes from antral follicles were divided into one class with highly compacted chromatin surrounding the nucleolus, forming a complete ring around the nucleolus and where the chromatin was thread-like. The other class had the chromatin more homogeneously widespread and the chromatin surrounding the nucleolus was less defined (Zuccotti *et al.*, 1998; Bouniol-Baly *et al.*, 1999). Zuccotti *et al.* (1998) stained freshly recovered mouse oocytes with Hoechst to see the nuclear chromatin, cultured the oocytes and re-stained the oocytes after culture and fertilisation. Of the oocytes with chromatin surrounding the nucleolus, 81.2% reached metaphase II or beyond compared to 45.1% of the oocytes with no chromatin surrounding the nucleolus (Zuccotti *et al.*, 1998). Thus both chromatin configurations were capable of resuming meiosis although the latter less

efficiently. The formation of a chromatin ring around the nucleolus appeared to be a prerequisite for germinal vesicle break down (Zuccotti *et al.*, 1998). Presumably, the configuration with no chromatin ring around the nucleolus preceded the form with the surrounded nucleolus in time and that the chromatin surrounding the nucleolus led to the formation of chromosomes of the first meiotic metaphase (Zuccotti *et al.*, 1998). This meant that the oocytes with no surrounded nucleolus were at a more immature stage which would mature by going through the surrounded stage and then move on to the germinal vesicle break down stages (Zuccotti *et al.*, 1998). In the present study most of condensed chromatin contained a clearly visible nucleolus in the middle of the chromatin, which seemed to be similar to the phases of chromatin condensation described in mice (Mattson *et al.*, 1990).

The chromatin and cumulus data seemed to support the classification of the chromatin configurations with the fluorescent nucleus being the most immature stage of prophase or perhaps a post mortem decondensation of the immature LCC. Other reports indicated that FN was correlated with viable follicles and lower maturation rates due to this configuration possibly being present in juvenile oocytes (Hinrichs *et al.*, 1997 and 2000). Loosely condensed chromatin appeared to be the main immature stage, but was found in both compact and expanded cumulus oocytes. This configuration in its varying sizes may be the transition from immature to a step towards acquiring meiotic competence. Supporting this was the finding that LCC oocytes likely matured in vitro as more oocytes matured than there were CC oocytes, and therefore LCC may have added to numbers of maturing oocytes (Hinrichs *et al.*, 2000). From these stages the chromatin appeared to condense, making individual chromatin visible, progressing via diakinesis to metaphase I and II.

Alternatively the chromatin may further condense from the LCC stages, leading to the very condensed chromatin, which then aligned on the metaphase plate to form metaphase I and then separated via anaphase to metaphase II. This pathway seemed plausible, as condensed chromatin oocytes in mares appeared to increase in vitro maturation rates, and seemed further progressed than loosely condensed chromatin (Hinrichs *et al.*, 2000). The information from mouse studies indicated that condensed chromatin may be a normal feature of atresia in the follicle rather than an in vitro induced artefact. Zuccotti *et al.*, (1998) hypothesised in mice that the antral follicles, with oocytes with highly condensed chromatin and nucleolar chromatin, were ready to ovulate. After ovulation had occurred, the oocytes with the nucleolar chromatin still present in non-ovulated follicles were incapable of development and may have been an atretic form (Zuccotti *et al.*, 1998). The surrounded nucleolus

configuration may have been a step towards atresia as 66% of the condensed perinucleolar configuration was atretic in monkeys (LeFevre, 1989). The initial stages of meiotic resumption, until metaphase I, appeared not to be particularly affected by the presence of nucleolar chromatin, but the progression towards metaphase II was affected by presence or absence of nucleolar chromatin (Zuccotti *et al.*, 1998).

Perhaps there were 2 pathways: one for the oocytes, which could reach metaphase, and another for oocytes, which had become atretic before reaching metaphase and therefore reached an end stage with the atretic-looking condensed chromatin. The hypothesis of condensed chromatin being an end-stage was certainly not the only end-stage according to the findings of this study as old oocytes (degenerating ooplasm) from small, very atretic follicles would contain metaphase chromatin. In horses, most studies pointed towards condensed chromatin being a normal feature during the preparation for meiosis (Hinrichs *et al.*, 1997 and 2000). Condensed chromatin was thought to represent meiotic competence, and was either obtained by follicles becoming atretic and losing meiosis-inhibiting effect or by a signal received in relation to preovulatory growth during normal maturation of the healthy follicle (Hinrichs *et al.*, 1997 and 2000). In summary, condensed chromatin may be a step in the normal progression towards meiotic maturation.

3.6 Conclusion

Based on the results of this study, it can be recommended to keep equine ovaries at temperatures between 35-37 °C for less than 2 hours if the oocytes are to be used for assessing in vivo conditions, bearing in mind that other parameters may be more sensitive to temperature and time than chromatin configuration and cumulus morphology. When using the oocytes for IVM, the increase in expansion and change in chromatin may aid nuclear maturation, although the capacity for developing beyond the blastocyst stage may be reduced. Oocytes should be fixed in either paraformaldehyde or buffered formalin in large volumes and stained afterwards.

Cumulus morphology and chromatin configuration were correlated and so was chromatin configuration and atresia of the follicle. The equine oocyte appeared to follow established pathways of oocyte maturation with the addition of a configuration of unknown relevance (condensed chromatin), which may be a form of oocyte atresia, a normal step during acquisition of meiotic competence or may be an induced configuration, which does not necessarily prevent in vitro maturation.

4 Analysis of atresia in equine follicles

4.1 Introduction

Around 70-99% of ovarian follicles depending on species are destined to undergo atresia (Byskov, 1979). There are approximately 35,000 primordial follicles in the mare ovary, of which 100 are growing at any given time (Driancourt *et al.*, 1982a). As only 1-2 follicles ovulate per cycle, the fate of the majority of the follicles in the ovary is atresia. Atresia is a physiological method of regulating the size of the follicle cohort for ovulation and may be the result of cells undergoing apoptosis. Apoptosis was the molecular mechanism by which oocytes and developing follicles are eliminated from the ovary in the species studied (rats (Nahum *et al.*, 1996; Boone *et al.*, 1997a), sheep (Jolly *et al.*, 1997a), cattle (Jolly *et al.*, 1994a; Blondin *et al.*, 1996), and pigs (Hughes, Jr. 1991; Tilly *et al.*, 1991; Guthrie *et al.*, 1994; Pesce *et al.*, 1994)). Apoptosis is a form of physiological cell death in contrast to necrosis, which is "accidental" (pathological) cell death caused by harmful conditions (Kerr *et al.*, 1972; Schwartzman *et al.*, 1993; Darzynkiewicz *et al.*, 1998). Apoptosis in contrast to necrosis appears to require gene expression (Schwartz *et al.*, 1993; Schwartzman *et al.*, 1993; Hale *et al.*, 1996). Physiological cell death occurs in tissues, which undergo pronounced morphological alterations such as the periodical growth and regression of large structures like follicles.

Distinguishing between healthy and atretic follicles has been done in a variety of ways. Follicles have been classified as healthy or atretic based on surface opacity (Grimes *et al.*, 1987), translucency and vascularisation of the follicle (Moor *et al.*, 1978), on follicular fluid hormone content (Kenney *et al.*, 1979; Guthrie *et al.*, 1994 and 1996; Jolly *et al.*, 1994b; Nahum *et al.*, 1996), as well as by histologic means. Equine follicles have been characterised by histology (Kenney *et al.*, 1979; Driancourt, 1979, 1982a and 1982b). Sections of follicular wall stained with haematoxylin and eosin allowed histologic estimation of numbers of pyknotic and mitotic nuclei in the granulosa cells. None of the studies on equine follicle histology has described apoptosis of granulosa cells.

Morphologically, apoptosis is characterised by loss of cell volume (cytoplasmic condensation), nuclear condensation resulting from margination of the chromatin and redistribution against the nuclear envelope in dense masses (Kerr *et al.*, 1972). Cell remnants are contained within a membrane after budding and separating from the cell surface, leaving

apoptotic bodies of varying sizes. Only the larger apoptotic bodies are seen with a light microscope. The residual bodies from apoptosis are phagocytosed. In rats is has been shown that neighbouring granulosa cells engulf the apoptotic cells (Boone *et al.*, 1997b). Cells undergoing apoptosis may form apoptotic bodies and completely disappear within 24 hours (Kerr *et al.*, 1972).

Biochemically, apoptosis is characterised by loss of DNA integrity following endonuclease-mediated fragmentation (Wyllie, 1980a). The calcium/magnesium dependent endonuclease causing the apoptotic DNA degradation has been identified as caspase-activated deoxyribonuclease (CAD) (Enari *et al.*, 1998). The internucleosomal cleavage of DNA results in mono- and oligonucleosomal fragments which are multiples of 180 - 200 base pairs, which will show as "DNA ladders" when visualised by agarose gel electrophoresis (Wyllie, 1980a; Tilly *et al.*, 1991). By contrast, necrosis is characterised by random fragmentation of DNA by multiple endonucleases, which produces a continuous spectrum of DNA fragment sizes and shows up as even staining in the lane after agarose gel electrophoresis (Afanas'ev *et al.*, 1986; Gold *et al.*, 1994).

Methods for detection of apoptosis include ethidium bromide staining of DNA, end labelling with [^{32}P]dideoxy-ATP which labels the free 3'-end of DNA fragments followed by radiography (Tilly *et al.*, 1993), or end labelling of DNA fragments in individual cells in situ (Ansari *et al.*, 1993), allowing for histological detection, including to some degree also quantitative determination of apoptosis. Electron microscopy of granulosa cells gives information about the ultrastructural changes that occur during apoptosis (Kerr *et al.*, 1972). Flow cytometry has been used to detect and quantify apoptosis in follicles (Susin *et al.*, 1999). This method assesses the percentage of cells found at each stage of the cell cycle and detects cells with less DNA (less fluorescence after excitation), presumably resulting from apoptosis (Guthrie *et al.*, 1994; Blondin *et al.*, 1996). Detection of translocation of phosphatidylserine to the surface of cell membranes with Annexin V staining of fresh granulosa cells specifically detects apoptotic cells (Van Blerkom *et al.*, 1998). Phosphatidylserine is located on the inner leaflet of the cell plasma membrane in normal cells. In apoptotic cells the distribution of phospholipids is changed and they also appear on the outside of the plasma membrane. Simultaneous staining with propidium iodide, which crosses the damaged plasma membrane of necrotic cells, detects necrosis or pyknosis. Staining granulosa cells with Hoechst 33258 visualises the DNA in non-viable cells, but not in viable cells by becoming highly fluorescent after binding to DNA. This is a way of

morphologically evaluating the nuclei for apoptosis. Due to the time constraints of this study, only a preliminary study on detection of apoptosis with Annexin V, propidium iodide and Hoechst 33258 was performed. See Appendix B.

DNA from murine thymocytes incubated with dexamethasone was used as an apoptotic control. Glucocorticoids have been shown to induce apoptotic cell death by activation of endonucleases in lymphoid cells (Wyllie, 1980a).

In order to study follicle dynamics, selection and regression in the equine ovary, it was necessary to develop methods of determining whether follicles were healthy or atretic. In the previous chapter, oocyte chromatin configuration and cumulus morphology were investigated to obtain knowledge for use in the following chapters. In this chapter the focus was on the granulosa cells as another indicator of follicle health. The aim of this chapter was to validate a method of characterising the health of follicles and of detecting apoptosis in granulosa cells. First of all, detection of apoptosis by electrophoresis and ethidium bromide staining and by end labelling of DNA fragments were compared in order to see if the two methods were in accordance. The necessary amount of DNA for use with ethidium bromide gels was established. The classical method of haematoxylin and eosin staining of sections of the follicular wall was used as the standard for detecting atresia. Apoptosis as detected by DNA laddering was determined in the same population of follicles to get an indication of how well the two methods of evaluating follicle health were correlated. Granulosa cell morphology as determined by stereomicroscopy was compared with histologic evaluation and gel electrophoresis to find out if this method would give an indication of the state of the follicle immediately upon dissection of the follicle. This would be useful as an extra indicator of follicle health, which would also be an immediate and easy indicator, unlike the other methods, which were more laborious. Once established that the gel laddering may indeed be a true indicator of apoptosis in horses, the effect of temperature and time on presence of apoptosis in granulosa cells was examined in order to find the optimum storage conditions without inducing apoptosis for the benefit of the following chapters. The overall aim was to find a reliable way of determining whether a follicle was atretic.

4.2 Materials and methods

4.2.1 Ovary processing

The present study was a retrospective study and hence the numbers of follicles in each group varied greatly. Ovaries were obtained from horses and ponies after slaughter or after ovariectomy. The exteriorised ovaries were kept at 20 - 30 °C or 35 - 37 °C in M199 with Hanks salts and 25 mM Hepes for 0.5 - 10 hours. A 5x5 mm section of the follicular wall was dissected out and fixed in 10 % buffered formalin for 24 hours for subsequent histological evaluation of the follicular wall. The follicle inner wall was scraped with a bone curette to release the granulosa cells. The granulosa cells were flushed off with M199 with Hank's salts and evaluated with a stereo microscope. The granulosa cells were pelleted by centrifugation at 2,000 *g* for 30 seconds at room temperature, snap frozen and stored at –70 °C until DNA extraction. Follicles ranged from 2 – 60 mm (the effect of size on follicular health will be discussed in chapter 6).

4.2.2 Detection of apoptosis

The principle in detection of apoptotic fragments is that low molecular weight DNA increases and high molecular weight DNA decreases in apoptotic cells. Apoptotic DNA which has undergone fragmentation (low molecular weight DNA) may easily be separated from very large, genomic DNA by centrifugation as part of DNA extraction. After agarose gel electrophoresis, DNA fragments were separated based on the different molecular weights. Low molecular weight DNA (180 base pairs) run the furthest on the gel, whereas the larger fragments are slower to get through the agarose gel. For specific details in recipes, see chapter 2.

4.2.2.1 DNA extraction

Granulosa cells were lysed with sodium dodecyl sulphate, sodium chloride, tris, EDTA and digested with proteinase K at 57 °C for 6 hours. Protein precipitation was initiated with potassium acetate and chloroform. Further extraction and precipitation was performed with isopropanol and finally ethanol. After removal of the ethanol, the samples were air dried and resuspended in deionised, filter sterilised H_2O and stored at -20 °C. DNA concentration in the samples was quantified spectrophotometrically.

4.2.2.2 Ethidium bromide staining

Ethidium bromide is a fluorescent dye, which stains DNA. For detection of apoptosis, ethidium bromide was incorporated in agarose gels. In samples with 6 μg or more DNA, the DNA fractions were separated by agarose gel electrophoresis, using 6 - 10 μg DNA from one follicle per lane in a 1.5% agarose gel. The DNA was stained with ethidium bromide incorporated in the gel (1 mg/ml) and visualised with ultraviolet light using a transilluminator connected to an image analysis system.

4.2.2.3 Apoptotic controls

On each gel, DNA from freshly isolated murine thymocytes was used as negative (non-apoptotic) controls and DNA from murine thymocytes incubated for 24 hours with dexamethasone (100 μg dexamethasone/ml culture medium) was used as a positive (apoptotic) control (Wyllie, 1980a; Cohen *et al.*, 1984).

4.2.2.4 End-labelling

Terminal deoxynucleotidyl transferase is an enzyme, which detects the free 3'-end of DNA fragments and adds a molecule of [^{32}P]dideoxy-ATP to it. This molecule is radioactive and will make a print on a radiographic film, thereby allowing for detection of localisation of apoptotic fragments. Samples with 2 - 5 μg DNA were analysed this way. DNA samples were labelled at the 3'-end with [^{32}P]dideoxy-ATP by incubation with Terminal Transferase, reaction buffer (Na, Tris HCl, BSA at pH 6.6) and cobalt chloride. The incubation was terminated with EDTA, the samples were centrifuged on spin columns for purification of the radio-labelled DNA. The fragments were separated with agarose gel electrophoresis. The gel was vacuum dried, sealed in plastic wrap and exposed to Kodak X-ray film.

End-labelling is not specific for apoptosis only, because extensive DNA degradation with breaks in the internucleosomal linker DNA, exposing the 3'-ends, can be seen during necrosis and perhaps autolysis too, but the random fragmentation seen in necrosis does not create the ladder pattern after electrophoresis. The laddering due to the specific enzyme cuts is specific for apoptosis (Tilly *et al.*, 1993).

4.2.2.5 Apoptosis grading systems

Follicles were classified as apoptotic when the characteristic laddering of internucleosomal DNA fragments were detected (Wyllie, 1980a). Follicles with no laddering were classified as non-apoptotic. Two grading systems were used. One simply stated whether laddering was present or not and whether the laddering was distinct or not (Figure 4.1).

Apoptosis grade 0: no apoptosis.
Apoptosis grade 1: some laddering, indicative of apoptosis and some smearing.
Apoptosis grade 2: distinct laddering, indicative of apoptosis and very little smearing.

The other grading system described the non-apoptotic follicles in more detail (see Figure 4.1).

Apoptosis grade 10: Only high molecular weight DNA was detected, i.e. there was only signs of DNA initially in the lane. No apoptosis.
Apoptosis grade 20: Primarily high molecular weight DNA, but a trace of DNA was detected down the whole lane. No apoptosis.
Apoptosis grade 30: A mix of high and low molecular weight DNA was present. The lane was covered with an even smearing in its whole length. No apoptosis.
Apoptosis grade 40: The smearing was stronger than in apoptosis grade 30, and seemed primarily to be localised in the two thirds of the lane furthest away from the well. No apoptosis.
Apoptosis grade 50: Some smearing and underlying laddering. Apoptosis.
Apoptosis grade 60: Less smearing and more laddering. Apoptosis.
Apoptosis grade 70: Very distinct laddering. Apoptosis.

The apoptotic control mouse thymocytes were graded as apoptosis grade 70. The non-apoptotic control mouse thymocytes were graded between 10 and 30. For evaluation of the 10 – 70 grading system, which was carried out in conjunction with other parameters in order to estimate the validity of this system, see Appendix A.

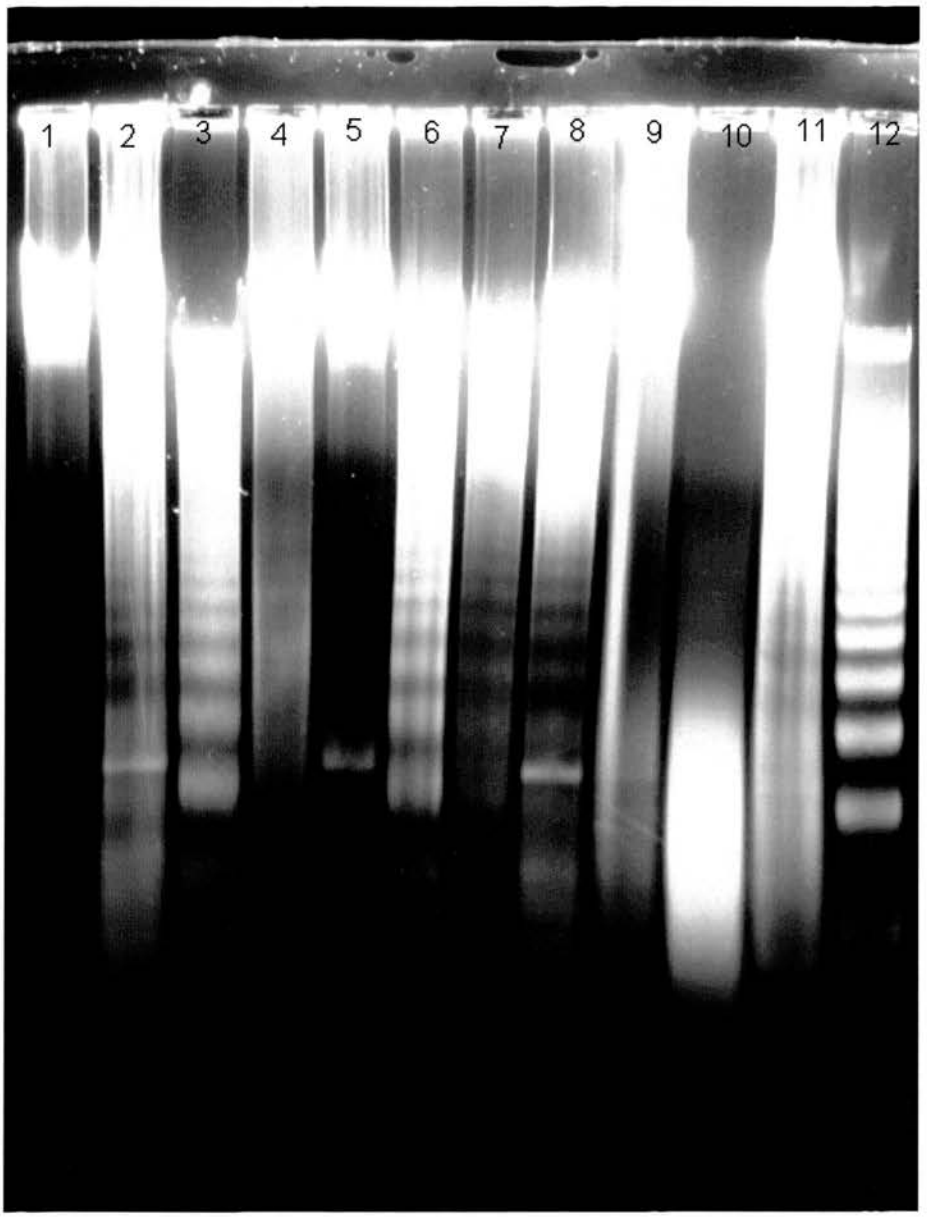


Figure 4.1. Apoptosis grades. Lane 1: apoptosis grade 0 and apoptosis grade 10 (mouse thymocyte negative control); lane 2: 1/50; lane 3: 2/60; lane 4: 0/30; lane 5: 0/10; lane 6: 2/60; lane 7: 1/50; lane 8: 1/50; lane 9: 0/30; lane 10: 0/40; lane 11: 1/50; lane 12: 2/70 (mouse thymocyte positive control).

4.2.3 Detection of atresia

4.2.3.1 Preparation of sections for histology

Samples were fixed in buffered formalin for 24 hours and stored in 70% alcohol until processing. Samples were dehydrated by alcohol followed by cedar wood oil. The samples

were transferred to toluene and finally embedded in paraffin wax. Consecutive sections (5-7 μm) were cut using a microtome, mounted on gelatin-coated slides and dried overnight in an oven before staining with haematoxylin and eosin. The sections were stained with Harris haematoxylin, washed in water, and washed with Scott's Tap Water Substitute. Counter staining was done with Putt's eosin. The samples were washed in water, dehydrated, cleared and mounted in DPX mountant. For details in the recipe, see chapter 2.

4.2.3.2 Atresia grading

Sections of follicular wall were evaluated with a light microscope at x20, x40 and x100 (oil). One hundred cells were differential counted in 10 different sections per follicle making a total of 1000 counted cells per follicle in different areas of the cut sample. A method by (Kenney *et al.*, 1979) for evaluating atresia in the follicular wall was used, but with some modifications with regards to apoptotic cells.

Atresia grade 0 (Figure 4.2): no atresia, viable follicles. The granulosa cells were active and mitotic figures were seen. The nuclei were stained light blue or blue with the chromatin diffusely distributed within. The cytoplasm was light pink. The granulosa cells were arranged with a basal layer on top of the basement membrane, separating it from the theca cells, and with 2 or more layers depending on follicle size towards the lumen of the follicle. No hyaline membrane was seen. The odd apoptotic or pyknotic granulosa cells may have been present. The theca cell layer was well vascularised and could contain luteinised cells.

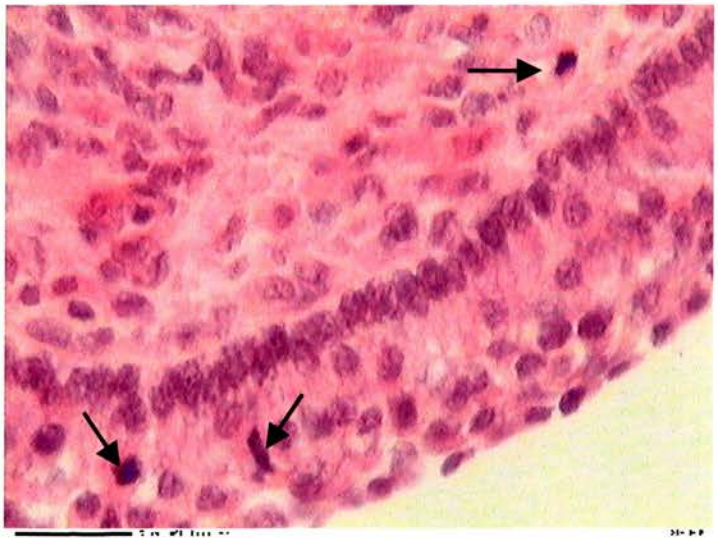


Figure 4.2. Atresia grade 0. Arrows: mitotic cells. H&E stain, X40 objective, bar = 25 μm.

Atresia grade 1 (Figure 4.3): initiation of atresia (incipient atresia). Pyknotic and apoptotic granulosa cell nuclei were seen at regular intervals. The nuclei appeared darker blue resulting from condensing chromatin. There was no change or perhaps luteinisation of the theca cells. Pyknosis and mitosis could occur simultaneously in the granulosa cells of the same follicle, but the granulosa cell nuclei were smaller, darker blue and not as active.

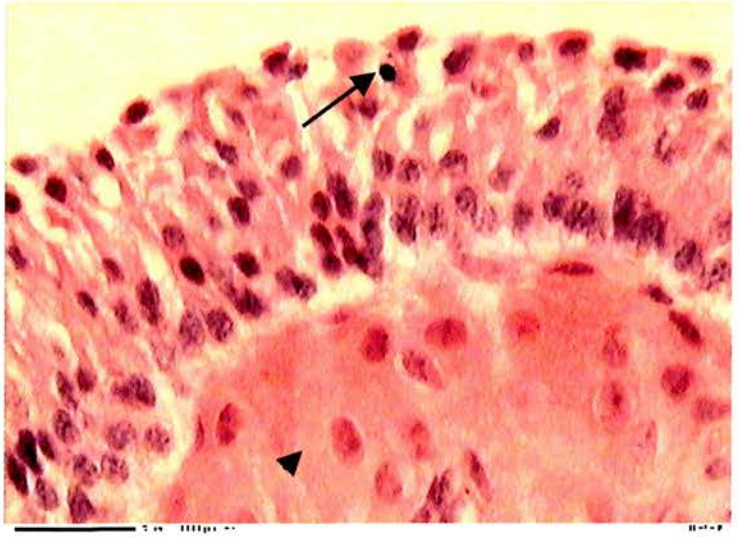


Figure 4.3. Atresia grade 1. Arrow: pyknotic cell. Arrow head: luteinised theca cells. H&E stain, X40 objective, bar = 25 μm.

Atresia grade 2 (Figure 4.4): Degeneration of both granulosa and theca cell layers, and basal membrane thickening forming the hyaline membrane. The granulosa cells underwent massive pyknosis and apoptosis followed by a gradual loss with the result that only a mono- or bilayer of rather small, inactive appearing granulosa cells remained. The theca cells disappeared.

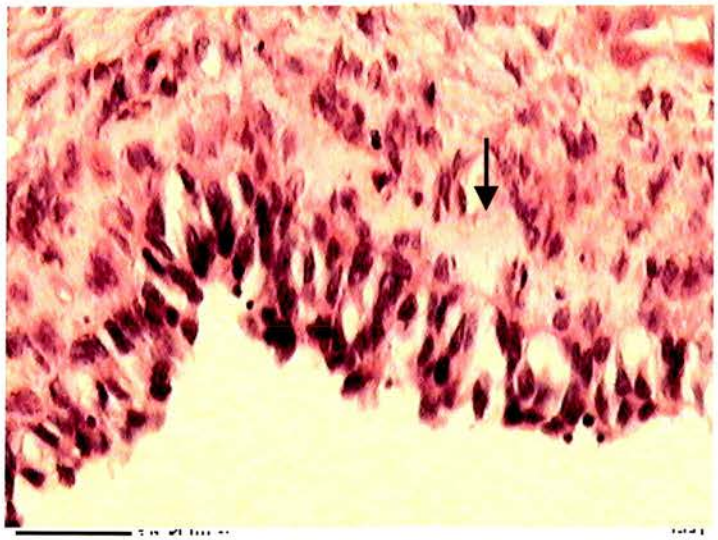


Figure 4.4. Atresia grade 2. Many pyknotic and apoptotic granulosa cells. Arrow: hyaline membrane. H&E stain, X40 objective, bar = 25 μm.

Atresia grade 3 (Figure 4.5): disappearance of cells. Separate identity of the granulosa and theca cells was lost and a few connective tissue cells, fibroblast-like cells and a thick basal (hyaline) membrane were the only relics.

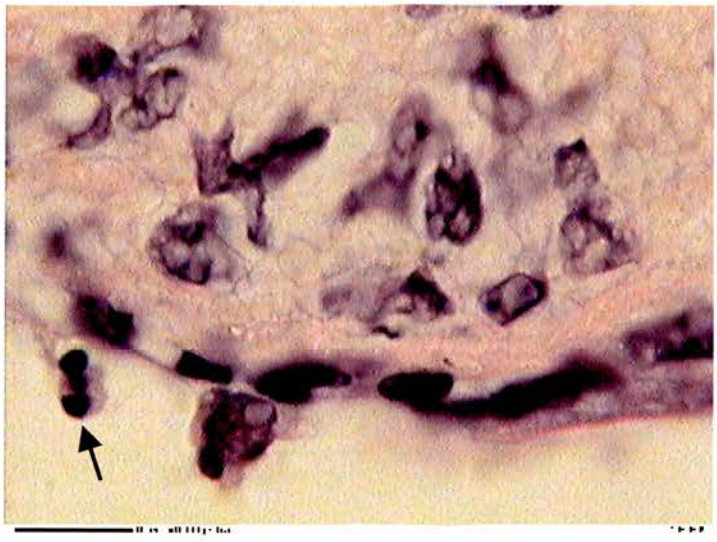


Figure 4.5. Atresia grade 3. Arrow: Apoptotic granulosa cell. H&E stain, X100 objective, bar = 10 μm.

4.2.4 Morphology of freshly retrieved granulosa cells

Granulosa cells were evaluated with a stereo microscope at the time of retrieval, before snap freezing. No cell count was performed. See details of the grading system in chapter 2.

4.2.5 Experiments related to method of determining follicle health

4.2.5.1 Comparison of end labelling and ethidium bromide staining

In samples where too little DNA was extracted for using the ethidium bromide technique, the DNA was end labelled because less DNA was needed with this technique. Twenty-six follicles were subjected to end labelling as well as ethidium bromide staining in order to establish the congruity between the two methods. For end labelling, 2 μg DNA was used and for ethidium bromide staining 10 μg was used.

4.2.5.2 Determination of necessary DNA content for ethidium bromide staining

With the aim of determining the necessary amount of DNA needed for detection of apoptosis, DNA from an apoptotic follicle and DNA from a non-apoptotic follicle was dispensed in decreasing concentrations on gels containing ethidium bromide.

Non-apoptotic follicle: 10 μg, 9.5 μg, 9.0 μg, 8.8 μg, 8.5 μg, 8.3 μg, 8.0 μg, 7.8 μg, 7.5 μg, 7.0 μg, 6.5 μg, 6.0 μg.

Apoptotic follicle: 9 μg, 8.5 μg, 8.3 μg, 8.0 μg, 7.8 μg, 7.5 μg, 7.0 μg, 6.5 μg, 6.0 μg, 5.5 μg and 5.0 μg DNA.

The gel with the non-apoptotic follicle was done at first. As DNA was easily seen in concentrations as low as 6 μg, lower concentrations were tried for the apoptotic follicle.

4.2.5.3 Histology of the follicular wall in relation to ethidium bromide staining

In order to correlate histology with ethidium bromide staining of DNA, follicular wall and granulosa cells were collected from 20 follicles. The follicles had all been held in the ovary for less than 4 hours and the ovaries had been kept at 30 °C after slaughter or ovariectomy. Atresia grading was compared with apoptosis gradings on these 20 follicles.

4.2.5.4 Morphology of freshly retrieved granulosa cells in relation to histology of the follicular wall

Follicular wall and freshly isolated granulosa cells were collected from 33 follicles with the intent to compare histology with morphology of the granulosa cells as estimated with stereomicroscopy of the cells immediately after recovery. The follicles were held in the ovary for less than 4 hours and the ovaries were kept at 30 °C.

4.2.5.5 Morphology of freshly retrieved granulosa cells in relation to ethidium bromide staining

In order to examine the correlation between the morphology of the granulosa cells and the DNA fragmentation, freshly isolated granulosa cells from follicles (n = 351) held for less than 4 hours in the ovary were evaluated under a stereomicroscope and then snap frozen, DNA extracted and gel electrophoresis performed.

4.2.6 Experiments related to the effect of temperature and holding time on granulosa cell health

4.2.6.1 Effect of temperature on apoptosis

Follicles (n = 197) with holding time less than 2 hours were kept at 20 °C, 30 °C or 35-37 °C in the ovary with the objective of examining the effect of temperature on granulosa cell apoptosis as determined by ethidium bromide staining and end-labelling.

4.2.6.2 Effect of ovary holding time on apoptosis

To examine the effect of holding time on apoptosis, follicles (n = 236) were kept at 30 °C for 0-1 hours, >1-2 hours, >2-3 hours, >3-4 hours, >4-6 hours or >6-10 hours before scraping the granulosa cells out and snap freezing. Apoptosis was determined by ethidium bromide staining and end-labelling.

4.2.6.3 The effect of suboptimal incubation on granulosa cell health

A time study was carried out to assess the effect of time on granulosa cells taken from the same follicle to investigate whether suboptimal conditions would induce apoptosis in equine non-apoptotic follicles. The study was an extension of experiment 4.2.6.2, where the initial

state of the follicle was not known. In the present experiment, the initial state was known and any changes with time could be detected. The experiment was performed with follicles (n = 17) ranging from 18 – 55 mm. Granulosa cells were scraped out and a proportion of the cells were snap frozen immediately. The remaining granulosa cells were resuspended in Medium 199 with Hank's salts (n = 12) or follicular fluid (n = 5). The granulosa cells were left at room temperature for 1, 2, 3, 5, 12 or 24 hours. There were not enough granulosa cells from each follicle to cover all time points. Holding time of ovaries before follicle processing was less than 3 hours.

4.3 Statistical analysis

Fisher's exact probability test was used to describe the association between the different methods of assessing either atresia and/or apoptosis. The methods tested were end labelling versus ethidium bromide staining, ethidium bromide staining versus histology and histology versus granulosa cell morphology. The data was put into a 2x2 test with a non-apoptotic and an apoptotic group for each method or in the case of histology, a non-atretic or an atretic group. The null hypothesis was that the distribution of the data was random. If the P-value was equal to or less than 0.05, the null hypothesis was rejected, and an association between the methods existed.

A Chi-square test was applied when testing for an association between ethidium bromide staining and granulosa cell morphology, with the granulosa cells grouped as 0+0.5+1 and 1.5+2+3. The null hypothesis was that the data was randomly distributed. A Chi-square test with Yates correction was used to test for significance between follicles held at different temperatures and storage times.

4.4 Results

4.4.1 Comparison of end labelling and ethidium bromide staining

In 25 of the 26 follicles (96.2%) there was agreement between the end labelling and ethidium bromide staining ($P<0.001$). Sixteen follicles were classified as non-apoptotic and 9 were apoptotic with both methods, and 1 follicle was classified as apoptotic after end labelling whereas the ethidium bromide technique did not detect apoptosis in this follicle. In 3 out of 9 (33.3%) apoptotic follicles, the end labelling showed more distinct apoptosis than with the ethidium bromide staining. As the methods are comparable with regards to presence or

absence of apoptosis, results obtained from end labelling and ethidium bromide staining were treated as one group in the following chapters.

4.4.2 Determination of necessary DNA content for ethidium bromide staining

Concentrations of DNA from the same two follicles, ranging from 10 μg to 5 μg, were electrophoresed with the aim of determining the lowest concentration of DNA that could be detected. Apoptosis was easily detected in each of the concentrations (see Figure 4.6). The non-apoptotic follicle's DNA print also was easily seen in each of the concentrations used. Based on these results it was decided to use ethidium bromide staining on follicles with DNA content as low as 6 μg.

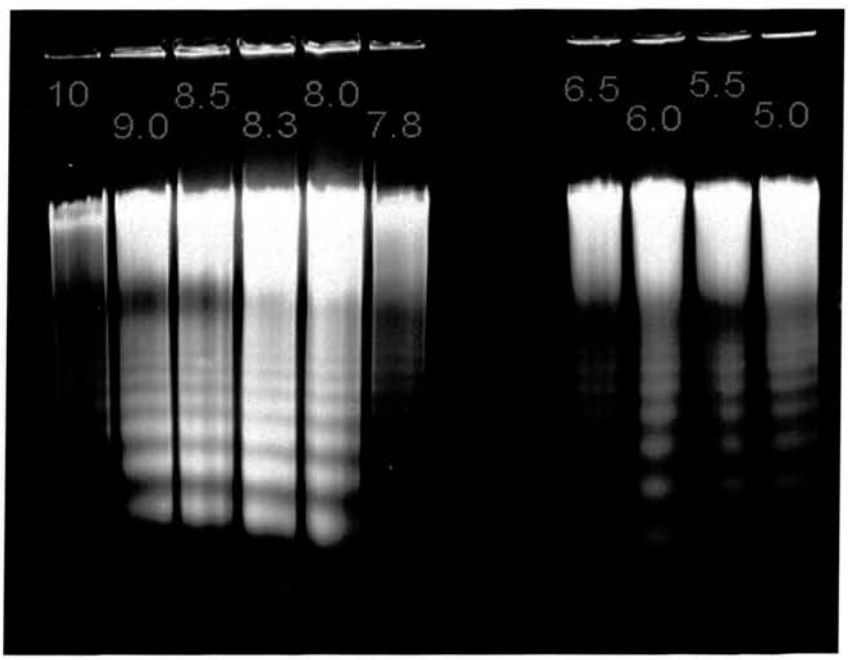


Figure 4.6. Decreasing concentrations of DNA from an apoptotic follicle. Apoptosis is easily detected in all concentrations. Numbers in lanes indicate concentrations (μg).

4.4.3 Histology of the follicular wall in relation to ethidium bromide staining

Histological atresia and ethidium bromide detection of apoptosis was significantly correlated ($P<0.001$), with 18 of the 20 processed follicles (90%) receiving corresponding grades in to the two methods. Twelve follicles were non-atretic, 3 were atresia grade 1, none was atresia grade 2 and 5 were atresia grade 3. See

Table 4-1 and Figure 4.7. Originally 4 other follicles participated in the experiment, but they were atresia grade 3 and were so degenerated that they contained too few granulosa cells to yield enough DNA for testing for apoptosis.

All follicles (n = 12) classified as non-atretic by histology, were classified as non-apoptotic by ethidium bromide staining. As determined by histology these follicles contained maximum 1.9% apoptotic granulosa cells, maximum 1.7% pyknotic cells and up to 5.8% mitotic cells.

Three follicles were classified histologically as having grade 1 atresia. Two of the 3 follicles were classified as apoptotic by ethidium bromide staining. The remaining follicle was classified as non-apoptotic. In this follicle, 7.9% of the granulosa cells were apoptotic. In the 2 follicles where apoptosis was detected, there were 14.8% or 15.5% apoptotic cells. This indicated that when numbers of apoptotic granulosa cells were below a certain percentage, the ethidium bromide staining did not detect the follicle as apoptotic.

None of the follicles were classified as grade 2 atresia. In stage 3 atresia, 4 of the 5 follicles were classified as apoptotic by ethidium bromide staining. These 4 follicles had 58 – 75% apoptotic granulosa cells on histology. The remaining follicle was classified as non-apoptotic. On histology this follicle contained 100% fibroblast-like cells rather than granulosa cells. The 4 follicles with too little DNA contained more than 95% fibroblast-like cells. Once the follicles got to this stage of advanced atresia, it appeared that the granulosa cells for the most part had perished or what remained was apoptotic or pyknotic. See colour atlas page 81.

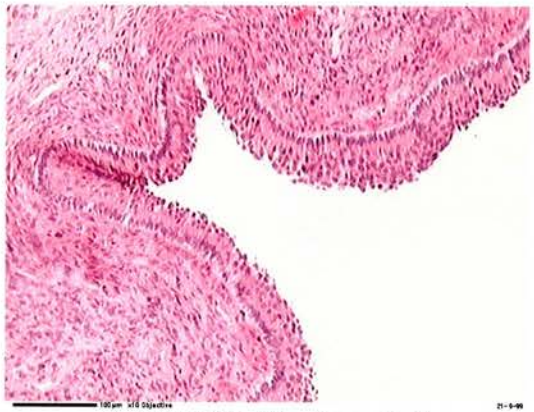
In summary, a total of 6 follicles were classified as apoptotic after ethidium bromide staining. None of these were classified histologically as being healthy. Four follicles contained 14.8%, 15.5%, 58.0% and 75.4% apoptotic granulosa cells respectively. The remaining 2 follicles contained 99% and 100% fibroblast-like cells, but apoptotic fragments must have been present to cause the DNA laddering. Histological evidence of apoptosis with more than 14% apoptotic granulosa cells seemed to correspond very well with apoptosis in ethidium bromide gels.

Table 4-1. Histology versus ethidium bromide staining. The data shows the presence or absence of apoptosis within different atresia grades in follicles. No follicle was atresia grade 2.

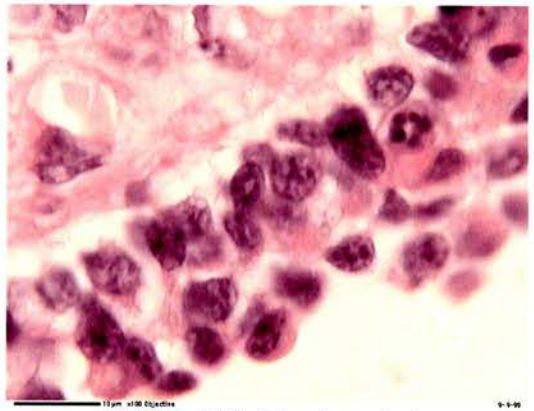
Atresia grade (histology)	n	No apoptosis (%)	Apoptosis (%)
0	12	100	0
1	3	33.3	66.7
2	0	0	0
3	5	20	80

Chi-square: $P < 0.001$

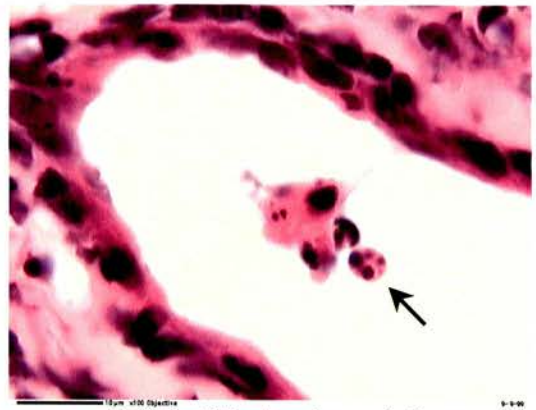
Figure 4.7. Histological detection of atresia compared to presence of apoptosis detected by ethidium bromide staining



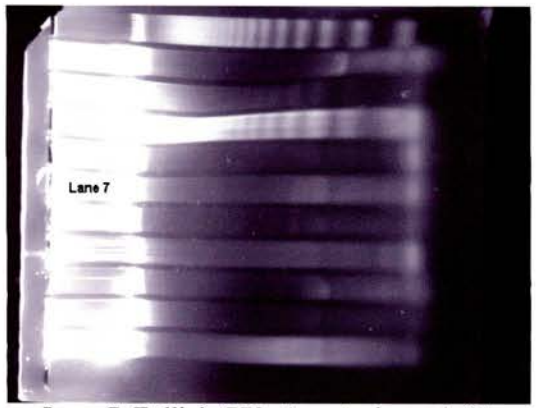
Follicle 772: Atresia grade 0



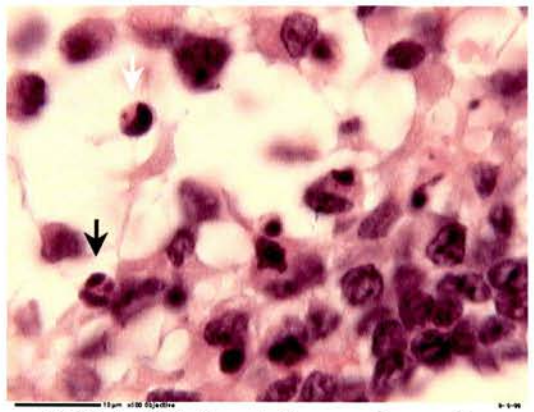
Follicle 587: Atresia grade 1



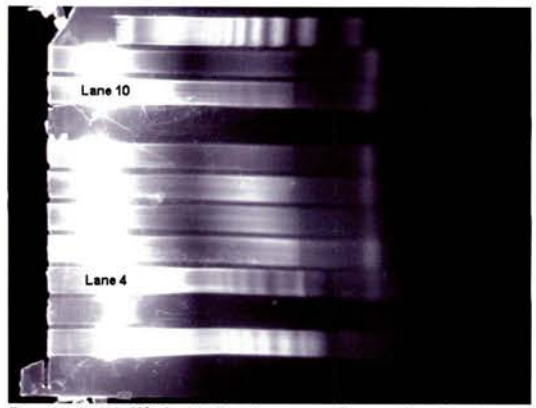
Follicle 579: Atresia grade 3. Arrow: apoptotic cell.



Lane 7: Follicle 772: Apoptosis grade 30



Follicle 587: Apoptotic granulosa cells. White arrow: half moon shaped nucleus. Black arrow: Fragmented nucleus.



Lane 4: Follicle 579: Apoptosis grade 70. Lane 10: Follicle 587: Apoptosis grade 50

4.4.4 Morphology of freshly retrieved granulosa cells in relation to histology of the follicular wall

A total of 33 follicles were evaluated. Seventeen follicles were classified as atresia grade 0, 5 follicles were atresia grade 1, 2 follicles were atresia grade 2 and 9 follicles were atresia grade 3. The stereo-microscopic evaluation of the fresh granulosa cells was significantly correlated ($P<0.001$) with the histological findings. See Figure 4.8.

Atresia grade 0: Seventeen follicles were classified as non-atretic by histology and in 16 (94.1%) of these, the granulosa cells were graded as healthy (granulosa cell grade 0). In the remaining follicle was a mixed population of healthy looking and expanding granulosa cells and the histology showed decreased mitotic activity and fewer large, active granulosa cells, the emergence of the glassy membrane, but the follicle was still non-atretic.

Atresia grade 1: Three of 5 follicles with grade 1 atresia had granulosa cell grade 0 under the stereomicroscope. This indicated that early atresia was not easily detected when examining the fresh cells under a stereomicroscope. The remaining 2 follicles were graded as granulosa cell grade 0.5 and granulosa cell grade 1, which was consistent with the early changes seen in grade 1 atresia.

Atresia grade 2: Two follicles showed grade 2 atresia and they were both starting to degenerate as detected with the stereomicroscope (granulosa cell grade 0.5 and granulosa cell grade 2).

Atresia grade 3: Nine follicles showed grade 3 atresia and none of these were classified as healthy follicles according to the granulosa cell grading. Eight (88.9%) of these were granulosa cell grade 2 with the stereomicroscope. The remaining follicle was noted as having a thin granulosa cell layer (granulosa cell grade 0.5).

Only 3 out of 19 (15.8%) granulosa cell grade 0 follicles were from histologically atretic follicles. In granulosa cell grades 0.5 – 3, only 1 out of 13 (7.7%) follicles were not histologically atretic.

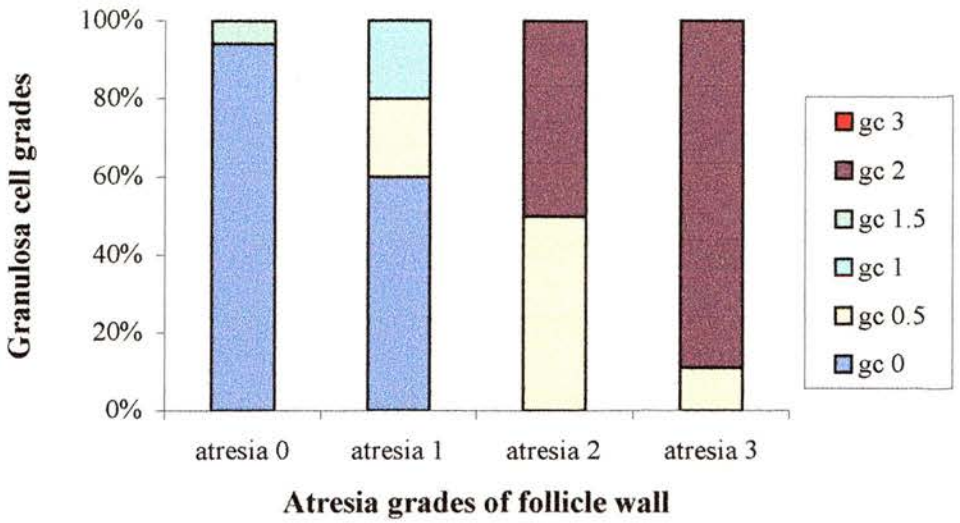


Figure 4.8. Morphology of freshly isolated granulosa cells in relation to histologic evaluation of sectioned follicular wall. Compact granulosa cell sheets decreased with increasing atresia ($P < 0.001$). Atresia 0 – 3 was determined by histology after sectioning of follicle wall, and gc 0 – 3 was determined by stereomicroscopy of freshly isolated, unstained granulosa from the same follicles. Compact granulosa cells were primarily found in non-atretic follicles, and expanded granulosa cells were mainly present in atretic follicles. Numbers of follicles in each group: Atresia 0 (n = 17), Atresia 1 (n = 5), Atresia 2 (n = 2), Atresia 3 (n = 9).

4.4.5 Morphology of freshly retrieved granulosa cells in relation to ethidium bromide staining

In total 351 follicles were examined for granulosa cell morphology and presence of apoptosis. Two hundred and seven follicles were non-apoptotic (apoptosis grade 0), 101 follicles were apoptosis grade 1 and 43 follicles were apoptosis grade 2. See Figure 4.9.

Apoptosis grade 0 (no apoptosis): Of the 207 non-apoptotic follicles, 88 (42.5%) were graded as granulosa cell grade 0. The granulosa cell grades 0.5 and 1 consisted of primarily healthy looking granulosa cells and when these groups were added together, 54 of the 207 follicles (26.1%) of the non-apoptotic follicles contained granulosa cells, which were mainly healthy looking. 65 of the 207 follicles (31.4%) contained apparently degenerating granulosa cells (granulosa cell grades 1.5, 2 and 3).

Apoptosis grade 1: Of the 101 apoptosis grade 1 follicles, 44 (43.6%) were found to contain healthy looking granulosa cells (granulosa cell grade 0). 8 out of 101 follicles (7.9%) contained granulosa cell grade 0.5 – 1. 49 of the 101 follicles (48.5%) contained degenerating granulosa cells (granulosa cell grade 1.5 – 3) which is an increase from apoptosis grade 0.

Apoptosis grade 2: Of the 43 apoptosis grade 2 follicles, 6 follicles (14%) contained healthy looking granulosa cells (granulosa cell grade 0), 6 out of 43 (14%) were granulosa cell grade 0.5 and 1, and 31 (72.1%) had degenerating granulosa cells (granulosa cell grades 1.5, 2 and 3) which is a further increase from apoptosis grade 1. Of the 31 follicles with degenerating granulosa cells, 26 follicles contained granulosa cell grade 2.

Granulosa cell grade 3: 5 out of 6 follicles (83.3%) with very few granulosa cells (granulosa cell grade 3) were classified as non-apoptotic, suggesting that the very degenerated follicles are classified as non-apoptotic.

In the previous section it appeared that the granulosa cell grades above grade 0 were correlated with atresia. In this experiment, it seemed that granulosa cell grades 0, 0.5 and 1 were correlated with non-apoptotic follicles. In the definition of grades, grades 0, 0.5 and 1 contained almost only normal appearing cells: in group 0.5 the sheets of cells were a little thinner than usual, and the cells in group 1 were for the most part normal looking, but a few were expanded. Grades 1.5, 2 and 3 contained 50% or more expanded cells. In the statistical test, the groups 0, 0.5 and 1 were put together and 1.5, 2 and 3 were grouped together and tested against apoptosis. The above results showed that there was a significant difference ($P<0.01$) between distribution of granulosa cell grades within apoptosis grade 0 and grade 1, and between apoptosis grade 1 and 2 ($P<0.05$).

When only comparing the granulosa cell grades 0 and 2 (the two grades comprising the largest proportion of the follicles) with apoptosis grades, it emerged that there was no significant difference ($P>0.5$) between apoptosis grades 0 and 1. The distribution of granulosa cell grades suggested that the major change from apoptosis grade 0 to 1 was in the increase of granulosa cell grade 1.5 and a reduction in grades 0.5 and 1. In other words, there were only subtle changes in granulosa cell morphology from apoptosis grade 0 to 1, and no change in granulosa cell grades 0 or 2. The change in granulosa cell grade 0 and

granulosa cell grade 2 was pronounced (P<0.001) between apoptosis grades 0+1 and 2, indicating that distinct apoptosis was reflected morphologically in the granulosa cells.

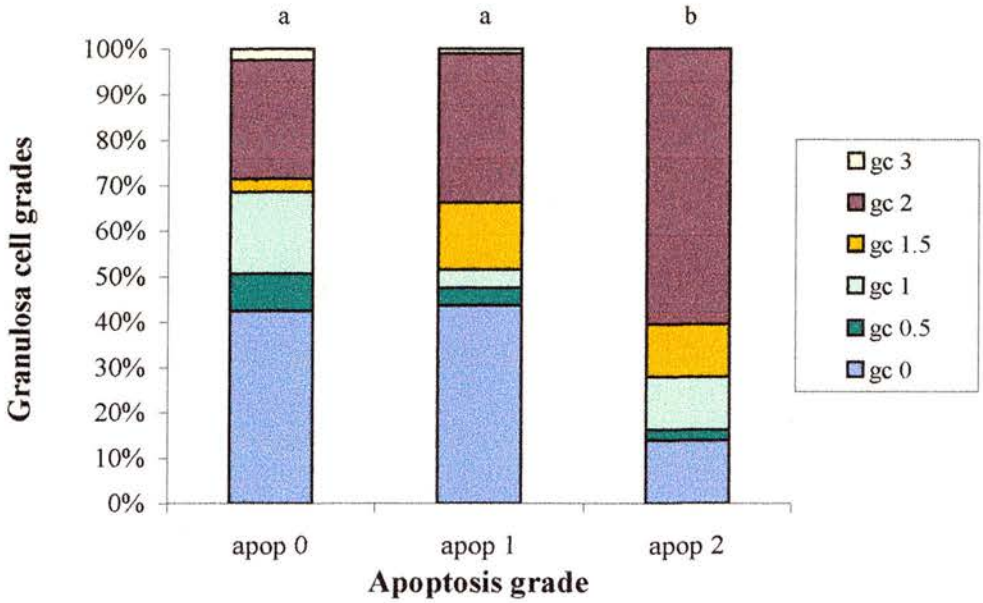


Figure 4.9. Relationship between apoptosis and granulosa cell morphology of freshly isolated cells. The granulosa cell morphology of freshly retrieved granulosa cells was related to apoptosis. Follicle numbers in each group: Apoptosis 0 (n = 207), Apoptosis 1 (n = 101), Apoptosis 2 (n = 43). Letters a and b denote difference (P < 0.001).

4.4.6 Effect of temperature on apoptosis

Follicles were held at 20 °C (n = 16), 30 °C (n = 86) and at 35-37 °C (n = 95). There was no difference (P > 0.5) in proportion of non-apoptotic follicles in the 2 groups below body temperature (20 °C and 30 °C), but there was a difference (P < 0.001) between follicles kept at 20-30 °C and at 35-37 °C. This indicated that decreased temperature may have delayed the apoptotic process. See Figure 4.10.

4.4.7 Effect of ovary holding time on apoptosis

Follicles were kept in the ovary at 30 °C for 0-1 h (n = 41), >1-2 h (n = 46), >2-3 (n = 52), >3-4 (n = 42), >4-6 h (n = 34) and >6-10 h (n = 21). There was no significant change (P>0.5) in proportion of apoptosis between the hours of holding time, although there was a tendency for apoptosis to increase with time. See Figure 4.11.

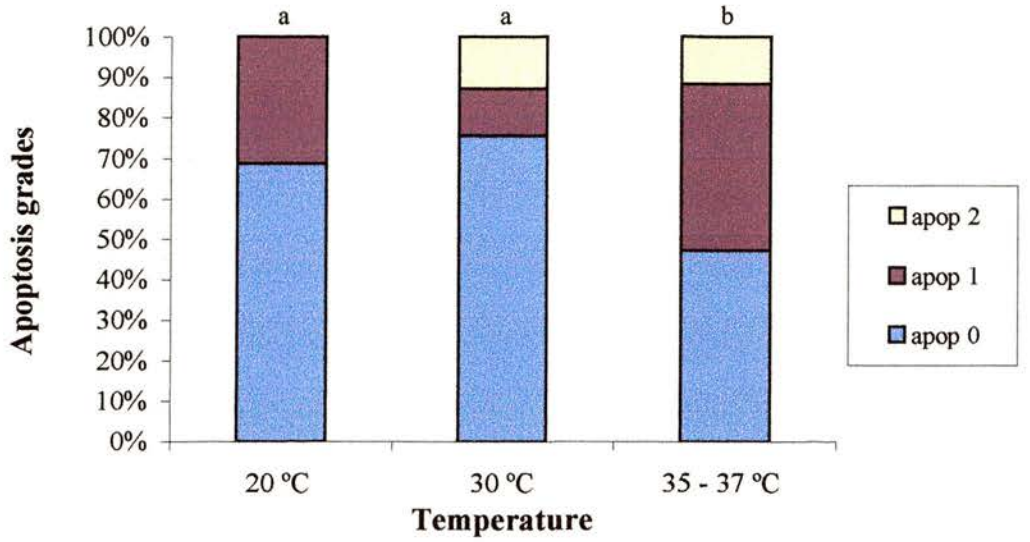


Figure 4.10. Effect of temperature on granulosa cell apoptosis. Follicles in each group: 20 °C (n = 16), 30 °C (n = 86), 35 - 37 °C (n = 95). Letters a and b denote difference ($P < 0.001$).

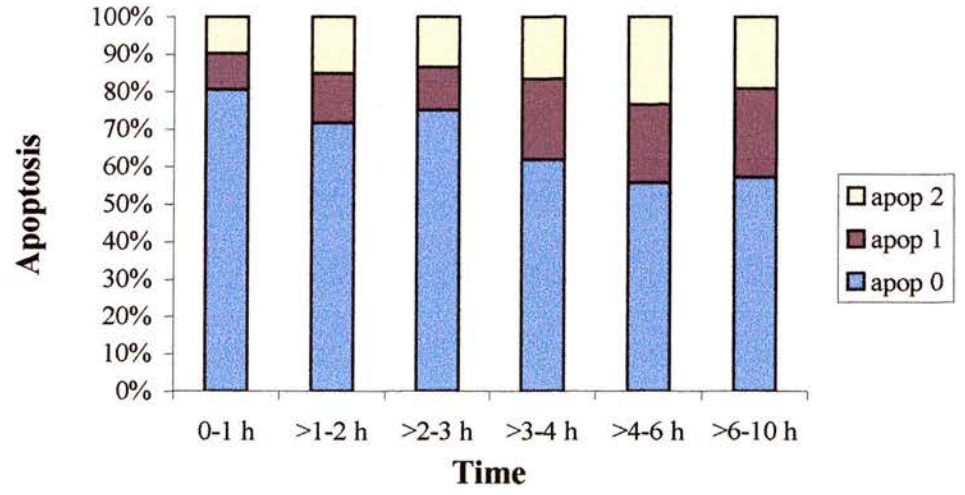


Figure 4.11. Effect of holding time on granulosa cell apoptosis. There was a non-significant tendency for apoptosis to increase with time. Follicle numbers in each group: 0-1 h (n = 41), >1-2 h (n = 46), >2-3 h (n = 52), >3-4 h (n = 42), >4-6 h (n = 34), >6-10 h (n = 21).

4.4.8 The effect of suboptimal incubation on granulosa cell health

Seventeen follicles were subjected to the time study. Two of the 17 follicles were apoptotic at time point 0 and were excluded from the analysis. No change in follicle apoptosis status was observed within the first 4 hours in any of the follicles, but from 5 hours onwards there was a significant change ($P<0.001$). See Figure 4.12. At 5 hours 22.2% (2 out of 9), at 12 hours 50% (3 out of 6) and at 24 hours 77.7% (7 out of 9) follicles were apoptotic. The 2 follicles which had apoptosis at 5 hours, were the ones that had been out of the mares for the longest (2.7 and 2.9 hours, respectively). At 5 hours only faint apoptosis was seen in the apoptotic follicles, but at 24 hours 4 out of the 7 (57.1%) apoptotic follicles exhibited distinct apoptosis.

The notion that apoptosis became more distinct with time was supported by the 2 follicles, which were apoptotic (grade 1/grade 50) at time point 0. At 5 hours they both showed distinct apoptosis (grade 2/grade 60), and the one follicle which had time point 12 and 24 showed apoptosis grade 2/grade70 at these time points. The 2 follicles with no apoptosis after 24 hours both had started to show strong smearing, which may have obscured apoptotic laddering. It did not appear to make any difference whether the granulosa cells were kept in follicular fluid or medium 199.

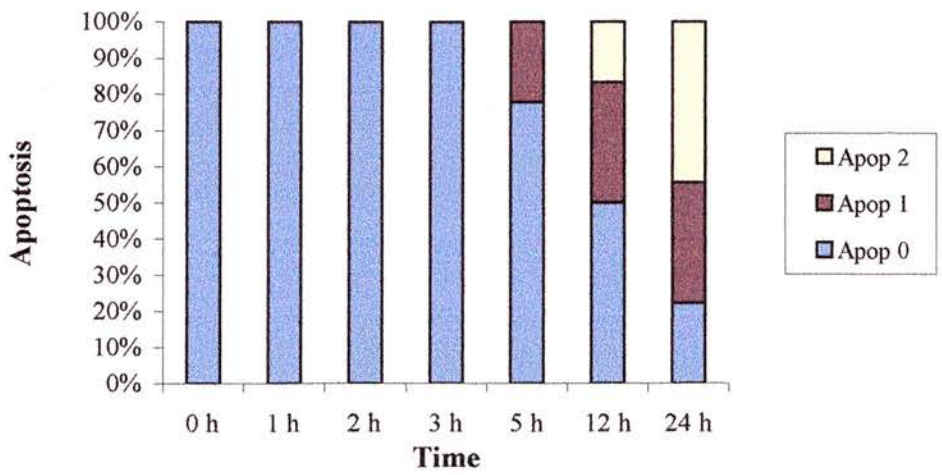


Figure 4.12. Induction of apoptosis in granulosa cells through suboptimal incubation. There was a significant change in proportion of follicles with apoptosis after 5 hours ($P < 0.001$).

4.5 Discussion

The two methods of end labelling and ethidium bromide staining detected the same follicles as apoptotic or non-apoptotic in the majority of the follicles. There was a tendency to better distinguish the apoptosis in the end labelled samples as the end labelling showed more distinct apoptosis than the ethidium bromide staining. End-labelling specifically visualises the 3' ends of broken DNA strands, which makes the method more sensitive for apoptosis than the ethidium bromide staining, which stains DNA in general. In agreement with this study, Jolly *et al.*, (1994b) reported that the end labelling method detected apoptosis in some samples, which were graded as non-apoptotic after ethidium bromide staining. End labelling was also more sensitive than ethidium bromide staining in the sense that much less DNA is needed. Tilly *et al.*, (1993) demonstrated that as little as 15 ng DNA could be detected by end labelling, and there was a dose-related increase in the overall labelling of the DNA with increasing concentrations of [^{32}P]dideoxy-ATP. In the present study, the difference in sensitivity could have biased the results if primarily small follicles were examined with 3' end-labelling. However, there was no clear pattern as to which size range of follicles would yield sufficient DNA for ethidium bromide staining. The only pattern seen was that follicles with a thick layer of granulosa cells, regardless of follicle size, would contain enough DNA for ethidium bromide staining. In conclusion the ethidium bromide staining method was almost as good at detecting apoptosis as the end labelling method, but in some follicles the laddering was more pronounced with end labelling.

Detection of atresia by histology and detection of apoptosis by ethidium bromide staining in the present study, was significantly correlated, a finding which was supported in a bovine study that compared histology with flow cytometric detection of apoptosis (Blondin *et al.*, 1996). The bovine study demonstrated that with increasing degree of atresia, percent apoptotic cells increased. In another study in ewes, the same granulosa cells were stained at first with H&E, washed and then stained by TUNEL, which specifically stains apoptotic fragments. The results showed that the granulosa cell morphologies classified as apoptosis in H&E sections contained fragmented DNA and was indeed apoptosis (Jolly *et al.*, 1997a).

In this study, no biochemically apoptotic follicles were non-atretic, but some of the early atretic follicles did not show apoptotic laddering. Perhaps at this stage there were too few apoptotic cells to be detected by ethidium bromide staining. It is unknown whether DNA laddering in agarose gels was recognisable before or after histological changes in the follicle. Histologically, it may not be possible to detect apoptosis in its very first stages as

histological evaluation involves assessment of nuclear chromatin condensation, which occurs in the later stages of apoptosis (Kerr *et al.*, 1972). In rats, DAPI staining of granulosa cell nuclei allowed detection of apoptotic nuclei at the same time point as DNA fragmentation was detected, but a major increase in oligonucleosome formation happened 24 hours earlier than a major increase in apoptotic nuclei as detected morphologically in hypophysectomised rats (Nahum *et al.*, 1996). This means that there is not much time difference between detection of apoptosis with the two methods, which would support the high correlation between the two methods in this study. Activation of an endonuclease may not be an early event in morphologically recognisable apoptosis (Oberhammer *et al.*, 1993), and endonuclease activation in apoptosis may not be necessary to induce chromatin condensation, suggesting that DNA fragmentation and chromatin condensation may be triggered through separate pathways during apoptosis in mouse liver cells (Sun *et al.*, 1994).

Studies in the cow and ewe reported that apoptotic death of granulosa cells may have occurred in healthy follicles and early in the atretic process before other morphological or biochemical signs of degeneration were evident (Jolly *et al.*, 1994b and 1997b; Blondin *et al.*, 1996). DNA fragmentation was correlated with morphological atresia, follicular fluid hormone content and aromatase activity. These authors concluded that granulosa cells may have died by apoptosis before there was an appreciable decrease in the capacity of the granulosa cell layer to respond to gonadotrophins or to produce oestradiol. Jolly *et al.*, (1994b) found DNA laddering in all bovine follicles that were classified as morphologically atretic, but also in 76% of follicles classified as healthy. In the present study there was laddering in only 31.1% (64 out of 206) of the morphologically healthy granulosa cells (granulosa cell grade 0, 0.5, 1) and in none of the histologically non-atretic follicles. Jolly *et al.*, (1994b) did not use histology or morphology of the granulosa cells for assessing atresia in the follicles but rather theca colour, health status of the oocyte and presence of cellular debris in the follicular fluid. These seem to be less precise methods, and therefore possibly not good enough indicators to assume that the follicles truly were healthy as the authors themselves pointed out. Care needs to be taken with their conclusion that apoptotic death of granulosa cells may have occurred before other morphological or biochemical signs of degeneration were evident. This hypothesis may still be true, though, but was not convincingly shown in the above-mentioned study.

Another possible explanation for the discrepancy between the histology and the ethidium bromide staining method in the early atretic follicles is that atresia is characterised in only a

section of the follicular wall. Because pyknosis (and apoptosis) was also seen to some extent in dominant, viable follicles (Ireland *et al.*, 1982) the onset of atresia is difficult to estimate. Furthermore, apoptosis and mitosis can be present in the same follicle, and therefore atresia is determined by whichever predominates. Kenney *et al.*, (1979) reported that in follicles with mild atresia detected by histology, atretic granulosa cells were not uniformly distributed, but rather were found in foci of atresia among areas of normal looking granulosa cells. This could lead to inaccuracy in the determination of presence of atresia using a histological method where only a small section of the follicle wall is examined. Opposing this view was a report which found that apoptotic cells were not localised in groups but rather widely disseminated throughout the granulosa cell layer in sheep (Jolly *et al.*, 1997a). Detection of apoptosis in granulosa cells by ethidium bromide staining in the present study was based on the use of a large proportion of the granulosa cells in the follicle, although the exact number of cells was not known. Bias from using only few cells from the follicle was therefore not an issue. In conclusion, with the methods used in this study, there was good agreement between methods, and histology appeared to be very sensitive for estimating the health status of the follicle.

A very crude estimate of percentage apoptotic granulosa cells necessary to detect apoptosis in a follicle by ethidium bromide staining was reached: histological evidence of apoptosis with more than 14% apoptotic granulosa cells corresponded very well with apoptosis detected in ethidium bromide gels. In a study of apoptosis in porcine follicles, the percentage of A_0 cells (apoptotic cells) was greater in morphologically atretic follicles (45.9%) than in morphologically healthy follicles (5.3%) (Guthrie *et al.*, 1994). Follicles were biochemically atretic when more than 11.2% A_0 cells were present, which was in good agreement with our crude estimate of 14%.

In this study, there was a clear difference in histologically early apoptotic granulosa cells and histologically advanced apoptosis. In the histologically early apoptosis, the nuclei were half moon shaped within the granulosa cell and with no fragmentation of the nucleus. In more advanced apoptosis, fragmentation of the nucleus and the cell was seen and it was often not possible to see the outline of the granulosa cell. The period of time separating the different morphologies is not known. The material and methods present were not suitable to attempt to correlate early fragmentation of DNA with the early apoptosis seen on histology. It may well be that fragmentation of DNA happened long before any histological signs as discussed above.

In the very atretic follicles (atresia grade 3) ethidium bromide staining did not show any laddering. Either the granulosa cells had perished completely and DNA was extracted from the fibroblast-like cells present or the DNA had been broken down further and thus did not show the characteristic laddering any longer. It has been shown that the difference between the modes of cell death is ill defined and after apoptosis, cells can undergo secondary necrosis (Zamzami *et al.*, 1997). Apoptotic cells can disappear within 24 hours (Kerr *et al.*, 1972) making the disappearance of the granulosa cells a possibility. The thickness of the granulosa cell layer was reduced progressively between 24 and 72 hours after hypophysectomy in rats (Nahum *et al.*, 1996). To reach a very advanced stage of atresia where the granulosa cells have disappeared and only fibroblasts are seen took 4 days in mice (Byskov, 1974). In the mare the duration of the atretic process from viable follicle to pronounced atresia has not been established, but it should in theory be possible to find an answer with ultrasound scanning and the use of follicle aspiration (2-follicle model) or GnRH-antagonism causing atresia in the follicles.

There is a potential error in classifying follicles only by ethidium bromide staining as some advanced atretic follicles could be classified as non-apoptotic or healthy. In order to avoid this potential bias, it is necessary to evaluate a non-apoptotic follicle by parameters other than DNA fragmentation. Sections 4.4.4 and 4.4.5 investigated the validity of using gross morphology of granulosa cells at the time of retrieval in order to estimate e.g. if the follicle is in stage 3 atresia or truly a healthy follicle.

The stereomicroscopic evaluation of the fresh granulosa cells was significantly correlated with the histological findings. The best correlation was in cases of non-atretic follicles and highly atretic follicles. Discrepancy between the methods was seen in the follicles with early atresia (atresia grade 1) were the granulosa cell morphology was relatively insensitive. In agreement with the present study, Hinrichs *et al.*, (1997) also found good correlation between granulosa cell expansion and histological atresia in equine follicles. The finding that almost all follicles with some granulosa cell expansion (granulosa cell grades larger than 0) were histologically atretic, suggested that once the granulosa cells no longer were in close contact with each other in large sheets, the follicle of origin had started to undergo atresia. A characteristic of cells undergoing apoptosis is that junctions with neighbouring cells are severed (Duvall *et al.*, 1986).

The stereomicroscopic evaluation of fresh granulosa cells was also correlated with ethidium bromide staining. An interesting finding was that non-apoptotic follicles consisted of a mix of follicles with healthy and degenerating granulosa cells but mostly follicles with healthy cells. This indicated that in the cases of morphologically degenerating granulosa cells, either the granulosa cells were not apoptotic or there were too few apoptotic cells to be detected on the gel. Apoptosis grade 1 also contained follicles with a mixed population of granulosa cells, but now considerably more follicles contained granulosa cells, which were degenerating than in the non-apoptotic group. The apoptosis grade 2 group contained the most follicles with degenerating granulosa cells. The presence of predominantly granulosa cell grade 2 (few, expanded cells in a gel-like substance) in the group of follicles with apoptosis grade 2, indicated that this granulosa cell grade was more strongly associated with distinct apoptosis than the other granulosa cell grades. Similarly, a bovine paper found that numbers of recovered granulosa cells was inversely correlated with DNA fragmentation (Jolly *et al.*, 1994b).

Granulosa cell grade 2 and 3 were only found in histologically very atretic follicles (atresia grade 2 and 3). An interesting finding was that most follicles with granulosa cell grade 3 (very few, almost no granulosa cells) were found in follicles classified as non-apoptotic. This was in agreement with the histological findings where, in atresia grade 3, the granulosa cells had almost perished and therefore did not show any laddering when run on a gel. This potentially is a major source of error if only presence or absence of apoptosis is used to classify a follicle as healthy or not. These findings suggested that apoptosis grade 0 was a mixed population of truly healthy follicles and highly atretic follicles, which did not show DNA laddering. There are several hypothetical explanations for this. Either the follicles became atretic through pathways other than apoptosis or the apoptotic DNA fragments were degraded further to smaller DNA pieces of varying sizes and thus did not present as laddering or finally, the cells from which DNA was extracted was not granulosa cells as these had perished, but rather fibroblasts. To examine whether apoptotic DNA fragments were further broken down by enzymes and with time would fail to exhibit the ladder pattern due the large spectrum of DNA fragment sizes, granulosa cells could be incubated for a prolonged period of time in sub-optimal conditions. This would at first induce apoptosis as shown above, and leaving the cells for even longer would give the enzymes time to work for a longer period of time.

In conclusion, granulosa cell morphology changes were related to presence of apoptosis, but DNA strand breaks could be present with no morphological change in the granulosa cells. Evaluation of freshly retrieved granulosa cells is a quick way of getting a rough indication of the health status of a follicle and should be used as a supplement to methods for detecting apoptosis, as it can be done immediately and will give necessary additional information, particularly in cases where follicles are highly atretic, but not apoptotic.

There was a significant effect of temperature on presence of apoptosis with fewer apoptotic follicles when held at 20 and 30 °C compared to 35 – 37 °C. Holding temperatures lower than 35-37 °C may have delayed the apoptotic process. Perhaps the endonucleases, as with most enzymes in the warm-blooded animals, work most efficiently at body temperature, and by lowering the temperature, the enzymes do work (as shown in the time study, section 4.2.6.3), but with some delay. The observation that granulosa cells from an initially non-apoptotic follicle were apoptotic after 24 hours, and that the granulosa cells kept at room temperature were apoptosis grade 2, whereas the granulosa cells from that same follicle kept at 5 °C were apoptosis grade 1, also supported the theory that lower temperatures may have delayed apoptosis.

There was a non-significant tendency for apoptosis to increase when leaving ovaries at 30 °C for more than 3 hours. The time study elaborated on these findings and showed that granulosa cells removed from the follicles and left at room temperature in either follicular fluid or medium 199 with Hank's salts started undergoing apoptosis after 5-12 hours of incubation. Apoptosis became more prevalent as incubation time increased, and the apoptosis became more distinct with time. This may have been a reflection of relatively more granulosa cells becoming apoptotic, and therefore it became easier to detect the apoptosis because a veil of non-laddered DNA from presumably normal cells no longer obscured it. This hypothesis was supported by (Wyllie, 1980a), where thymocytes incubated with dexamethasone showed stronger laddering at 5 hours than at 3 hours. Care needs to be taken when assessing degree of laddering. The amount of degraded, low molecular weight DNA, which can be extracted from a single cell, varies depending on the stage of apoptosis. Early during apoptosis only a small fraction of DNA is degraded but when apoptosis is more advanced nearly all DNA is fragmented (Darzynkiewicz *et al.*, 1998). Theoretically the DNA fragments in this study could stem from few, very apoptotic cells, but the use of more than 90% of granulosa cells from the follicles will diminish chances of only a few cells being very apoptotic and the remainder not apoptotic at all.

Tilly *et al.* (1993) found spontaneous onset of apoptosis in granulosa cells cultured in serum free medium for 24 hours, consistent with the result of this study. Another study supported further findings of the present study by reporting that bovine follicles showed no signs of oligonucleosome formation up to 5 hours after ovariectomy (Imig *et al.*, 1993). Follicles became progressively apoptotic after hypophysectomy of rats, with DNA fragments starting to appear between 4 and 8 hours after hypophysectomy (Nahum *et al.*, 1996). The progressive appearance of apoptotic fragments in the rat study also supported the findings of this study, that apoptosis became more prevalent with incubation time. The apoptosis induced in vivo by hypophysectomy was interesting as it suggested an immediate effect of lack of hormones from the hypophysis on apoptosis. Perhaps the breakdown of DNA in follicles removed from the mares was not simply post mortem change due to lack of blood supply but also a direct result of hypophyseal hormone withdrawal.

The present studies suggested that follicles should be held for no more than 5 hours before processing. The holding medium should be calcium and magnesium free, as the endonuclease responsible for apoptosis is dependent on these minerals.

4.6 Conclusion

Studies on atresia in equine follicle populations have been conducted using histological material to classify follicles as growing or regressing (Kenney *et al.*, 1979; Driancourt *et al.*, 1982a). In this chapter ethidium bromide staining and radioactive end labelling of the free 3'-end of DNA fragments were used to identify apoptosis. Histology was used for detecting atresia and apoptosis in a section of the follicular wall and finally granulosa cell morphology was used as an immediate indication of follicle health. There are a number of other methods to detect apoptosis and atresia, but the temporal and economic limits of this project did not allow for more methods to be used. The conclusion of the present chapter was that the apoptosis detection and grading system was supported by the other ways of detecting follicular health, but it had potential pit falls. Each of the methods to detect atresia and apoptosis has advantages and limitations. The more parameters that are used in conjunction with each other, the more accurate is the interpretation of the state of the follicle. This knowledge was crucial for the following chapters of the thesis.

5 Follicular growth and regression in relation to plasma gonadotrophin levels during the unmanipulated oestrous cycle

5.1 Introduction

Follicular development has been studied for several years using a variety of methods. Studies on equine follicle populations involved rectal palpation (Aitken, 1926; Bergfelt *et al.*, 1985) and sectioning of whole ovaries at different periods in the oestrous cycle (Driancourt, 1979, 1982a, 1983, and 1984). In the eighties ultrasonography became available and the first report on equine ovaries was published in 1980 (Palmer *et al.*, 1980). In the horse, studies on follicular growth have been performed with ultrasonography alone (Sirois *et al.*, 1989; Ginther, 1990a, 1992c and 1993a), combined ultrasonography with follicle stimulating hormone measurements (Ginther *et al.*, 1992a and,1993c; Bergfelt *et al.*, 1992 and 1993), and with physical manipulation of the follicles by aspiration (Gastal *et al.*, 1997b, 1999a, 1999b and 1999c), and aspiration combined with hormone manipulation (Gastal *et al.*, 1999d, 1999f, 2000). Follicle dynamics have been studied intensively in cattle (Savio *et al.*, 1988; Murphy *et al.*, 1990; Driancourt *et al.*, 1991a; Sunderland *et al.*, 1994) and to a lesser extent in sheep (Fry *et al.*, 1996; Evans *et al.*, 2000). In pigs follicular growth has been studied more commonly by excision of the ovaries at different stages of the cycle rather than scanning (Grant *et al.*, 1989; Guthrie *et al.*, 1990; Miller *et al.*, 1998).

Scanning has been used to monitor follicle changes during spring transition (Ginther, 1990a), during the oestrous cycle (Sirois *et al.*, 1989; Bergfelt *et al.*, 1993; Ginther, 1993a), and during pregnancy (Bergfelt *et al.*, 1992; Ginther *et al.*, 1992a and 1992c). Most studies on follicular growth in mares have focused on follicles larger than 15 mm (Sirois *et al.*, 1989; Bergfelt *et al.*, 1993), due to the difficulty in tracking smaller follicles. Follicular waves have been defined by the simultaneous emergence and development of several follicles and it has been established that follicular growth occurs in waves in mares (Sirois *et al.*, 1989; Bergfelt *et al.*, 1993), and in cattle (Ginther *et al.*, 1996). Most mares had one wave, which gave rise to the ovulatory follicle, but some had 2 waves, where the dominant follicle of the dioestrous wave either ovulated during dioestrus or regressed. One study attempted to describe the dynamics of smaller follicles (2-20 mm) in the unmanipulated cycle (Ginther *et al.*, 1993b). More waves were found in the smaller size groups than in the largest follicles. Because of the difficulty in tracking individual small follicles Gastal *et al.*, (1997a) developed the 2-

follicle model, a technique of aspirating all follicles on the ovaries, allowing only two follicles to grow, and periodically ablating any other follicles, which grew up. This enabled them to follow the growth of only the dominant and the subordinate follicle, although having created a somewhat artificial environment for the study of growth.

Associations between elevations in mean daily FSH concentrations and emergence of follicular waves during the interovulatory interval in heifers (Adams *et al.*, 1992), sheep (Campbell *et al.*, 1999), during pregnancy of mares (Bergfelt *et al.*, 1992; Ginther *et al.*, 1992a) and during the equine oestrous cycle (Bergfelt *et al.*, 1993; Ginther *et al.*, 1993b and 1993c) were reported. An increase in mean concentrations of FSH was shown to occur a few days before the dominant follicle of a wave appeared in mares (Bergfelt *et al.*, 1993). Individual follicles larger than 15 mm were monitored during days 0 - 50 of pregnancy, and periodic emergence of follicular waves occurred in association with mean increases and decreases of FSH concentrations (Bergfelt *et al.*, 1992). In horses, the importance of FSH in relation to follicular development was demonstrated by diminished follicular development following treatment with a proteinaceous fraction of equine follicular fluid, which lowered FSH concentrations (Bergfelt *et al.*, 1985).

Reports in mares (Gastal *et al.*, 1997a and 1999c) and cattle (Ginther *et al.*, 1998) suggested that deviation was preceded by an increase in LH concentrations. In follicle aspirated mares, an early increase in LH induced by prostaglandin treatment did not speed up the follicle deviation compared with follicles in mares that had natural luteolysis 4 days later, indicating that LH did not increase growth rate of small follicles (Gastal *et al.*, 2000). In the cow the early stages of follicle development (follicles less than 4 mm) were not dependent on gonadotrophins, but FSH was needed for growth to 7 – 9 mm and LH pulses were necessary for follicle development beyond 9 mm in diameter (Gong *et al.*, 1996).

Very few comparisons have been made between horses and ponies. Two studies focused on differences in progesterone production up to and during pregnancy, and found that follicle growth rate and maximum follicle diameter before ovulation and luteal diameter were not different between mare types (Bergfelt *et al.*, 1996; Townson *et al.*, 1989). During the luteal phase however, progesterone concentrations tended to be higher in ponies than in mares, but this was non-significant (Bergfelt *et al.*, 1996). Differences in cycling characteristics, which have been reported in ponies, were longer oestrous cycle, shorter ovulatory season and fewer double ovulations and dioestrous ovulations (Ginther, 1974). In a study of sectioned ovaries,

no differences between pony and horse mares were found with regards to number of growing follicles and the distribution into size classes (Driancourt *et al.*, 1982b).

Despite the many studies on follicular growth, there are no detailed studies in mares examining levels of gonadotrophins, E2 and P4 throughout the cycle in relation to follicular growth patterns in the unmanipulated cycle. The aim of this experiment was to characterise the relationship between changes in hormone levels during the cycle in relation to the appearance of follicular waves and ovulatory follicles as well as to compare hormone levels and follicular growth patterns in ponies and horses. These data were needed for chapter 6, in order to try to answer the overall question of when does selection occur and how is this reflected in the dominant and the subordinate follicles? The previous chapters (chapter 3 - oocytes, cumulus and chapter 4 - follicle health) and the present chapter provide the background for the final experimental chapter. It was necessary to know at which stage of cycle follicles were growing and regressing, and at which point deviation took place in order to establish when to retrieve the ovaries for determining health of the follicles at a microscopic and biochemical level. In chapter 6, the ovaries were removed from the animal, but in the present chapter the follicles were followed over a full cycle, providing a picture of what the health status of the follicles in the animal may have been at the time of retrieval as judged by ultrasonography.

5.2 Materials and methods

Eight pony mares and 6 horse mares were scanned daily from 4-8 days before the first ovulation until the second ovulation, equivalent to one full cycle plus 4-8 days. The ponies' cycles lasted from 6.5.98 to 28.8.98 and the mares' cycles lasted from 30.7.98 to 20.9.98. Ponies were defined as mares shorter than 148 cm measured over the withers, and horses were larger than 148 cm. The weights and ages of the mares are shown in table 1.

Figure 5-1. Weight and age of ponies and mares

	Pony, mean ± sem	Pony, range	Horse, mean ± sem	Horse, range
Weight	305 ±12.3 kg	240-325 kg	464 ±32.8 kg	383-584 kg
Age	9.8 ±1.9 years	2.5 – 16 years	11.4 ±1.4 years	8 – 16 years

5.2.1 Ultrasound scanning

The cervix, uterine body and horns and ovaries were palpated to examine tone and size. A 5 MHz linear array transrectal probe was inserted into the rectum and the vagina, cervix, uterus and ovaries were visualised on an ultrasound scanner. The vagina was examined for presence of fluid, the cervix for density, the uterus for presence of fluid and oedema and the ovaries for follicular activity and presence of corpora lutea. Care was taken to distinguish other non-echogenic areas from follicles. Cross-sections of blood vessels could be mistaken for small follicles and cystic vestiges of embryonic structures associated with the oviducts could mimic follicles, although they could be identified just outside the ovary. Maps were drawn at the time of scanning of each ovary's structures. Each ovary was scanned from one end to the other several times to make sure that all follicles had been recorded. The diameter of the follicles was measured on the screen, and presence or absence of an echogenic rim along the inside of follicle was noted, as well as any signs of luteinisation such as trabeculae in the lumen. Diameters of non-spherical follicles were obtained by averaging length and width. See Appendix D for follicle map and scanning pictures.

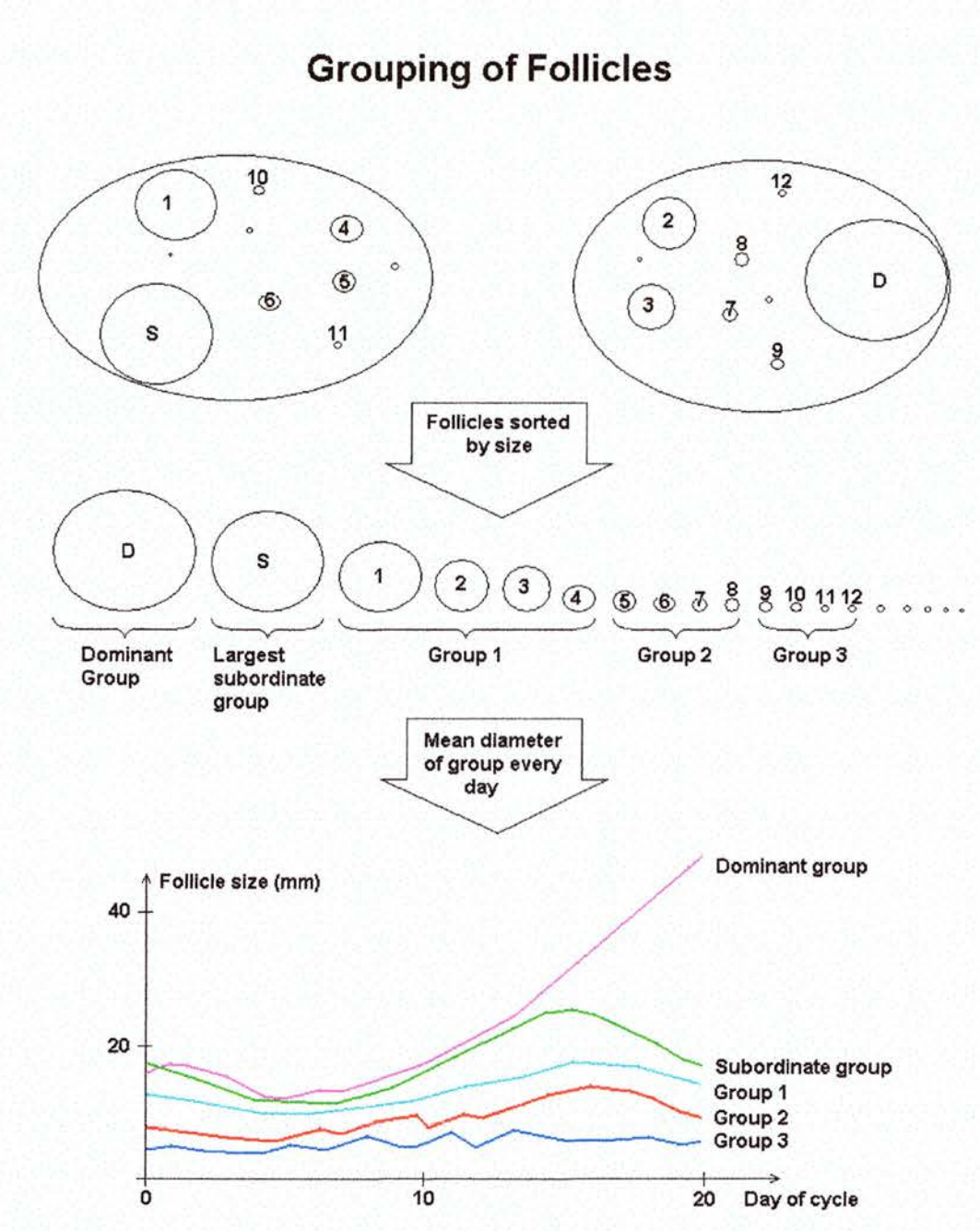
5.2.2 Hormones

Jugular blood samples (2 x 7.5 ml) were collected daily at the time of scanning. Circulating plasma concentrations of FSH, LH and E2 were measured in all mares on all days, and progesterone was measured in all horses and in 2 ponies. Initially, progesterone measurements were not part of this project, but the data from the mares and the 2 ponies were used in another study, for which progesterone was measured, and the progesterone data was included from these animals in the present study. For details of hormone assays, refer to chapter 2.

5.2.3 Follicle maps

The individual mare's follicle maps were analysed for each day during the cycle. For each mare the dominant and the largest subordinate follicle were traced retrospectively. The dominant follicle was the follicle, which went on to ovulate and which could be traced back in time as the largest follicle on its ovary. The largest subordinate follicle was the largest follicle on either ovary, which was not the dominant follicle. The remaining follicles were combined for both ovaries and ranked from largest to smallest without regard to day to day identity of the individual follicles, because small follicles could not be identified in a

consistent manner from day to day. See diagram page 102. The ranked follicles were divided into groups with the 4 largest follicles in group 1, the 4 next largest follicles in group 2, the four following follicles in group 3, until all follicles had been designated. When combining data from different animals, each animal contributed 4 follicles from each follicle group per day. For 8 ponies this would mean that group 1 consisted of 8 ponies times 4 follicles, equivalent to 32 follicles. The 32 follicle diameters were averaged to obtain mean follicle diameter in group 1 on that day. This resulted in one dominant follicle group, one largest subordinate follicle group, group 1 (4 largest follicles apart from the dominant and largest subordinate follicle), group 2 (next 4 largest follicles), group 3 (smaller than group 2), group 4 (smaller than group 3), group 5 (smaller than group 4) and group 6 (smallest follicles). In some cases too few follicles were present in the two smallest groups to make 4 follicles per mare, and therefore they were added together to form group 5+6. A mean follicle diameter was obtained for each group on each day. When plotted against days, mean diameter of a particular group on a particular day was visualised, depicting the waves of follicle growth (see appendix D). A follicular wave was defined as a significant increase in size from the smallest follicle diameter of a group, followed by a decrease where the follicle diameter was no longer significantly different from the initial small follicle diameter. The day of wave emergence was defined by the day of the lowest mean preceding the increase. The day of divergence was the first day of a significant size difference between the dominant and subordinate follicle. The beginning of a phase of regression was the first day of negative growth rate. The identity of the individual follicles from day to day was determined only in the dominant and the largest subordinate groups but not in the groups 1-5+6. Follicles, which were less than 2 mm were not measured, and for ranking the follicles, these were arbitrarily set to be 1 mm. The follicle data handling was based on a method in (Ginther *et al.*, 1993b) but with modifications.



The dominant and the largest subordinate follicle were kept separate each day. The remaining follicles were combined for both ovaries and ranked from largest to smallest without regard to day to day identity of the individual follicles. The ranked follicles were divided into groups with the 4 largest follicles in group 1, the 4 next largest in group 2 until all follicles had been designated. The results from each day were plotted in a graph and depicted the waves of growth during the cycle.

5.2.4 Statistical analysis

Data from each mare was normalised to the mean cycle length in order to avoid error from the difference in cycle lengths. At first mean cycle length was determined. A full cycle from day of ovulation until the day before the next ovulation was the equivalent of 1. The days of the mean cycle were expressed as fractions of 1. Each mare's day of cycle was transformed into a fraction of 1. The fractions of the individual mare were fitted to the fractions of the mean cycle. In case of more days in the individual mare's cycle, the average of the measured factor (hormone concentrations, follicle diameter) over 2 days was calculated. In case of a shorter cycle than the mean cycle, the mare would have missing data in some cycle fractions (arbitrary cycle days). By normalising cycle lengths, an arbitrary cycle was obtained in which the changes in e.g. E2 was relative to the ovulation in each animal, thereby eliminating error from differences in cycle lengths. If not normalising the cycles, the mean E2 concentrations would result in a preovulatory rise spread out over many days as each pony approached ovulation. Furthermore, the data at the end of the cycle in a mare with a long cycle, would not be analysable, as there would only be data from this particular animal.

As some of the tests in the following required that data were from a normally distributed population, a hypothesis of normal distribution of the data was tested and accepted (see Appendix C). Several of the tests used were parametric tests. A non-parametric test was also applied. Parametric data can validly be tested with non-parametric tests, only the power of the test is not as good as the parametric tests.

To compare the difference between hormone levels and follicle growth in ponies and horses, a regression analysis was performed. Mean levels of hormones or follicle means per day in ponies was plotted against mean levels in mares. The resulting plot indicated the degree of correlation. The correlation was only descriptive. No statistical test was performed on the correlation, as the data were not independent as is required to perform a statistical test on regression data. The data were not independent because the same animals were sampled from day to day. Instead a Wilcoxon signed rank test was used to test whether there were any differences between the hormonal patterns throughout the cycle and furthermore to test the actual concentrations through the cycle in horses and ponies. To examine patterns, the concentrations on a specific day were expressed as a percentage of the total concentration of the whole cycle to eliminate potential differences in concentrations between horses and ponies. This percentage was obtained for both ponies and mares. The percentages from ponies were subtracted from percentages from horses for each day, summed for the whole

cycle and tested as median = 0.00 versus median not = 0.00, showing how close the sum of differences was to zero. If the sum of differences was close to zero as determined by the test, the horses and ponies were not significantly different. To test the actual concentrations, the levels per day in horses and ponies were subtracted and the differences were summed and tested for closeness to zero. When a significant difference was detected with the Wilcoxon signed rank test, which only detects an overall difference, confidence intervals were used to localise the specific days of difference. In order to locate days of significant changes of mean hormone levels or mean follicle diameter, the difference between two sample means was analysed and tested with confidence intervals for each day. When there was no overlap between means and confidence intervals, there was a significant difference. This test was crude in terms of detecting significant changes, but it was excellent at depicting the changes through the cycle. A t-test was applied to find the exact day on which concentrations or sizes became significant. In some cases the t-test involved creating a baseline mean against which single points were tested. The confidence intervals and the t-tests were used in conjunction because of the different capacities. Because of the changing diameter of the follicles through the cycle, there was no baseline, and only confidence intervals in connection with means were tested. The rates of growth and regression of follicles were evaluated by linear regression.

5.3 Results

One horse mare was excluded from the study because her cycle was non-ovulatory and was terminated with prostaglandin on day 26 after the dominant follicle had started to regress and uterine oedema had been absent for several days. Examination of her hormone data showed that the dominant follicle was oestradiol producing, but that no LH surge appeared, perhaps due to incomplete luteolysis (progesterone > 3 ng/ml). One pony mare was excluded because of ovulation of a 27 mm follicle (day 0/day 24) followed by another ovulation (day 6/day 30). Both mares went on to have normal ovulations at the expected time in subsequent cycles. Data from 7 pony mares and 5 horse mares were analysed. The mean cycle length of the ponies was 21.0 ± 0.98 days and for the mares 21.8 ± 0.92 days. The mean length of luteal phase of 5 horses and 2 ponies was 15 days and the follicular phase was 6 days.

5.3.1 Luteinising hormone

Mean LH concentrations were higher in horse mares around the time of the LH surge, but this difference failed to reach significance (P = 0.62) (Figure 5-2). There was a strong

correlation ($R^2 = 0.82$) between LH levels in ponies and horses, indicating that the changes followed the same pattern during the cycle. The slope of the regression line (2.3) reflected the (non-significant) higher concentrations in horses.

As there were no significant differences between levels of LH in ponies and horses, data were added together. When combining data from ponies and horses LH concentrations reached a maximum on day 0 – 1, decreased after day 1 and reached a minimum concentration on day 11. After day 11 the concentration gradually increased until the preovulatory surge. The change in concentration became significant ($P < 0.05$) from day –3 until day 4, where concentrations returned to low levels, suggesting that the LH surge lasted 8 days, and that the low mid cycle period lasted from day 5 until day 14 (see Figure 5-3).

5.3.2 Follicle stimulating hormone

There was no significant difference between ponies and horses either in patterns through the cycle ($P = 0.77$) or in concentrations ($P = 0.19$). See Figure 5-4.

When combining data from horses and ponies a trough in FSH levels were found from day -4 until day –1 (Figure 5-5). A wave started after day –1 with maximum on day 3, remaining elevated and fluctuating until after day 14, where a steady decline began to form the preovulatory trough. The concentrations were significantly increased from day 1 until day 14 ($P < 0.05$). There was a significant decrease after day 14 ($P < 0.01$).

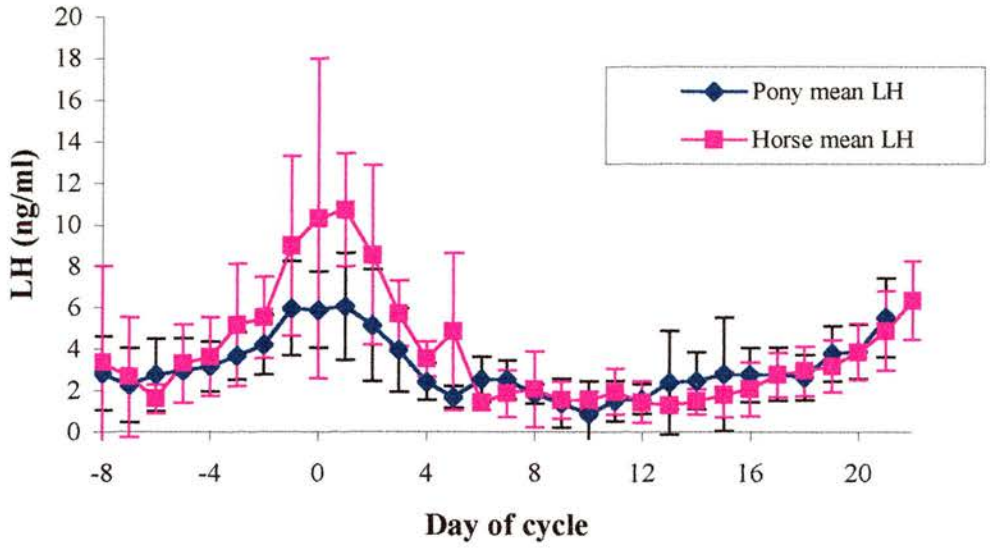


Figure 5-2. LH concentrations during the cycle in ponies and mares. Data from pony mares (n = 7) and horse mares (n = 5) are shown with 95% confidence interval bars. There was no significant difference between ponies and horses.

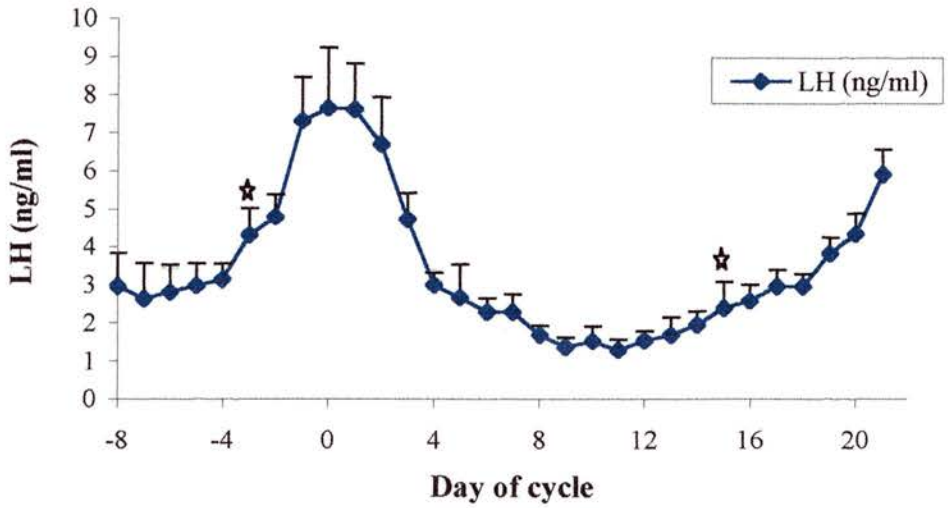


Figure 5-3. LH concentrations during the cycle. Stars indicate the beginning of a significant change from dioestrous values ($P < 0.05$). Bars indicate SEM. Data were obtained from 12 mares.

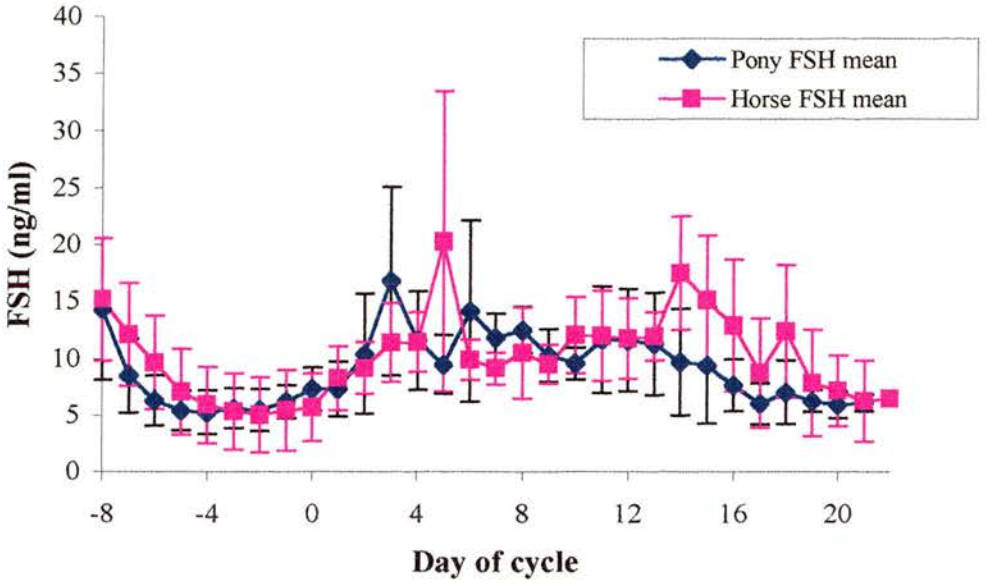


Figure 5-4 FSH levels in ponies and mares during the cycle. Data from pony mares (n = 7) and horse mares (n = 5) are shown with 95% confidence intervals. Differences between the two equine types were not significantly different with a Wilcoxon ranked test or with the more crude 95% intervals, which are shown here.

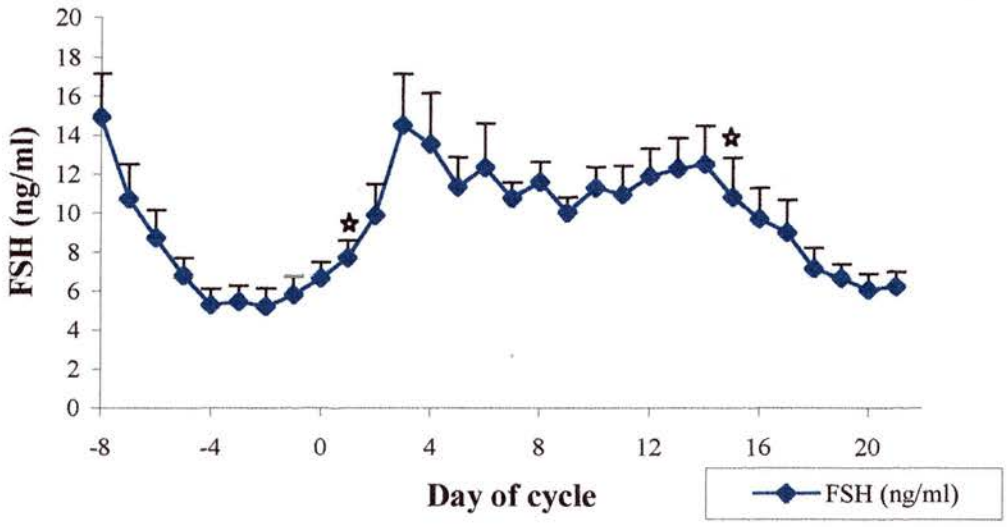


Figure 5-5. FSH during the cycle. Stars indicate days of significant change ($P < 0.05$). Bars indicate SEM. Data were obtained from 12 mares.

5.3.3 Oestradiol

The mean oestradiol levels for ponies and horses appeared to follow the same pattern during the cycle ($R^2 = 0.81$), with no significant difference in changes through the cycle ($P = 0.92$). Oestradiol levels from day 0 to day 14 did not vary significantly ($P > 0.05$), but in ponies the preovulatory oestradiol wave started earlier than in the horses, which was reflected by the mean for ponies being significantly higher on days 14, 16 and 17 ($P < 0.05$). In the previous cycle (days –8 to –1), the ponies also appeared to have higher concentrations of oestradiol, but the variation in the individual pony was so large that there was no significant difference to the horses. This difference between the two preovulatory periods could be due to some animals having received prostaglandin before the first ovulation. See Figure 5-6.

The data for horses and ponies were added together despite the difference in concentrations on days 14, 16 and 17. The reasoning was that the pattern of change through the cycle was the same for both ponies and horses. Adding the data together would increase standard deviations in the data on these particular days, but after consultation with a statistician it was decided that the data would benefit from the stabilisation through more measurements (12 animals rather than either 5 or 7) despite the differences on the 3 days. The choice was made to add the two groups together, in the knowledge that these data were statistically different on 3 days.

Combined data for ponies and horses showed that E2 was significantly elevated above the low dioestrous levels from day –6 to day 0 ($P < 0.05$), at base line values from day 1 until day 13 where levels started to increase ($P < 0.05$), reaching maximum 2 days before ovulation. See Figure 5-7.

5.3.4 Progesterone

Progesterone levels increased significantly on day 1 after ovulation ($P < 0.05$) to a maximum on day 5, fluctuating until day 12, when a steady and significant decrease began ($P < 0.05$). See Figure 5-8.

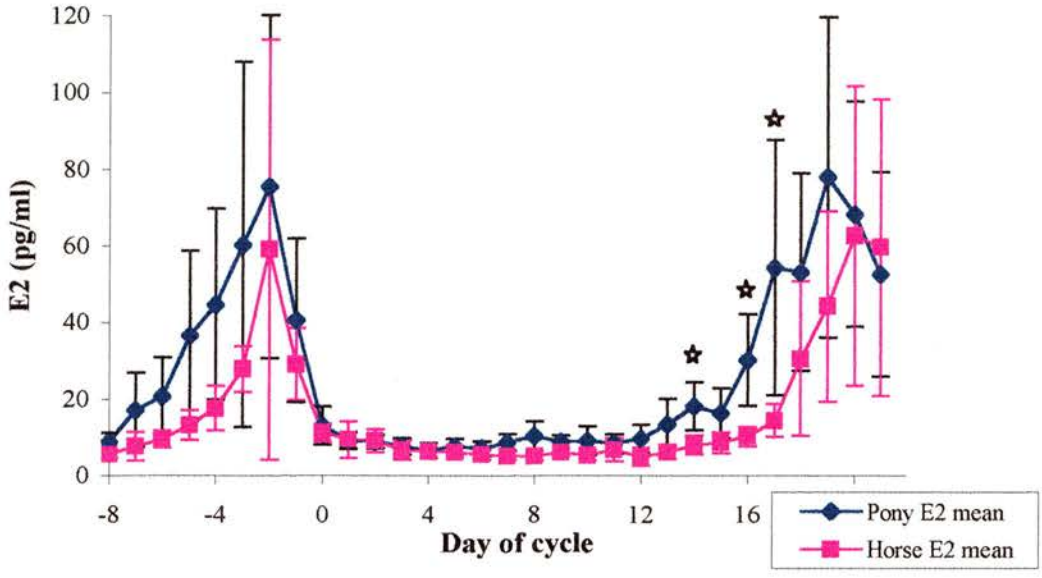


Figure 5-6. E2 in horses and ponies during the cycle. Data from pony mares (n = 7) and horse mares (n = 5) are shown with 95% confidence intervals. There was a significant difference between the two mare types in connection with the rise during oestrus. Stars indicate a difference ($P < 0.05$).

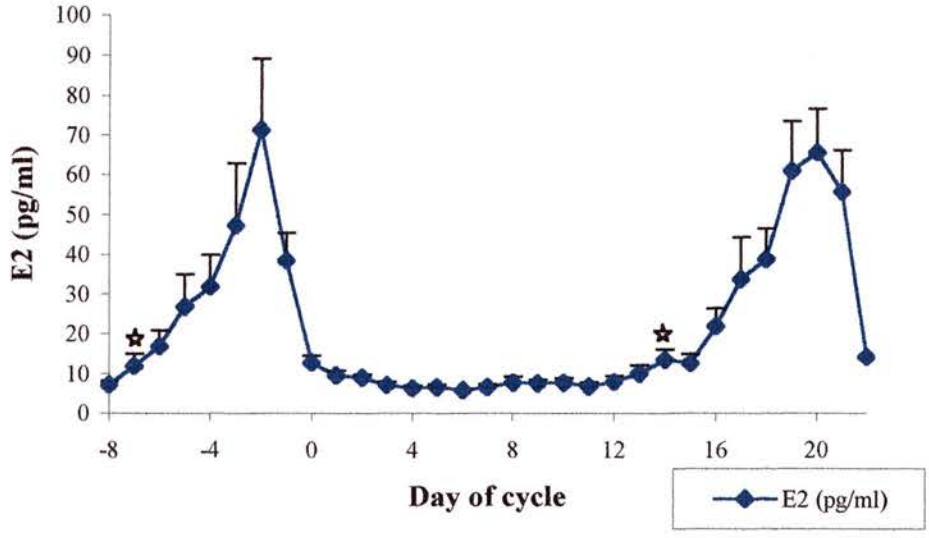


Figure 5-7. E2 concentrations during the cycle. Stars indicate first day of significant increase in concentration ($P < 0.05$). Bars indicate SEM. Data were obtained from 12 mares.

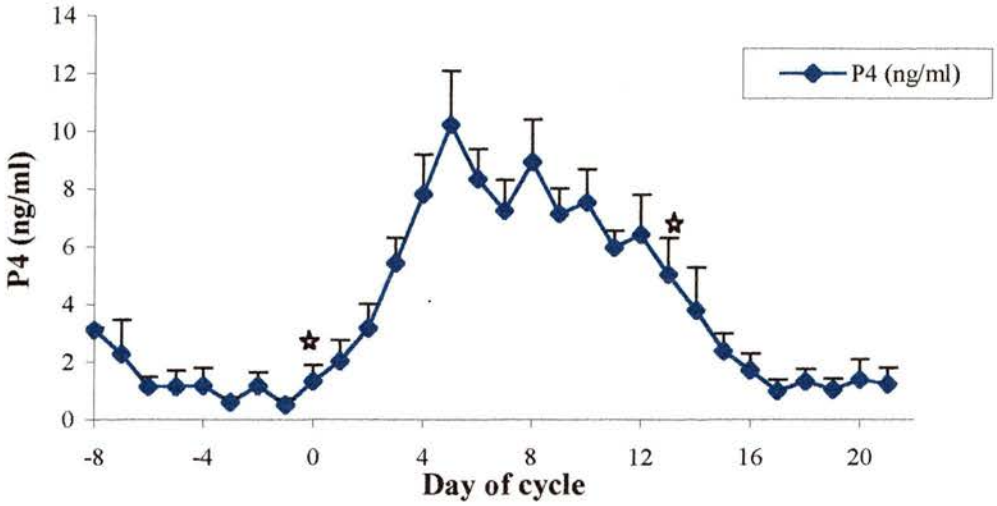


Figure 5-8. Progesterone during the cycle. Stars indicate day of significant change in concentration ($P < 0.05$). Bars indicate SEM. Data were obtained from 7 mares.

5.3.5 Follicle maps

Figure 5-9 shows combined follicle map data for ponies and horses.

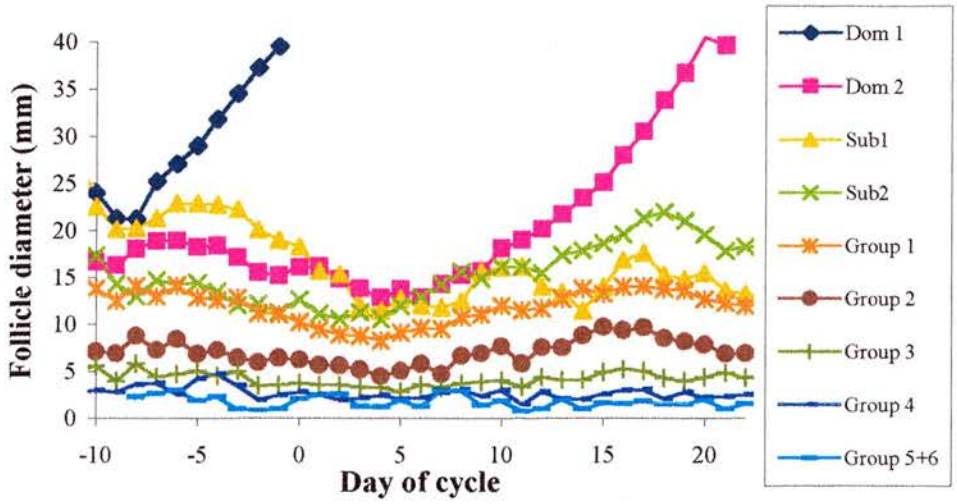


Figure 5-9. Combined follicle maps for horses and ponies. Scanning started 10 days before the first ovulation and continued until one day after ovulation on day 21. Data were obtained from pony mares (n = 7) and horse mares (n = 5).

5.3.6 Dominant follicle

There was no significant difference ($P > 0.05$) in diameter of the dominant follicle between ponies and mares, although the horse follicles tended to be smaller until 2 days before ovulation. The dominant follicles of ponies and horses were highly correlated ($R^2 = 0.87$) in their growth pattern. Because there were no differences between ponies and horses, data were added together. During the cycle the ovulatory follicle appeared on day 6 as a 12.9 ± 1.0 mm follicle, increased in size and became significantly larger when it was 19.0 ± 2.0 mm on day 11 ($P < 0.05$). See Figure 5-10 and Figure 5-11.

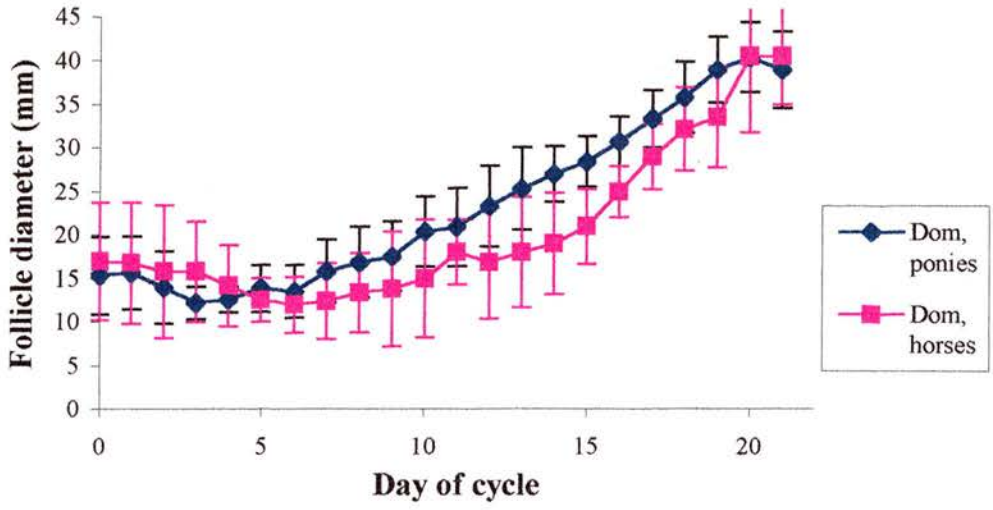


Figure 5-10. Dominant follicle mean diameter in horses and ponies. Data from pony mares (n = 7) and horse mares (n = 5) are shown with 95% confidence intervals. There were no significant differences between horses and ponies in growth rate and size of the dominant follicles. The average diameter increase from day 10 was 2 mm in ponies and 2.5 mm in horses. From day 0 until day 6, the depicted follicles were regressing follicles from the previous cycle.

5.3.7 Largest subordinate follicles

The diameters of the largest subordinate follicles in ponies and horses were not significantly different ($P > 0.05$), although the horse follicles tended to be smaller than those of the ponies. The subordinate follicle appeared on day 4 as a 10.4 ± 1.4 mm follicle. From day 9 the subordinate follicle did not grow as fast as the dominant follicle, but it did increase in size until day 18 when the largest diameter (22 ± 1.5 mm) was reached and from then on the subordinate follicle decreased in size. The decrease was not significant until after ovulation. See Figure 5-12.

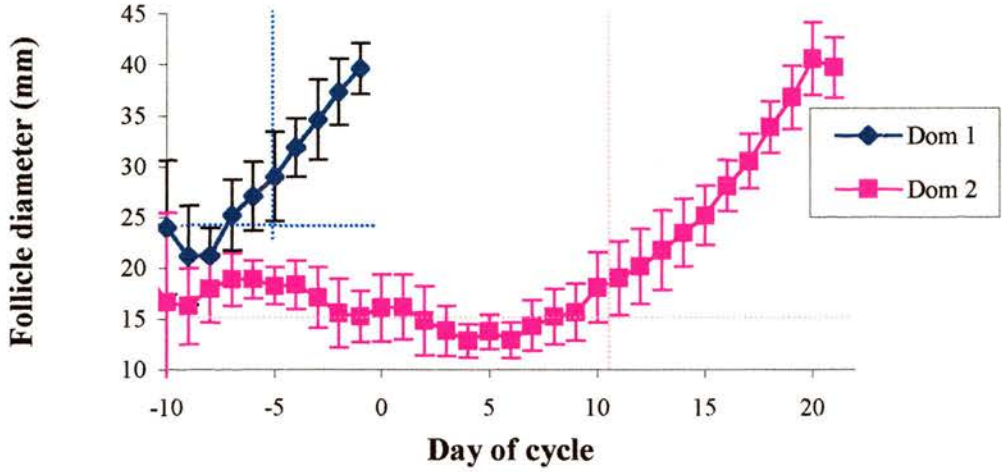


Figure 5-11. Dominant follicle diameter during the cycle. Horizontal lines show the upper limit for the 95% confidence interval. Vertical lines indicate point of significant increase in diameter ($P < 0.05$). Average increase in diameter from 8 days before ovulation was 2.3 mm for dominant follicle 1, and 2.0 mm for dominant follicle 2. N = 12 mares.

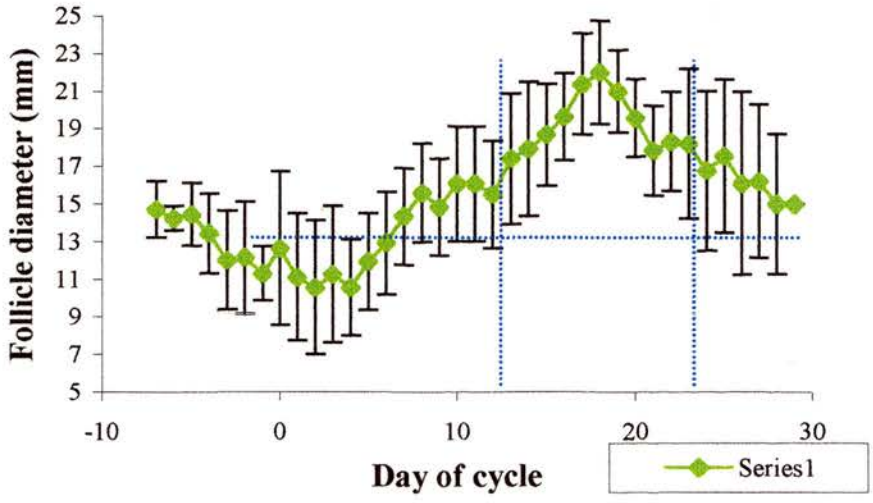


Figure 5-12. The largest subordinate follicle's diameter changes during the cycle. Data are shown with 95% confidence intervals (n = 12 mares). Horizontal lines show the upper limit for the 95% confidence interval. Vertical lines indicate point of significant increase and decrease in diameter ($P < 0.05$), delineating a wave. The mean cycle length was 21 days, but the first 6 days of the following cycle are depicted, with day 22 being equivalent to day 0 of the new cycle.

5.3.8 Groups of subordinate follicles

The first group of subordinate follicles in ponies and horses followed the same pattern during the cycle and were significantly correlated ($P < 0.001$) but were different size-wise ($P < 0.05$). From day 3 until day 15 the mare follicles were smaller than the ponies. The same was true for the second group of subordinate follicles, although the correlation was less significant ($P < 0.01$). Despite the size difference on some days between horses and ponies, the data were added together, because the patterns through the cycle were the same. The effect on follicle groups would be larger standard deviations, and a curve, which reflected the mean of the two types of horses, but it was chosen to accept the error imposed by this to obtain a better overview of the data.

One wave of growth was found in group 1 subordinate follicles. The follicles decreased in size until day 4 (8.3 ± 0.4 mm) then significantly increased in size from day 8 ($P < 0.05$) with maximum (14.1± 0.8 mm) on day 17. Group 2 followed the exact same pattern of growth of one wave, but the follicles were smaller with a mean minimum of 4.5 ± 0.3 mm and a maximum of 9.7 ± 0.5 mm (Figure 5-13). Group 3 also showed one follicular wave. The minimum mean diameter for group 3 follicles was on day 5 (2.8 ± 0.2 mm), with a significant increase ($P < 0.05$) after day 11, reaching maximum diameter (5.3 ± 0.4 mm) on day 16 (Figure 5-14). Group 4 encompassed 2 follicular waves (Figure 5-15). The first had a minimum diameter on day 2 (2 ± 0.2 mm), a maximum diameter on day 8 (3 ± 0.2 mm), and the second wave started on day 11 (1.5 ± 0.2 mm) with a maximum diameter on day 17 (3 ± 0.4 mm). Group 5+6 had 4 follicular waves. The follicles fluctuated between <2 and 3 mm with maximum diameters on day 2, 7, 13 and 20 (Figure 5-16). The wave emergence in the groups was approximately parallel. The follicles in groups 1 - 5+6 emerged 1-2 days before the day of the emergence of the dominant follicle. The range of follicular mean diameters on the day of wave emergence was <2 mm (group 5+6) to 8.3 ± 0.4 mm (group 1).

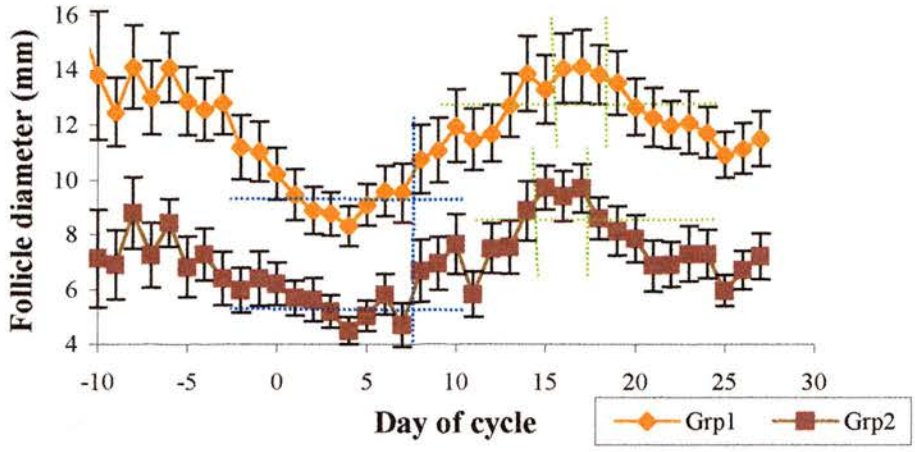


Figure 5-13. Groups 1 and 2 follicle diameters during the cycle. Data are shown with 95% confidence intervals. Horizontal lines show the upper or lower limit for the 95% confidence interval (n = 12 animals). Vertical lines indicate point of significant increase and decrease in diameter ($P < 0.05$), delineating a wave. The blue lines delineate the limits for the wave trough, and the green lines delineate the limit for the wave top. There was one wave of growth in groups 1 and 2. Significant top-point in group 2 was reached a day before group 1. The mean cycle length was 21 days, but the first 6 days of the following cycle are depicted.

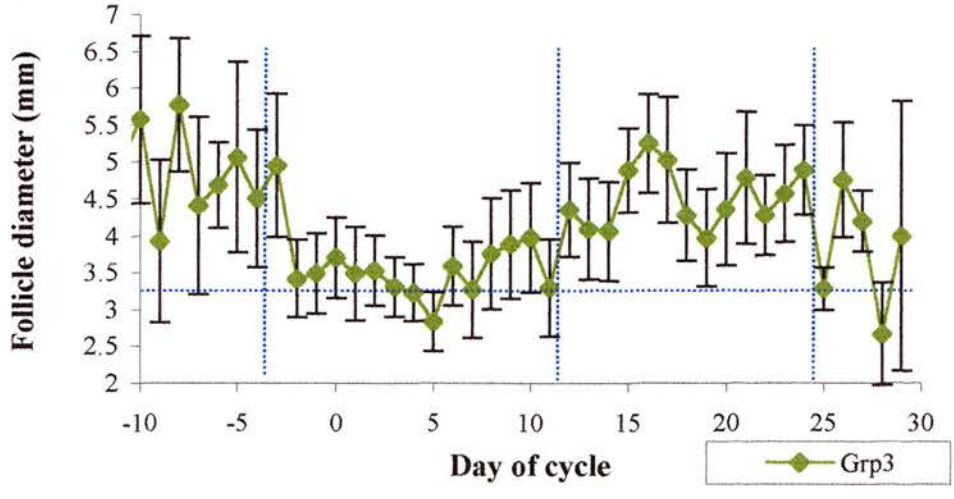


Figure 5-14. Changing diameter of group 3 follicles during the cycle. Data are shown with 95% confidence intervals (n = 12 animals). Horizontal lines show the upper limit for the 95% confidence interval. Vertical lines indicate point of significant increase and decrease in diameter ($P < 0.05$), delineating a wave. There was one wave of growth per cycle (day 0 – 21) in group 3.

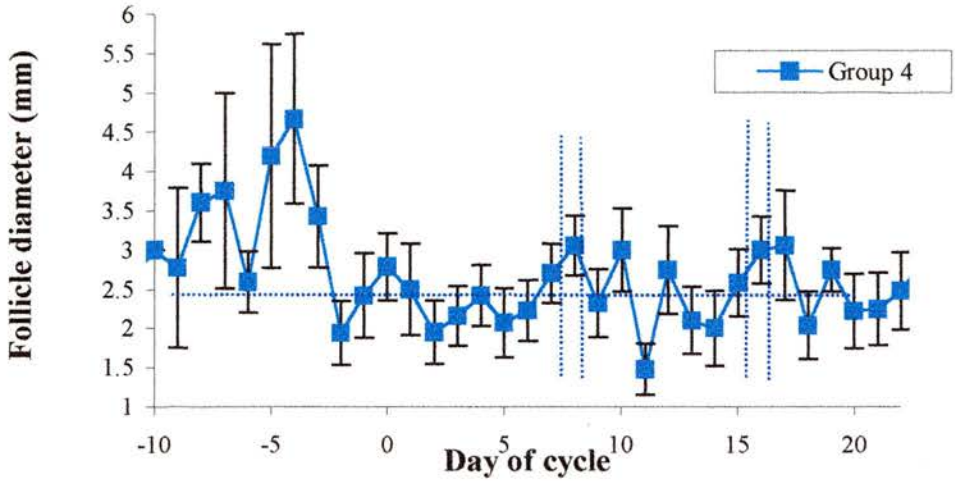


Figure 5-15. Changing diameter of group 4 follicles during the cycle. Data are shown with 95% confidence intervals (n = 12 animals). Horizontal lines show the upper limit for the 95% confidence interval. Vertical lines indicate point of significant increase and decrease in diameter ($P < 0.05$). There were 2 periods of significant increase in follicle size in group 4.

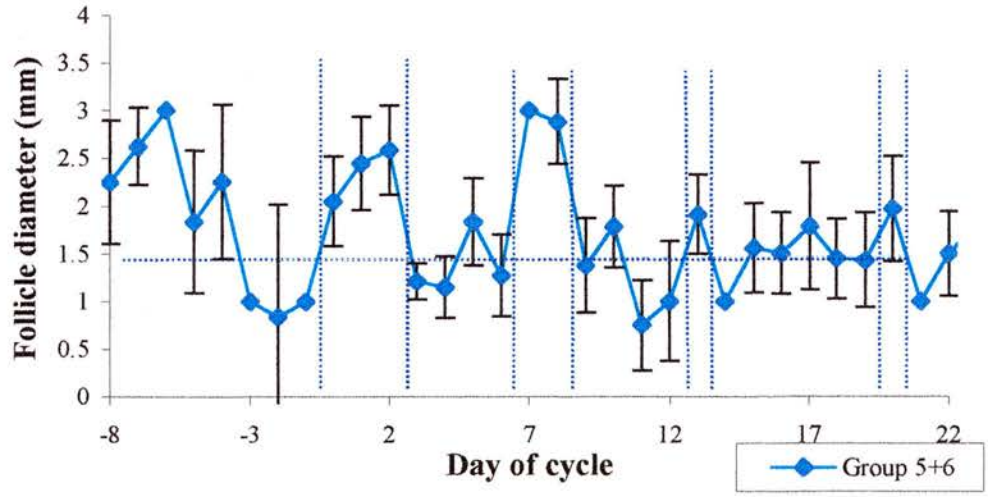


Figure 5-16. Changing diameter of group 5+6 follicles during the cycle. Data are shown with 95% confidence intervals (n = 12 animals). Horizontal lines show the upper limit for the 95% confidence interval. Vertical lines indicate point of significant increase and decrease in diameter ($P < 0.05$). There were 4 periods of significant increase in follicle size in group 5+6.

5.3.9 Growth rate

The growth rate of the dominant follicle from day 6 until day 13 was 1.3 ± 0.1 mm per day, and between day 14 and day of ovulation growth rate was 2.6 ± 0.2 mm per day (Figure 5-17).

5.3.10 Divergence

Divergence between the ovulatory and the largest subordinate follicle was first seen after day 9, but the difference between the follicles was not significant until day 12 (P = 0.04). See Table 5-1and Figure 5-18. The subordinate follicle did not decrease in size until after day 18 or 3 days before ovulation. The decrease was not significant until after ovulation.

5.3.11 Regression of follicles

The regression of the largest subordinate follicle started from day 18 and the follicle could be followed until day 4 after ovulation, when the follicle had regressed to 16.1 ± 2.7 mm from a maximum of 22 ± 1.5 mm (regression rate: 0.7 ± 0.1 mm per day). The subordinate groups 1 and 2 started regressing from day 17 with a regression rate of 0.8 ± 0.1 mm per day for group 1 and 0.4 ± 0.03 mm per day for group 2. See Figure 5-19.

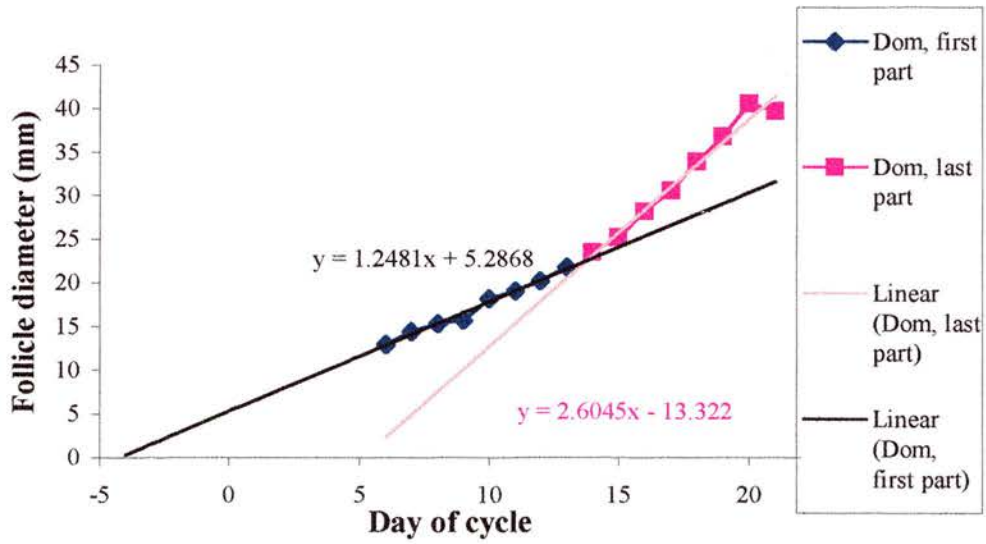


Figure 5-17. Growth rates of dominant follicle. The growth rate was slower between day 6 – 13 compared to day 14 – ovulation.

Table 5-1. Deviation of the dominant and largest subordinate follicle

	Dominant follicle (mm) ± SEM	Subordinate follicle (mm) ± SEM
Day 9	15.7 ± 1.6 [a]	14.8 ± 1.3 [a]
Day 12	20.2 ± 2.1 [b]	15.5 ± 1.6 [a]
Day 18	33.9 ± 1.4 [c]	22 ± 1.5 [b]

[A, b, c] Means with different superscripts across rows and columns are significantly different ($P < 0.05$). Twelve mares contributed with each a dominant and a subordinate follicle per day.

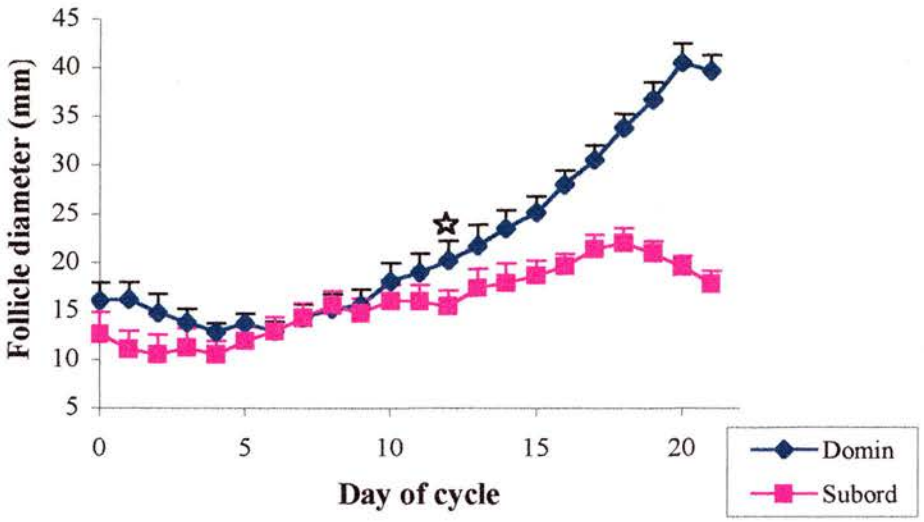


Figure 5-18. Divergence in follicle size of the dominant and largest subordinate follicle. The star indicates day of significant difference between the two ($P < 0.05$). The growth rate of the subordinate follicle decreased from the initial rate between days 6-8, and grew slower than the dominant follicle. Data were obtained from 12 mares.

5.3.12 Relation between gonadotrophin levels and growth of follicles

In ponies and horses the dominant and the subordinate follicles started increasing in size on day 6, at a time where LH was declining. LH reached minimum values on day 11, on which day the dominant and subordinate follicle started to deviate. LH increased as the dominant follicle grew larger and increased until 1 day after the dominant follicle had ovulated. The subordinate follicle both grew and regressed in the face of increasing LH. The increase in LH was correlated with dominant follicle growth ($R^2 = 0.87$). See Figure 5-20.

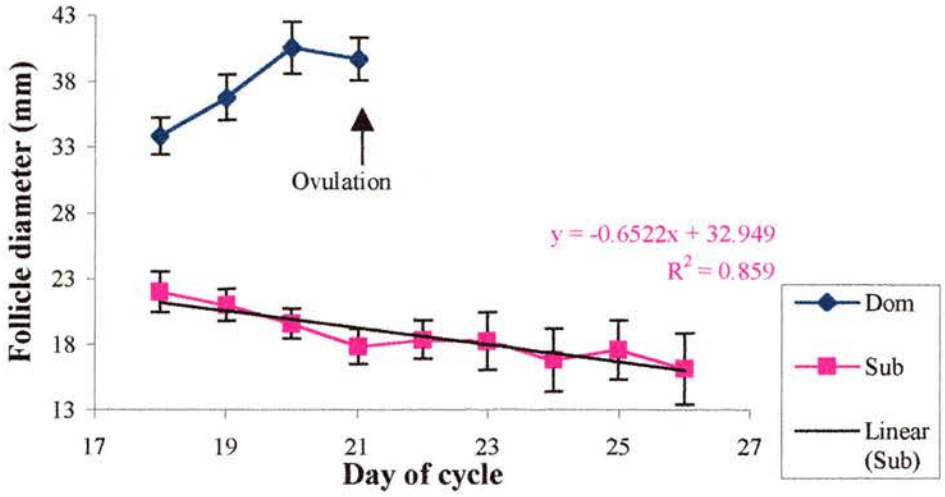


Figure 5-19. Rate of regression of the largest subordinate follicle. Data were obtained from 12 animals.

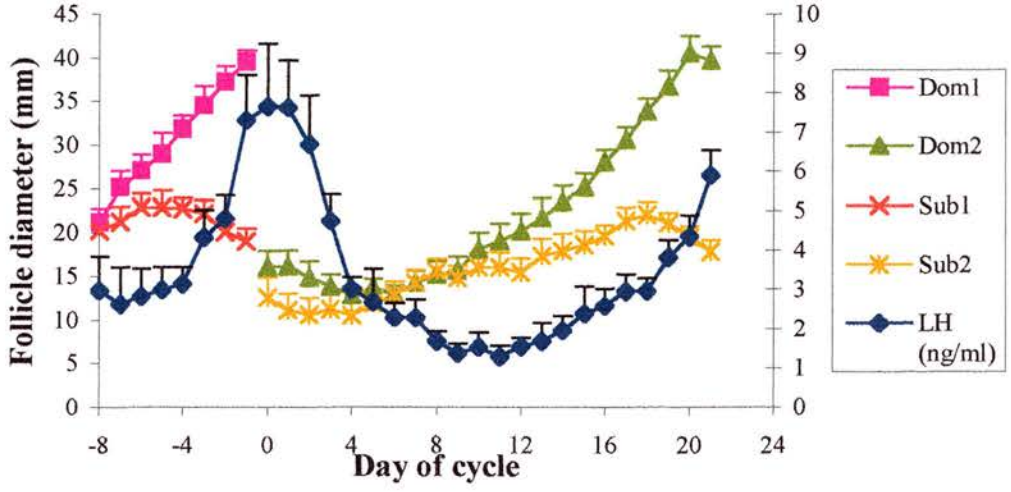


Figure 5-20. LH concentrations, dominant and largest subordinate follicle changes during the cycle. The preovulatory rise in LH was correlated with growth of the dominant follicle. Dom1, dominant follicle of the first ovulation; dom2, dominant follicle of the second ovulation; sub1, subordinate follicle of the first ovulation; sub2, subordinate follicle of the second ovulation. Second y-axis: LH concentrations (ng/ml). Data were obtained from 12 animals.

Immediately after ovulation both LH and group 1 and 2 follicles were declining, but after day 4 the follicles started growing and LH continued to decline. After day 10 LH and follicle groups 1 and 2 increased, but the follicles started regressing from day 17 whereas LH continued to increase. There was no correlation between LH and growth of 4-14 mm subordinate follicles ($P > 0.1$). Furthermore follicles in the groups 3-6 (1-6 mm) appeared to be growing independently of LH.

FSH peaked on day 3 after ovulation, and the dominant and subordinate follicles emerged on day 6. FSH increased from the day before ovulation, but the dominant and the subordinate follicles could not be discerned earlier than day 6, because of the pool of similarly sized follicles making it impossible to tell the individual follicles apart until they grew beyond a certain size. From day 14, as the dominant follicle grew larger than 23 mm, FSH declined until the day before the dominant follicle ovulated and was thus negatively correlated ($R^2 = 0.95$).

FSH was significantly correlated with growth of group 1 subordinate follicles. There was a delay of 8 days between the minimum of FSH (day –4) and the minimum of group 1 follicle size (day 4; 8.3 ± 0.4 mm), and a delay of 14 days between the maximum FSH (day 3) and maximum follicle size (day 17; 14.1 ± 0.8 mm). FSH started declining after day 14 and was followed by a decline in mean follicle size from day 17. When normalising FSH and group 1 to the day with the lowest point of each, a correlation between FSH increase and increase in follicle size was evident ($P < 0.01$). The same pattern was seen with the group 2 subordinate follicles.

As FSH declined after the high dioestrous concentrations, group 5+6 reached maximum on day –6 (3 mm), group 4 on day –4 (4.7 ± 0.6 mm) and group 3 on day –3 (5 ± 0.6 mm). This indicated a growth of the smallest follicles into the larger size groups with time. After the maximum in follicle sizes, which happened after the maximum in FSH concentrations, all follicle groups decreased in size as FSH decreased to a minimum on day –4 to –2. This decrease was significant for all groups ($P < 0.05$). The day of emergence of a wave of growth for each of the five groups was temporally associated with the highest mean of FSH concentration, which occurred on day 3 of the oestrous cycle. See Figure 5-21.

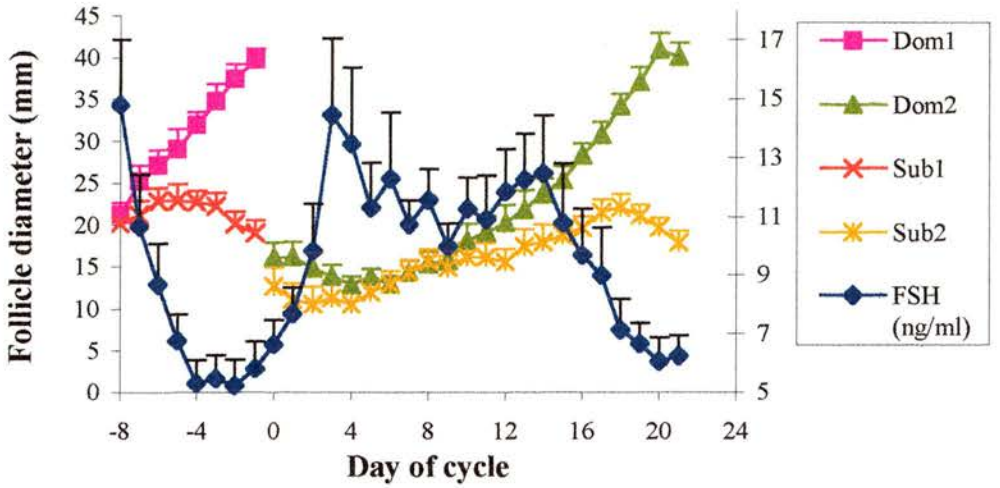


Figure 5-21. FSH, dominant and largest subordinate follicle during the cycle. FSH decreased as the dominant follicle grew. Dom1, dominant follicle of the first ovulation; dom2, dominant follicle of the second ovulation; sub1, subordinate follicle of the first ovulation; sub2, subordinate follicle of the second ovulation. Second y-axis: FSH concentrations (ng/ml). Data were obtained from 12 animals.

Growth of the dominant follicle and oestradiol production was highly correlated ($R^2 = 0.95$). The dominant follicle started to produce increasing concentrations of E2 from day 13 or 4 days after the start of deviation, when it grew beyond a mean of 21.8 ± 2.2 mm. Maximum E2 production was reached 2 days before ovulation. As E2 increased, group 1 and group 2 follicles decreased in size. See Figure 5-22.

Deviation between the dominant and the largest subordinate follicle occurred during the luteal phase, but the growth rate of the dominant follicle increased a few days later, at the time of luteolysis as seen by a decrease in progesterone concentrations. See Figure 5-23.

As the dominant follicle produced increasing concentrations of oestradiol from day 13, FSH started declining after day 14, and LH increased from day 15. Oestradiol decreased from day –2, FSH started increasing the following day and LH declined after day 1. Progesterone increased after ovulation until maximum on day 5, fluctuated until day 12 after which there was a steady decrease, at the time of an increase in LH (Figure 5-24).

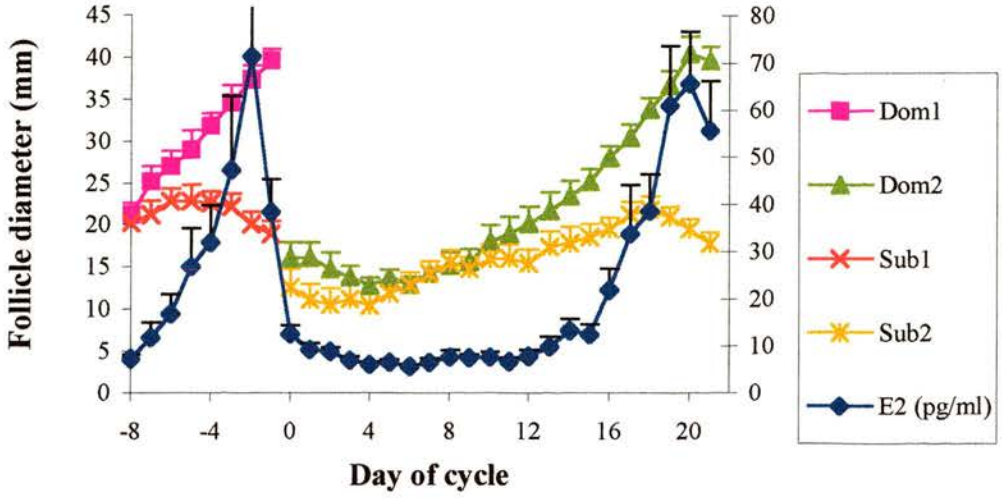


Figure 5-22. Changes in oestradiol concentrations in relation to growth of the dominant and subordinate follicle. The preovulatory rise in E2 was correlated with growth of the dominant follicle. Dom1, dominant follicle of the first ovulation; dom2, dominant follicle of the second ovulation; sub1, subordinate follicle of the first ovulation; sub2, subordinate follicle of the second ovulation. Second y-axis: E2 concentrations (pg/ml). Data were obtained from 12 animals.

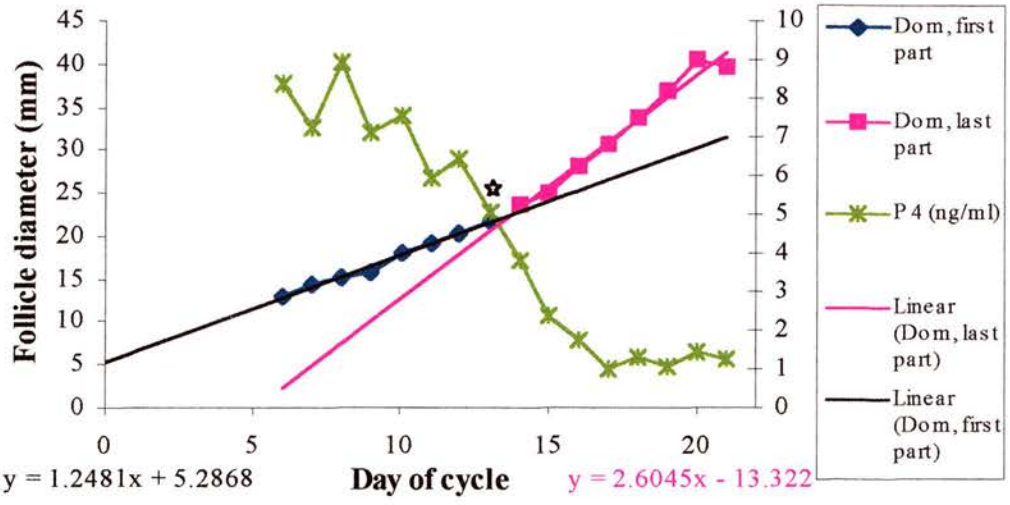


Figure 5-23. Change in growth rate of the dominant follicle in relation to decreasing P4 concentration. The daily average diameter increase of the dominant follicle increased from 1.2 mm to 2.6 mm per day, coinciding with the time of luteolysis. The star indicates day of significant change in P4 concentrations ($P < 0.05$). Follicle data were obtained from 12 animals and progesterone data were obtained from 7 animals.

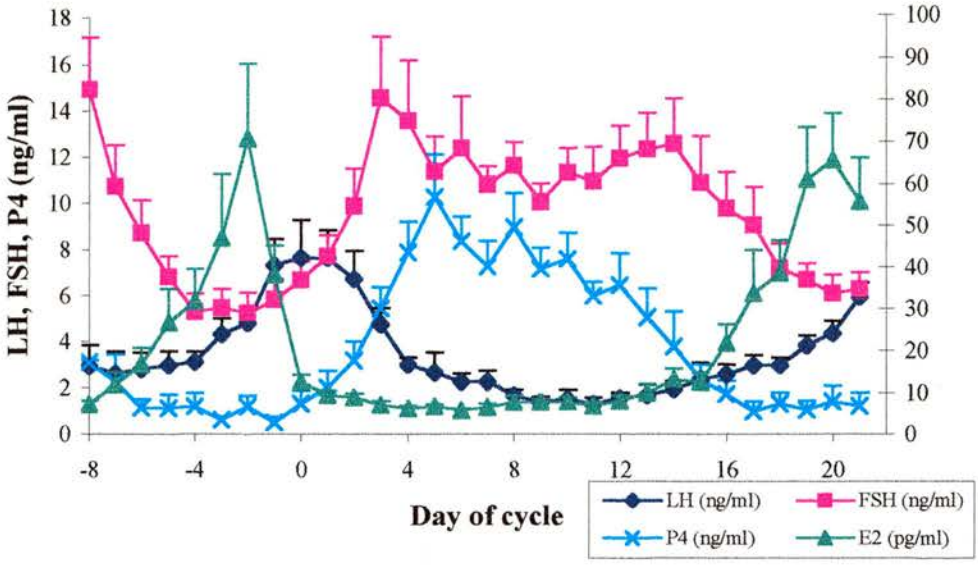


Figure 5-24. Changes of hormone concentrations during the cycle. There are almost reciprocal relationships between LH and E2 on the one hand versus FSH and P4 on the other. Second y-axis: E2 (pg/ml). Data were obtained from 7 pony mares and 5 horse mares.

5.4 Discussion

In this experiment the hormonal changes during the cycle in relation to follicular growth were investigated along with the differences between the cycles of horses and ponies. Ponies are often used as horse models because they are a more economical equine model than horses. Mean cycle length of the ponies was 0.8 days shorter than the horses' cycle. The ponies' cycles lasted from 6.5.98 to 28.8.98 whereas the horses' cycles lasted from 30.7.98 to 20.9.98 and thus was later in the year. In the literature there are great variations in cycle length from study to study. Sirois *et al.*, (1989) reported a mean cycle length of 19.2 days in Standardbred and Thoroughbred mares and Ginther (1974) reported a mean cycle length of 22.3 – 27.5 days dependent on month in ponies. The cycles are shortest around the longest day of the year (June) (Ginther, 1974), which may be the reason why the ponies' cycles were shorter than the horses' cycles. Furthermore, some of the small horses and large ponies were rather close in weight.

LH and FSH concentrations and patterns during the cycle were similar in horses and ponies, although there was a non-significant tendency to a difference. There was a large individual variation in hormone concentrations, particularly in the horses. In ponies the preovulatory

oestradiol wave started earlier than in the horses, although the pattern of cyclic changes and concentrations were otherwise similar. The dominant and largest subordinate follicles of ponies and horses were similar in sizes and growth patterns although the horse follicles tended to be smaller than in the ponies. These findings were in accordance with a study of pony and horse follicles from day –10 to day 0, where no difference was found in follicle growth rate and maximum follicle diameter (Bergfelt *et al.*, 1996). In the present study, the smaller subordinate follicles followed the same growth patterns during the cycle in ponies and horses but were significantly different size-wise. This is in contrast to a study using excised ovaries where no differences between pony and horse mares were found with regards to number of growing follicles and the distribution into size classes (Driancourt *et al.*, 1982b). Inspection of the data in this latter paper revealed a tendency towards higher numbers of 50 μm – 10 mm follicles in ponies, but equal numbers of follicles larger than 10 mm, which is in accordance with our data. Overall, ponies and horses appeared to be very similar, particularly in respect to timing of cyclic changes, but with a tendency to a difference in early antral follicle growth. Hormone concentrations showed a tendency to a difference at times of increased concentrations in LH, FSH and E2, but there were such large individual variations that the potential differences between horses and ponies were non-significant. Because of the high similarity, ponies appeared to be acceptable as general equine models for studying hormone levels and follicle growth, bearing in mind the possibility of a difference in growth of smaller follicles.

The ovulatory LH surge reached maximum concentrations on day 0 – 1, decreased after day 1 and reached a minimum on day 11. After day 11 the concentration gradually increased, significantly so from day 15 which coincided with luteolysis. These findings are in agreement with other reports, where concentrations increased progressively after luteolysis, reaching a maximum 1 - 3 days after ovulation and then diminishing both in concentration and pulse amplitude to dioestrous levels over the following 4 - 6 days (equivalent to days 5 – 9 after ovulation) (Whitmore *et al.*, 1973; Irvine *et al.*, 1994; Irvine *et al.*, 1998). In the present study, only daily sampling was used rather than intensive sampling, but it has been shown that the LH profile during the cycle was similar whether determined by sampling once daily or sampling every 4 hours (Irvine *et al.*, 1998).

A trough in FSH levels was present from day -4 until day –1. A wave started after day –1 with maximum on day 3, remaining elevated and fluctuating until after day 14, where a steady decline began to form the preovulatory trough. The concentrations were significantly

increased from day 1 until day 14. There was a significant decrease after day 14 or 7 days before ovulation at a time where the dominant follicle grew and started to produce increased levels of oestradiol. These results are in agreement with other studies, which reported low mean concentrations of FSH during oestrus, increased concentrations during dioestrus and a decrease, which began approximately 8 days before ovulation (Evans *et al.*, 1975; Bergfelt *et al.*, 1993). One study reported higher mean concentrations on day 4-5 and day 7-10 than in oestrus (Irvine *et al.*, 1998), whereas in this study the highest concentrations were found on day 3-4 and again on day 12-14. Cycle lengths in the two studies were similar. The FSH values of the preovulatory trough had small SEM, and the rise in FSH was concomitant with a large variation in the measurements, possibly due to the pulsatile release of FSH in dioestrus, which at this time in the cycle was reported to be slow and of high amplitude (Irvine *et al.*, 1998).

According to (Irvine *et al.*, 1998) once daily sampling is insufficient because of the marked pulsatility of FSH during dioestrus. Nonetheless the present data showed a pattern of low oestrous levels and high dioestrous levels. Finer detail in pulsatility would have been missed, but most likely follicles would have been exposed to more FSH during the period of high mean FSH levels irrespective of pulses.

E2 was elevated above the low dioestrous levels from day –6 to day 0, returned to dioestrous levels from day 1 until day 13 where concentrations started to increase, reaching maximum 2 days before ovulation. One study described a significant increase in concentrations 3 days before ovulation, remaining elevated until one day before ovulation (Meinecke *et al.*, 1987), whereas another was more in accordance with the present study describing an increase from day -7 until day –1 and returning to low levels on day 1 (Daels *et al.*, 1991).

The mean length of the luteal phase was 15 days and the follicular phase was 6 days, which was in agreement with the findings in other studies where the luteal phase ranged between 14.9-16.3 days and the follicular phase ranged between 4.3 – 7.1 days (Ginther, 1974; Sirois *et al.*, 1989). Progesterone levels increased significantly on day 1 after ovulation to a maximum on day 5, oscillating until day 12, where a steady and significant decrease began. These findings are in agreement with findings that progesterone increased within the first 24 – 36 hours after ovulation to high dioestrous levels by day 5 - 7, decreasing from day 13 - 14 or 3 days before onset of oestrus (Townson *et al.*, 1989).

The dominant follicle was defined as the largest follicle on one ovary, which would go on to ovulate. Until a mean of day 6, the largest follicle most likely was a regressing follicle from the previous cycle, but from a mean of day 6 the largest follicle was increasing in size and in many cases could be identified as one specific follicle from that day until ovulation. In some cases where the dominant follicle and the largest subordinate follicle were on the same ovary and they were close in size, they could have swapped identity if the smaller of the two grew to be the largest. Ginther *et al.*, (1993b) suggested that growth and regression of small follicles occurred throughout the interovulatory interval, and that the greater incidence of waves in the smaller size groups possibly was due to the smaller follicles being more likely to undergo atresia after a short period of growth than were the follicles in the larger size groups. This may be true, but another explanation may be that the smaller size groups have more atretic and regressing follicles passing through the group, and if a large atretic follicle enters a small size group, it will increase mean diameter of that group, despite it actually not growing. Wave-like changes in the smaller groups, could potentially be the result of movement of individual follicles from one group to another as well as by growth and regression of the whole group of follicles. The smaller size groups are more sensitive to these fluctuations as all follicles will pass through them, whereas this is not the case for the larger size groups.

The day of appearance was the day where regressing and growing follicles had reached the same size within a group and after this day, the diameters of growing follicles became larger than that of the regressing follicles meaning that the ratios of growing to regressing follicles had changed. At the time of a wave of follicles decreasing in size, the majority of follicles were regressing, and even though some follicles may have been growing and increasing in size, regressing follicles would have outnumbered them. The presence of a regressing follicle during a period of growth of a wave would lower the mean growth rates as the follicle passed through different size groups.

Bergfelt *et al.* (1993) used mean day of emergence of the dominant follicle to normalise the dominant and largest subordinate follicles and FSH to the day scale. This probably meant that they moved the individual mare's data either forwards or backwards on the day scale to fit the mean. The method in my study transformed all data to fit into a hypothetical cycle, whose length was determined by the mean of all mares. This way, the data was not manipulated around a specific day, but the individuality of each mare was kept because of the equal cycle length, and was then in a form, which was directly comparable to the other

mares. The present study focused on emergence of waves, changes in hormone levels and regression in relation to the whole cycle and not just a single day.

In some species, waves of follicle growth have been described as either individual follicles or a group of follicles growing up and regressing, followed by the growth of new follicles within the same cycle. In lambs, follicle growth occurred in 2 or 3 organised waves during the oestrous cycle with oestrous cycle lengths being longer the more waves were present (Evans *et al.*, 2000). When defining a follicular wave as a follicle that began to grow from 3 to more than 5 mm in diameter within a 48-hour period, waves were found to appear every 5 days in sheep (Bartlewski *et al.*, 1999). In mares, there was just one wave of growth in 71% of mares' cycles and 2 waves of growth (dioestrous wave either ovulatory or non-ovulatory) in the remaining mares (Sirois *et al.*, 1989). The mares in the latter study were thoroughbred mares, in which dioestrous ovulations are more common than in ponies (Ginther, 1974). In our study follicular waves were detected in all groups of follicle sizes, but there was only one wave of growth per cycle in the larger follicles. The number of waves per interovulatory interval was greater for group 5+6 (4 waves) than for the dominant group (1 wave) and groups 1-4 (1-2 waves). The findings of this study were in accordance with a similar study, where mean diameters for groups initially decreased and then on a mean of 6 days after ovulation, the mean diameters began to increase until day 15 (Ginther *et al.*, 1993b).

In cattle the growth patterns have been divided into phases of recruitment (growth of a group of follicles), selection (one follicle continues to grow and the others undergo atresia) and dominance (the selected follicle dominates morphologically). The results of the present study did not support this theory with these definitions in horses. Undoubtedly there was a phase of recruitment where groups of follicles started to grow (group 5+6 increased in size from 1-2 days before ovulation until day 2 coinciding with the initial increase in FSH). The equivalent of the recruitment phase would be the phase of growth, which is seen after day 6. The actual recruitment of the dormant follicle happens long time before this, and this subject is almost unstudied in horses, apart from studies by Driancourt *et al.* on early recruitment of follicles (Driancourt *et al.*, 1982a and 1982b). The selection phase as it is defined in cattle is apparently not present in horses. One follicle is clearly selected to be dominant (deviation beginning on day 9), and there is a phase of dominance, but the subordinate follicles did not undergo atresia as is part of their definition of selection. Rather the subordinate follicles kept growing until day 17, despite their being markedly smaller in size than the dominant follicle.

Tonic FSH and LH stimulation is necessary for the follicles to reach an immature antral stage but recruitment of the follicles for further growth requires additional stimulation by FSH (Hillier, 1994; Gong *et al.*, 1995 and 1996). If FSH support is removed, antral follicles will become atretic (Dufour *et al.*, 1979; Driancourt *et al.*, 1987). Ablation of the dominant follicle in heifers induced an increase in mean FSH concentrations, which resulted in a wave of follicular growth (Adams *et al.*, 1992). When treating the animals with follicular fluid, the FSH surge was obliterated and no follicle wave appeared. In the 2-follicle model described by Gastal *et al.*, (1997a) (see introduction), the presence of a dominant follicle suppressed the FSH surge and resulted in failure of other waves to emerge. The smaller of two follicles had no FSH suppressing capacity unless it grew to become dominant. If it became atretic, FSH increased (Gastal *et al.*, 1999c). When progesterone treatment prevented a rise in LH, the dominant follicle did not grow large enough or had not been exposed to sufficient LH to be capable of suppressing FSH (Gastal *et al.*, 2000). In our study, FSH peaked on day 3 after ovulation, and the dominant and subordinate follicles emerged on day 4 - 6. Ginther and Bergfelt reported that the FSH concentrations were highest the day before, on and after emergence of the single wave through all groups (Ginther *et al.*, 1993b), which was in good agreement with the present study. FSH was increasing from the day before ovulation, but the dominant and the subordinate follicles could not be discerned earlier than day 6, because of the pool of follicles present making it impossible to tell the individual follicles apart until they grew beyond a certain size. The follicles may have started growing from small sizes shortly after FSH started to increase, but more and larger follicles regressing at the same time may have obscured this growth. From day 14, as the dominant follicle grew larger than 23 mm, FSH declined until the day before the dominant follicle ovulated and was thus negatively correlated. Bergfelt *et al.*, (1993) found very similar changes in FSH: an increase in FSH 6-8 days before the emergence of a primary wave, mean concentrations of FSH remaining elevated although fluctuating for 6 days before emergence (i.e. from day 6-12), decreasing on day 14. Wave emergence in the present study happened earlier, but the discrepancy may partly be due to Bergfelt *et al.*, (1993) defining wave emergence as the time where the dominant follicle reached 15 mm.

The wave emergence in the different follicle groups was approximately parallel in time, possibly due to the effect of the rising FSH causing a period of growth in all groups. FSH started declining after day 14 and was followed by a decline in mean follicle size from day 17. These results indicate that a rise in FSH preceded growth of waves of follicles apart from in the small follicle groups, which seemed to be independent of FSH. This could either be

due to the apparent waves not being true waves due to regressing follicles entering the small groups, elevating the mean size of this group, or perhaps that follicles of this size are gonadotrophin independent. The size at which the ovine follicles become dependent on gonadotrophins is 2 mm (Dufour *et al.*, 1979; McNeilly *et al.*, 1991). In the mouse the preantral stages are gonadotrophin dependent (Hirshfield, 1991) and in cattle follicles less than 5 mm are independent of gonadotrophins (Ackerman *et al.*, 1993). In a study in mares, follicles of 2-3 mm appeared to respond to increasing FSH because the smallest follicle group showed wave emergence on the days with highest FSH concentrations and there was a frequent association between an increase in number of 2-3 mm follicles and an increase in FSH concentrations (Ginther *et al.*, 1993b). Transient increases in serum concentrations of FSH reached peak values on days that approximated to follicle wave emergence in sheep (Bartlewski *et al.*, 1999). This was not the case in our study as there was a time delay between peaks of FSH and maximum sizes of small follicles. There was no significant pattern with increases in FSH followed by increases in mean follicle diameter of the small follicles apart from in the initial FSH rise, which was followed by increases in all follicle group diameters. It seems unlikely that an increase in FSH should be followed by a ultrasonographically detectable increase in follicle size on that same day, because some time delay would be necessary for the follicle to react with increased mitosis of the cells and increased production of follicular fluid to increase diameter.

Hypothesising about how long it would take for a follicle to grow from the earliest stages to preovulatory follicle is very difficult with this or any model, as follicles can only be identified from the time that they reach 1-2 mm in diameter. Even at this time point they cannot be distinguished from each other. Also it is not known if they grow at a steady pace once they get started or if they can grow and then linger until a suitable hormonal environment for growth arises again. If indeed the growth rate before day 6 was equivalent to the growth between day 6 - 13, the dominant follicle would have been a 1 mm follicle on day –4, and 5 mm at the day of ovulation. In cows, 5-9 mm follicles were FSH dependent (Gong *et al.*, 1996), and if the same was true for horses, the future dominant follicle would have reached an FSH dependent size at a time where FSH was increasing. This hypothesis was supported by the finding that follicles as small as 3 mm were growing during the period extending from a few days before ovulation until at least the day of emergence of the primary wave (Ginther *et al.*, 1993b) which was also seen in this study.

The ovulatory follicle appeared on day 6 as a 12.9 ± 1.0 mm follicle. Other studies found that the dominant follicle appeared as a 15 mm follicle on day 6 (Sirois *et al.*, 1989) or day 12 (Bergfelt *et al.*, 1993). The time between the hemiovariectomy of the ovary with the dominant follicle between day 14 and oestrus and the next ovulation was 13.7 ± 1.3 days, which indicated that the presence of the dominant follicle aided in suppression of growth of the subordinate follicles to any considerable size. The appearance of the dominant follicle 6 days after hemiovariectomy in their study and 6 days after ovulation in this study may both be related to loss of suppressive activity caused by the presence of the dominant follicle amongst other factors. Their study only considered follicles larger than 15 mm due to difficulties in tracking smaller follicles. They found that the dominant follicle was the largest follicle for 9 days (Sirois *et al.*, 1989). This is in agreement with the present study's initial equivalent size and growth rate of the dominant and largest subordinate follicles until day 9, after which the dominant follicle was the largest because the subordinate follicle's growth rate slowed down. These findings are in accordance with those in a 2-follicle model where the growth rates for the two follicles between emergence and deviation were not different (Gastal *et al.*, 1997a). However, Gastal *et al.*, (1997a) showed that at the beginning of deviation the dominant follicle was larger in their study, whereas in the present study the follicles were of similar size. Their dominant follicle appeared to grow faster resulting in deviation, whereas our subordinate follicle appeared to grow slower than during the initial period. Perhaps the dominant follicle at that time would have reached a size where it started to produce inhibitory substances. After deviation had occurred, the dominant follicle of this study increased in growth rate. The growth rate of the dominant follicle from day 6 until day 13 was on average 1.3 ± 0.1 mm per day, and between day 14 and day of ovulation growth rate was 2.6 ± 0.2 mm per day, which was similar to 2.8 mm per day in another study (Sirois *et al.*, 1989). The change in growth rate coincided with luteolysis, suggesting that decreasing progesterone concentration may have increased growth rate.

Suggestions on the mechanism of selection of the dominant follicle have involved an early size advantage of the dominant follicle over the subordinate follicle, changing hormonal environment or factors secreted by the dominant follicle that inhibit the competing follicles. Divergence between the ovulatory and the largest subordinate follicle was first seen after day 9 or 3 days after emergence of the dominant follicle, but the difference between the follicles was not significant until day 12. The largest subordinate follicle did not decrease in size until after day 18 or 3 days before ovulation. One report found that divergence happened on day 15 or 3 days after wave emergence and that the subordinate follicle ceased to grow from day

16 or 4 days after emergence of the wave (Bergfelt *et al.*, 1993) whereas the subordinate follicle in our study grew for another 9 days after divergence before regressing. The discrepancy between the interval between emergence and regression of the subordinate follicle between the studies may be due to the other groups of workers only studying follicles larger than 15 mm, whereas our study detected the follicles earlier. Gastal *et al.*, (1997a) discussed the possibility of the dominant follicle having a small size advantage over the other follicle and therefore would be the first to reach a critical stage of 23 mm (15 – 16 mm at the time of deviation in the present study), where it seemed to inhibit the other follicle before it reached a similar size a day after. In cattle, the dominant follicle reached the size for deviation 6 hours before the subordinate follicle (Ginther *et al.*, 1997a). This is in agreement with the present study, where there was no difference in size until the deviation in growth rates started. The subordinate follicles started to grow slower, perhaps because of inhibition caused by the dominant follicle. Another study using the 2-follicle model described how subordinate follicles, which had reached a size of 6 mm at the time of ablation of the dominant follicle, underwent atresia at a later point, whereas follicles, which were smaller (2 mm) at the time of ablation of the dominant follicle, could go on to become the new dominant follicle and to ovulate (Gastal *et al.*, 1999c). This indicated that selection occurred at an early stage, but the findings are contrasted by the continued growth until day 18 of the largest subordinate follicle in this study and the growth of follicles in the same model until 23 mm in a previous study (Gastal *et al.*, 1997a). In cattle there is an indication that removal of the largest follicle before or soon after deviation allows the second largest follicle to become dominant. Apparently selection of the dominant follicle is not complete until deviation occurs (Ginther *et al.*, 1996) and even so selection does not necessarily mean that the competing follicles regress immediately, just that they are stunted in growth compared to the dominant follicle. It would be interesting to known what would happen to the largest subordinate follicle after removal of the dominant follicle at a time before the largest subordinate would start to regress in an unmanipulated cycle. Double ovulations are examples of two follicles being dominant without killing each other. This suggests that size may be important because the follicles often are close in size. Certainly size is not the only factor affecting which follicle will become the ovulatory follicle, as the smaller of two competing follicles can ovulate while the largest can undergo atresia in rare cases. This can be detected as the largest follicle losing the thick, healthy rim of granulosa cells as seen on ultrasound scanning.

At the time of divergence of the subordinate and dominant follicle, FSH concentration was still high and LH had not started to increase significantly in the present study, but shortly before the follicles started to regress, changes in the gonadotrophins started. These findings are in agreement with the following studies regarding the changing hormone levels causing changes in follicular growth, but interpreting their deviation as just a change in growth rate rather than immediate decrease in follicle size can not be confirmed by the present data. In cattle, the elevated concentrations of LH and reduced concentrations of FSH were present 16 - 32 hours before to at least 24 h after the beginning of follicle deviation (Ginther *et al.*, 1998). In mares the gonadotrophin dissociation (increasing LH, decreasing FSH) preceded follicle deviation by 3 or 4 days (Gastal *et al.*, 1997a and 1999c). When using a 2-follicle model in mares the deviation mechanism appeared to involve reduction in FSH below the levels required by the smaller follicles (Gastal *et al.*, 1999c), but with a positive effect of LH on the largest follicle after the beginning of deviation (Gastal *et al.*, 2000). The mechanism appears to involve a shift in responsiveness of the follicle from FSH to LH near the time of deviation. In sheep it has been shown that ovulatory follicles can transfer their gonadotrophic dependence from FSH to LH (Campbell *et al.*, 1999). LH receptors began to develop in the granulosa cells of the largest follicle at about the expected time of deviation in cattle and only in healthy follicles larger than 9 mm (Xu *et al.*, 1995; Bodensteiner *et al.*, 1996). This information is in agreement with a study showing that bovine follicles are dependent on LH for growth beyond 9 mm (Gong *et al.*, 1996). LH receptors were detected in granulosa cells in equine follicles as small as 5 mm, but there was a significant increase in concentration of receptors, as the follicles grew larger than 9 mm (Goudet *et al.*, 1999). These authors suggested that in the mare the gonadotrophin independent stage lasted until 9 mm. Another study found that on day 12 only the largest follicle (diameter not provided) had developed LH receptors on the granulosa cells and receptor density increased as the follicle grew closer to ovulation (Lawler *et al.*, 1998). In the present study 2-14 mm subordinate follicles were growing independently of LH changes, suggesting that not until follicles grew beyond 14 mm, would they enter the competition for dominance, if LH were a factor in one follicle becoming dominant. On day 9, when the future dominant follicle reached 15 mm, the first indication of this follicle becoming larger than the subordinate follicle was evident.

Two very recent studies found that neither decreased nor increased concentrations of LH changed the growth profile of the second largest follicle, suggesting that LH was not involved in the initiation of the deviation. This was similar to the present study's finding that the subordinate follicles both grew and regressed at the time of increasing LH

concentrations, seemingly unaffected by LH. The onset of deviation was not delayed by the decrease in LH but as the diameter of the dominant follicle was suppressed by the lack of LH, this hormone was required for the continued growth of the largest follicle after its initial expression of dominance (Gastal *et al.*, 1999d and 2000). The final maturation and development of antral follicles to ovulation after luteolysis was dependent on an increase in the pulsatile secretion of LH in sheep (Campbell *et al.*, 1999). LH alone cannot support the dominant follicle all the way to ovulation as immunisation against FSH receptors inhibits ovulation in sheep (Abdennebi *et al.*, 1999) and in Buserelin down-regulated ewes, administration of FSH only was capable of stimulating the growth of follicles to a preovulatory size (Picton *et al.*, 1990a and 1990b). In GnRH antagonist suppressed sheep, administration of FSH throughout the artificial follicular phase resulted in follicle development and ovulation in 50% of the animals, but pulsatile LH had little effect on ovulation rates in animals with FSH treatment. Withdrawal of FSH in the presence of LH resulted in ovulations but at a lower rate. When both FSH and LH were withdrawn, the ovulatory follicles became atretic (Campbell *et al.*, 1999).

That FSH is part of the selection process was further demonstrated by administering FSH to animals or by decreasing levels through oestradiol administration. Administration of FSH in cattle delayed selection of the dominant follicle and atresia in the subordinate follicles (Adams *et al.*, 1992; Mihm *et al.*, 1997), and administration of pituitary extract (FSH) prevented divergence and caused superovulation in mares (Ginther, 1992b). When daily injections of FSH were administered as the follicles reached 20 mm (presumably before deviation), deviation was overridden as indicated by an increased ovulation rate (Squires *et al.*, 1986). These data would support the finding in our study, that subordinate follicles are still active and growing until they reach 22 mm. Presumably FSH administration before this turning point would support further growth as suggested in the above studies.

In the present study growth of the dominant follicle and oestradiol production were significantly correlated as the granulosa cells produced oestradiol in the growing follicle. The dominant follicle started to produce increasing concentrations of E2 from day 13 or 4 days after the start of divergence at a point where dominance presumably had been established. At that point it grew beyond a mean of 21.8 ± 2.2 mm, which was similar to the findings in a two-follicle model in mares where an increase in systemic oestradiol concentrations occurred between the day that the largest of the two follicles reached 20 mm to 2 days after. When only the subordinate follicle was retained, oestradiol did not begin to

increase until two days after aspiration of the largest follicle, and it increased only in the cases where the retained follicle grew and became dominant (Gastal *et al.*, 1999c). This indicates that significant increases in oestradiol production only takes place at a time where a follicle has established dominance. In cattle these findings were confirmed as oestradiol concentrations were not higher in the dominant follicle than in the largest subordinate follicle until the day after the two follicles began to deviate in growth rates (Ginther *et al.*, 1997b). Another study in mares found that significant increase in systemic oestradiol occurred between the day before and the day of the beginning of deviation (Gastal *et al.*, 1999b). The difference in intrafollicular oestradiol concentrations between the two follicles before the detection of a change in differences in diameter made the authors suggest that oestradiol may be involved in deviation in follicle size in mares (Gastal *et al.*, 1999b). The deviation may be instigated by an increase in oestradiol resulting in decreased FSH concentrations and oestradiol-induced LH receptor formation caused by the dominant follicle (Gore-Langton *et al.*, 1994; Gastal *et al.*, 1999c). Again, deviation presumably is the time of regression of the subordinate follicles. Certainly increasing oestradiol levels were concomitant with decreasing subordinate follicle sizes and decreasing FSH in the present study. The increase in oestradiol in the present study also coincided with luteolysis. In cows, exogenous oestradiol administration during the development of uterine oestradiol responsiveness during the luteal phase initiated luteolysis (Salfen *et al.*, 1999). Oestradiol binding to its receptor in the endometrium initiated activation and synthesis of oxytocin receptors that resulted in prostaglandin release and luteolysis.

A study by (Gastal *et al.*, 2000) investigated the effect of progesterone on follicular growth. The absence of progesterone did not affect length of time for a follicle to grow up after ablation compared to presence of progesterone. This means that when we give prostaglandin to a mare, it is one of the large, healthy and at the time growing follicles, which will grow to ovulate, and not a follicle starting from less than 5 mm. In their study they removed all the healthy growing follicles, and thus it took equally long for the prostaglandin treated group and for the control group to grow up a follicle. This also seems to indicate that follicles are growing at least in the smaller groups whether progesterone is present or not. This is confirmed by the finding that neither shortening nor prolonging the luteal phase with either prostaglandin or progesterone affected the day of emergence of follicles after follicle ablation (Gastal *et al.*, 2000), but the growth of the two largest follicles was slower after administration of progesterone (Gastal *et al.*, 1999d). These data are in good agreement with our study, where deviation between the dominant and the largest subordinate follicle

occurred during the luteal phase, but the growth rate of the dominant follicle increased at the time of luteolysis in the present study.

The regression of the largest subordinate follicle started from day 18 and the follicle could be followed until day 4 after ovulation, when the follicle had regressed to 16.1 ± 2.7 mm from a maximum of 22 ± 1.5 mm (regression rate: 0.7 ± 0.1 mm per day). The rate of growth was faster than the rate of regression (1.3 – 2.6 mm per day versus 0.7 mm per day), which is comparable to another study (1.02 mm per day versus 0.56 mm per day) (Ginther *et al.*, 1993b). Driancourt *et al.* (1982a, 1982b) reported that there were more atretic than non-atretic follicles in general, but particularly so in the early luteal phase which is in agreement with the slowly regressing follicles up to and after ovulation in the present study.

The present study of unmanipulated cycles could not confirm the theory of declining FSH causing selection, as it appeared that selection of the dominant follicle and deviation in growth rate of the subordinate follicle happened earlier than the decline in FSH and the rise in LH. Both the largest subordinate follicle and the follicles in groups 1 – 3 continued to grow until day 16 – 18. This means that atresia did not appear to commence in the subordinate follicles until several days after the dominant follicle had been selected. Certainly it appeared that the declining FSH caused atresia in follicles, as mean diameters decreased 2-3 days after FSH began to decline. The dominant and the subordinate follicles started increasing in size on day 6, at a time where LH was declining. LH reached minimum values on day 11, two days after the dominant and subordinate follicle started to deviate. LH increased as the dominant follicle grew larger and increased until 1 day after the dominant follicle had ovulated. The subordinate follicle both grew and regressed in the face of increasing LH. These data suggested that a mechanism other than declining levels of FSH caused the divergence in growth rate. Apart from selection through decreasing FSH and increasing LH, the dominant follicles may inhibit the growth of the smaller follicles via secretion of paracrine factors. Follicular fluid growth factors or peptides are likely to affect follicle function or to modify gonadotrophin action. These factors may include inhibin, activin or IGFs. Administration of charcoal extracted follicular fluid (no progesterone, oestradiol or inhibin) after ovulation resulted in a delay in development of a follicular wave, prolonged the luteal phase and suppressed FSH in cattle (Turzillo *et al.*, 1990; Salfen *et al.*, 1999) as well as inducing atresia in follicles larger than 11 mm and initiating a new follicle wave in mares (Plata-Madrid *et al.*, 1992). In mares, mean concentrations of immunoreactive inhibin began to increase 9 days before ovulation, remaining high until 2 days before

ovulation, followed by a decrease when the LH surge was initiated (Nagamine *et al.*, 1998). Other studies described an increase between days 7-12, reaching high levels on day 0, whereas mean FSH concentrations significantly decreased between days 11-14, reaching low levels on days 18-20 (Bergfelt *et al.*, 1991 and 1993), indicating that inhibin regulates FSH. The authors suggested that perhaps the reduced FSH concentrations following emergence of the primary wave was attributable at least in part to the suppressive effect of increasing circulating concentrations of inhibin in association with the development of follicles. It is not known in detail to what extent follicles of various size categories contribute to systemic levels of inhibin. One study found that inhibin βA and βB was mainly secreted by the granulosa cells and the theca cells of large follicles (>30 mm), and granulosa cells of small follicles (<10 mm) may secrete the inhibin alpha subunit (Nagamine *et al.*, 1998). Another study reported that alpha-inhibin in granulosa cells was lower in follicles of 5-9 mm in diameter than in larger ones, but otherwise did not increase with increasing follicle size (Goudet *et al.*, 1999). In a study where mares where treated with inhibin antiserum, plasma levels of FSH, oestradiol and ovulation rates were increased (Nambo *et al.*, 1998).

There is a general agreement about the ability of a follicle to respond to the switch in gonadotrophic support being central to the mechanism of follicle selection and initiation of regression. The largest follicle may have interfered with the gonadotrophin supply to smaller follicles of the cohort, perhaps by producing paracrine substances, inhibin or oestradiol, which via negative feed back on the hypophysis would reduce FSH secretion to levels insufficient to support the subordinate follicles.

There appears to be some confusion related to the use of the term deviation. Ginther *et al.*, (1997a) defined deviation as the beginning of the greatest difference in growth rates between the dominant and subordinate follicles, but whether the change in growth rates included an actual decrease in the subordinate follicle size seems to differ from study to study. Assuming that deviation meant that the subordinate follicles actually started to regress, most studies are in agreement with our data. The main difference in the present study from other studies was that there was a clear divergence between growth rates of the dominant and the subordinate follicles at an early stage (day 9 or 3 days after emergence), but that the subordinate follicle, although inhibited in growth, was still viable and continued to grow until FSH declined. The difference from other studies may be caused by our recording follicles smaller than 15 mm in the present study. This allowed us to follow the growth of the smaller follicles until they reached the size for divergence, which was 15-16 mm. This was the size at which other

studies started to follow the follicles. The difference to the data published by Ginther and co-workers may first of all be caused by the 2-follicle model, but also that they normalised the data to either day of emergence of a wave of follicles or to day of deviation of growth. It is difficult to say which method is more accurate. Our data probably gives the most "natural" picture, but their method may be more exact at pinpointing specific changes.

5.5 Conclusion

In the larger species, ultrasonography of the ovaries allows for sequential information about the changes of the individual follicle diameters within the same animal over a period of time. This is an excellent tool for studying follicle dynamics and particularly in the horse due to the very large follicles compared to other species. When used in combination with plasma hormone levels, this experiment showed that the use of daily mapping of follicles made it possible to detect days of changes in follicular growth both of the individual large follicle and smaller follicles in groups in relation to hormone changes. In summary it appeared that the growth of the largest follicle to a point where it was capable of producing oestradiol, caused decreasing levels of FSH, which in turn caused the smaller follicles to regress. The oestradiol increased LH production and possibly induced LH receptor formation in the dominant follicle, as well as causing luteolysis, with resulting further increased levels of LH due to lack of negative feed back from progesterone. When the inhibiting effect of oestradiol disappeared just before ovulation, FSH started to increase, resulting in growth of a new wave of follicles. Divergence in growth rates occurred early in the cycle, when FSH levels were high, LH low and progesterone was still being produced. The cause of deviation in this study remains unknown. Only few studies have been conducted on follicular growth during unmanipulated cycles. It is necessary to do more studies with this model, because as much as the 2-follicle model is adequate for detecting the needs of a dominant follicle and its subordinate follicle, it is an invasive technique, which disrupts the normal cycle. The follicle maps are a reliable method of determining growth and regression, when accepting that there are influences from regressing, atretic follicles during periods of general growth and that it is not possible to monitor the individual small follicle.

The present chapter also provided information for determining timing of ovariectomy in chapter 6.

6 Follicle health status at different stages of the oestrous cycle

6.1 Introduction

From the day after ovulation until the next ovulation, one or more follicles will develop to the preovulatory stage (35-45 mm). During this period of time, many other follicles will also grow in an attempt to become the dominant follicle, but will eventually regress when not selected for dominance. The process and timing of selection as determined by biochemical parameters has not received much attention in the mare. Gastal *et al* (1999a, 1999b, 1999c, 1999d, 2000) recently started a series of studies on follicular selection using a two-follicle model combined with plasma and follicular fluid hormone levels. In these studies they found that the future dominant follicle appeared to have higher levels of oestradiol and that the follicle has a size advantage over its competitors. A study in mares found that follicular fluid of viable follicles contained significantly higher concentrations of oestrogen and progesterone, but lower concentrations of prostaglandin $F_{2\alpha}$ (Condon *et al.* 1979; Kenney *et al.* 1979). Studies by Driancourt *et al* (1982a, 1982b) focused on histological atresia at different stages of the cycle and in different sized follicles. They found that in mares, approximately 75% of follicles at a given time, regardless of size classification are undergoing atresia as determined by histology (Driancourt *et al.*, 1982b). They found most atresia on day 6, growth on day 14 and between day 14 and day 17 there was a significant increase in number of follicles undergoing early atresia. Just before ovulation all follicles apart from the preovulatory follicle were atretic (Driancourt *et al.*, 1982a). In the pig the total numbers of small (1-2 mm) and medium (3-5 mm) follicles were highest during the luteal phase, but there was a 90% decrease in these numbers during the follicular phase as the ovulatory follicles started growing (Guthrie *et al.*, 1995b). About 50% of follicles < 6 mm were atretic as determined by histology, whereas very few follicles > 6 mm were atretic in pigs (Guthrie *et al.*, 1995a). In pigs there did not seem to be a difference in growth and atresia during the cycle, indicating a continuous growth and regression independent of gonadotrophin changes (Guthrie *et al.*, 1996).

In chapter 3, oocyte chromatin configuration and cumulus morphology was studied. In chapter 4 the method of determining apoptosis was validated and compared with other indices of follicle health or death. In chapter 5 the dynamics of the follicles in the horse were examined in relation to hormone changes. In this chapter all the information gathered from

the previous chapters will be used to evaluate how a viable follicle differs biochemically from an atretic follicle. The overall aim of this study was to determine at which stage of cycle the dominant follicle was selected for survival and when the remaining follicles started to undergo atresia and regress as determined by follicle maps, apoptosis in granulosa cells, steroid hormone content in follicular fluid and state of oocyte health.

6.2 Materials and methods

6.2.1 Animals

Twelve cycling pony mares were ovariectomised on day 6 (n = 4), day 11 (n = 4), day 15 (n = 4) after ovulation. Slaughter house mares (n = 11) with plasma progesterone < 1 ng/ml, plasma oestradiol > 40 pg/ml and at least one follicle larger than 40 mm were selected in the assumption that they were in oestrus.

6.2.2 Daily monitoring of follicular growth

All mares apart from the slaughterhouse mares were scanned and blood sampled daily from ovulation until ovariectomy as described in chapter 2. Follicular growth was recorded daily on videotape and follicle maps were drawn from the videotapes at a later stage. The follicles were grouped for making graphs as described in chapter 5.

6.2.3 Ovary processing

The exteriorised ovaries were kept at 35-37 °C in M199 with Hanks salts and 25 mM Hepes. See Figure 6.1. All visible follicles on the surface were processed followed by the follicles found after slicing the ovary thinly. Each follicle was measured with a ruler before aspiration of all follicular fluid. The follicular fluid was stored at –20 °C until assaying for steroid hormones. The follicle was cut open and the inner wall was scraped with a bone curette to release both granulosa cells and the cumulus oophorus with the oocyte. The granulosa cells were pelleted by centrifugation at 2,000 *g* for 30 seconds at room temperature, snap frozen and stored at -70 °C until DNA extraction. After examination of the cumulus oophorus using a stereomicroscope, the oocyte was denuded by pipetting with smaller and smaller bore pipettes (0.2 - 0.6 mm in diameter) either with or without a solution of 0.25% trypsin/EDTA, fixed in buffered formalin and stained with a DNA-specific fluorescent Hoechst stain.

Granulosa cell DNA was extracted and agarose gel electrophoresis or DNA 3'-end labelling was performed to detect apoptosis. See further detail in chapter 2.

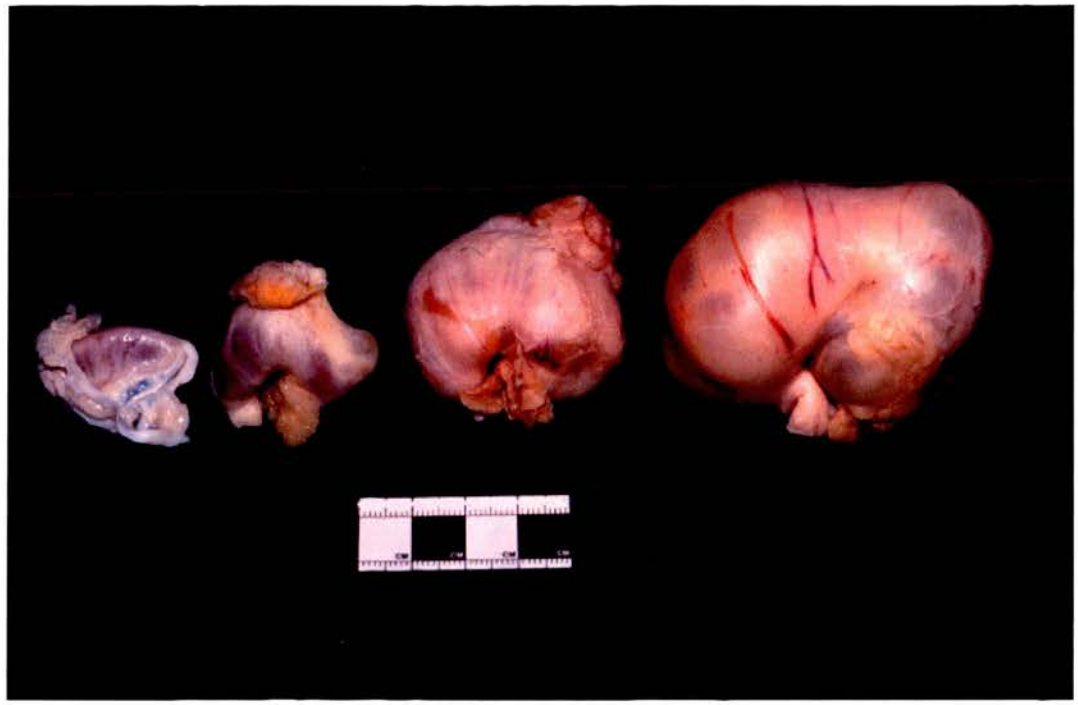


Figure 6.1. Equine ovaries. Ovaries of different sizes. The ovary to the left was from a foetus.

6.2.4 Hormone assays

Daily plasma samples were assayed for LH, FSH and oestradiol. Follicular fluid was assayed for oestradiol, progesterone and testosterone. See details of assays in chapter 2.

6.2.5 Experimental plan

Depending on size, each follicle would yield granulosa cells for detection of apoptosis, a cumulus-enclosed oocyte for evaluation of the cumulus and oocyte chromatin configuration and follicular fluid, which was assayed for oestradiol, progesterone and testosterone. These parameters were analysed to demonstrate any relation to each other and to day of cycle. Larger follicles could be identified individually from day to day on ultrasound scan, and finally were identified at the time of processing, giving a picture of the follicle's growth in the mare followed by a biochemical evaluation of health status. Not every follicle yielded information on all parameters as the oocyte occasionally was not found, or there was not

enough follicular fluid to do all steroid assays, or too little DNA was present in the follicle to determine whether it was apoptotic.

6.3 Statistical analysis

Differences in parameters caused by stage of cycle was analysed among groups using a test of randomness (single-tailed Chi-square) in cases where data were expressed as frequencies, e.g. frequency of chromatin configuration within apoptotic versus non-apoptotic follicles. Fisher's exact test was used for comparisons in which total number of measurements was less than 20 and when more than 20% of expected frequencies were less than 5. T-tests were performed to analyse means of different populations such as mean steroid concentration within a group of apoptotic versus non-apoptotic follicles. Relationship between continuous type measurements within a particular follicle, e.g. follicle size and follicle steroid concentrations, was analysed using a regression analysis.

6.4 Results

A total of 293 follicles from 23 mares (12.7 follicles per mare) were processed. A total of 139 oocytes were recovered yielding a retrieval rate of 47.4% and oocyte yield per horse was 6 oocytes.

6.4.1 Follicle diameter versus follicular fluid volume

The follicles were not completely spherical due to the follicles exerting pressure on each other, and therefore the diameter was checked against volume aspirated (Figure 6.3). In a perfect sphere, the volume can be calculated as

$Volume = 4/3 \times \pi \times r^3$

where r is radius of the sphere. Some follicles, including small follicles, would point towards the ovulation fossa inducing error when measured on the surface. The volume of follicular fluid was concordant with the diameter at the time of dissection of follicles less than 30 mm. In the larger follicles there was a tendency to overestimate the size of the follicle. There are several possible reasons: gravity would flatten the follicle, making it oval rather than round. The measurement of the follicle was done parallel with the surface on which the follicle was lying. The thick wall of large follicles would add to the diameter as I measured on the outside of the follicle, not subtracting the thickness of the wall. Finally, the large follicles

were more difficult to drain completely of follicular fluid because the collapsing follicular wall would fold over, creating pockets of fluid.

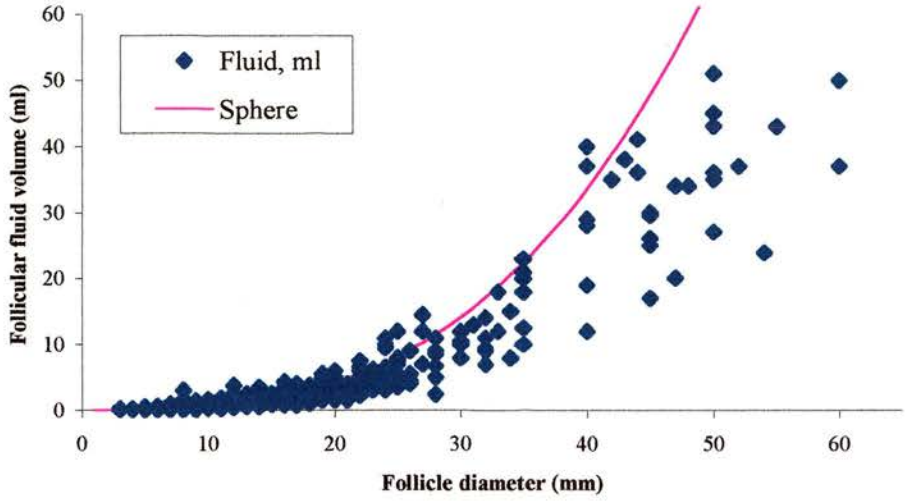


Figure 6.2. Follicle diameter versus follicular fluid volume. The pink line shows an ideal sphere. The blue dots are measurements of the follicles. There was a tendency to overestimate follicle diameter.

6.4.2 Follicle sizes during the cycle

The follicles were divided into size groups: 2-5 mm, 6-10 mm, 11-15 mm, 16-20 mm, 21-30 mm and 31-60 mm. The 2-5 mm group was largest (51.7%) on day 6, and gradually decreased through the oestrous cycle with minimum numbers during oestrus. The 16-20 and 21-30 mm groups increased from day 6 to day 15 and decreased from day 15 to oestrus as the follicles entered the largest follicle groups or started to regress. The 31-60 mm group was largest (16.3%) during oestrus, but also had a small peak (3.2%) on day 11. There were no 31-60 mm follicles on day 6. Follicle size distribution was significantly associated with day (Figure 6.3). The follicle map from chapter 5 supported the finding that follicle sizes generally increased as the cycle progressed as found when dissecting the ovaries on specific days (Figure 6.4).

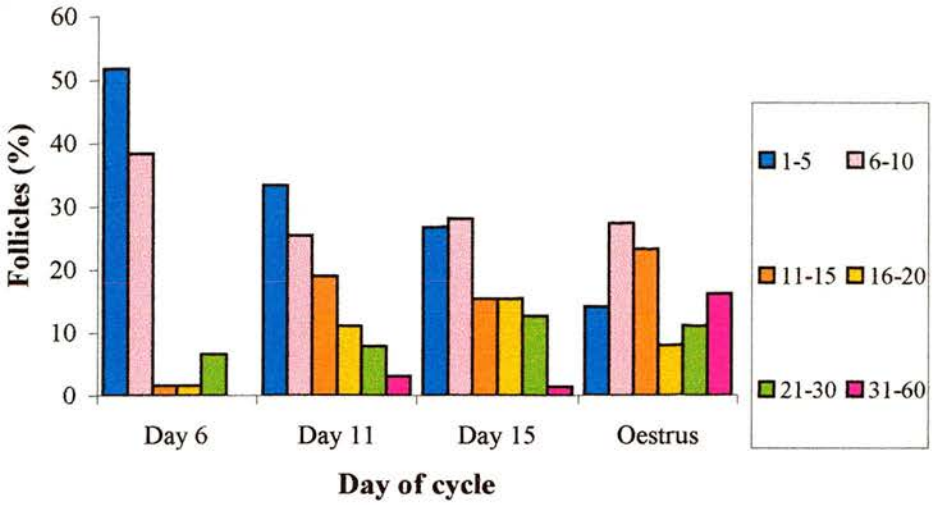


Figure 6.3. Follicle size distribution during the oestrous cycle. There was a significant difference between day 6 and day 11 ($P < 0.01$), day 15 ($P < 0.001$) and oestrus ($P < 0.001$), but no difference between day 11 and day 15. A significant difference was found in distribution of sizes between day 11 and oestrus ($P < 0.05$) as well as between day 15 and oestrus ($P < 0.01$). Follicles numbers on each day: Day 6 (n = 60), Day 11 (n = 63), Day 15 (n = 71), Oestrus (n = 99).

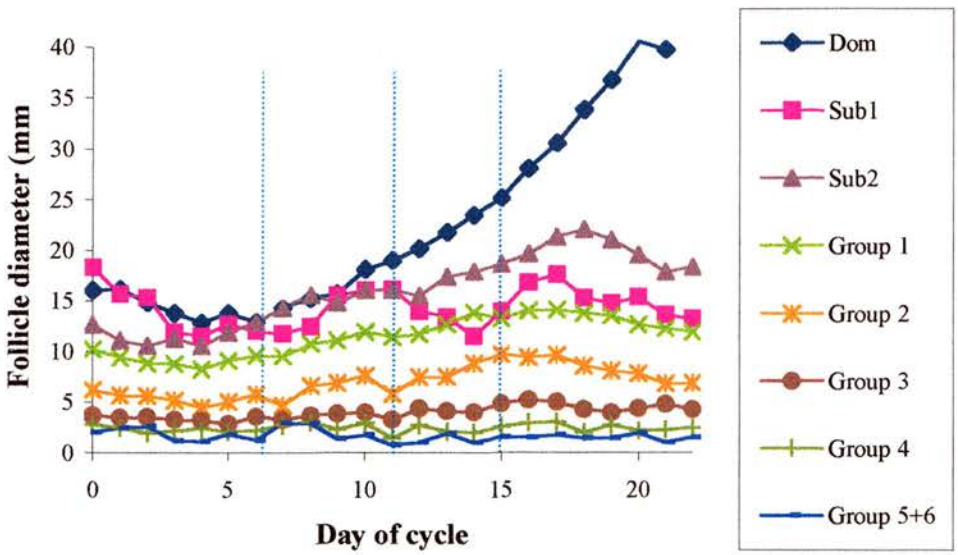


Figure 6.4. Follicle map from chapter 5. Follicular growth and regression during the cycle in different follicle groups. Vertical, blue lines mark days of ovariectomy in the present chapter. Oestrus is not marked, as the exact day of oestrus was not known. Dom, dominant follicle; Sub1, subordinate follicle 1; Sub2, subordinate follicle 2; Groups 1 – 6, follicles grouped together from largest to smallest (see chapter 5 for further detail).

6.4.3 Atresia during the cycle

A total of 272 follicles (92.8%) yielded enough DNA for analysis for apoptosis. The remaining 7.2% were either preserved whole or had too few granulosa cells. There was a significant difference ($P < 0.01$) in frequency of apoptosis between the days with most apoptotic follicles on day 6, followed by a gradual decrease through the cycle with least during oestrus. Results are shown in Figure 6.5.

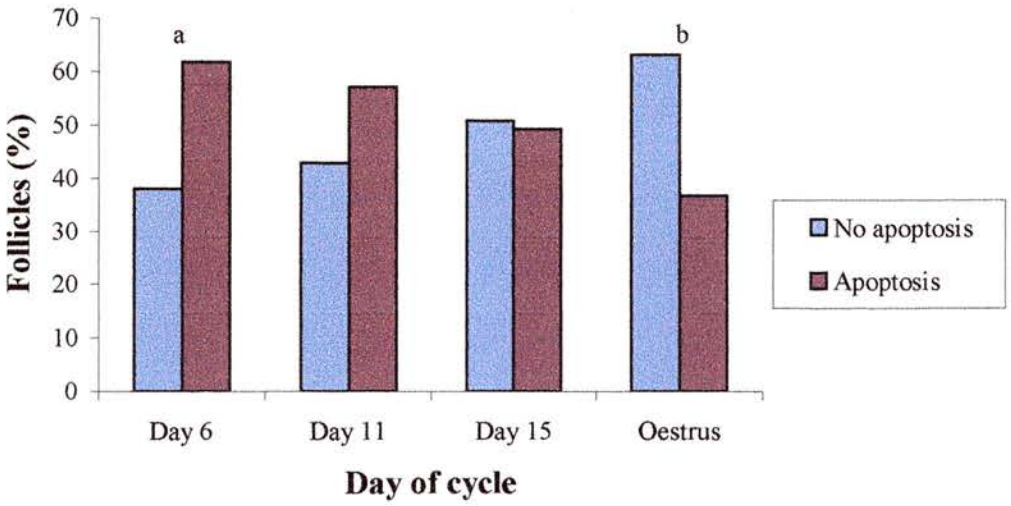


Figure 6.5. Distribution of apoptosis during the cycle. There was a change in frequency of apoptosis from day 6 to oestrus. Letters a and b denote a difference ($P < 0.01$). Numbers of follicles on each day: Day 6 (n = 55), Day 11 (n = 63), Day 15 (n = 67), Oestrus (n = 87).

6.4.4 Atresia in different follicle sizes

To examine the frequency of apoptosis within different size groups, follicles (n = 272) were divided into size groups of 1-5 mm, 6-10 mm, 11-15 mm, 16-20 mm, 21-30 mm and 31-60 mm (Table 6-1).

Table 6-1 Apoptosis in different size groups.

Size (mm)	n	No apoptosis (%)	Apoptosis (%)
2-5	70	42.9	57.1
6-10	84	47.6	52.4
11-15	44	38.6	61.4
16-20	27	63.0	37.0
21-30	28	53.6	46.4
31-60	19	94.7	5.3
Total	272	49.6	50.4

A comparison was made after the size groups were reduced to 1-15 mm, 16-30 mm and 31-60 mm after having examined for absence of differences within these groups. Frequency of non-apoptotic follicles increased with increasing follicle size ($P < 0.01$). See Figure 6.6.

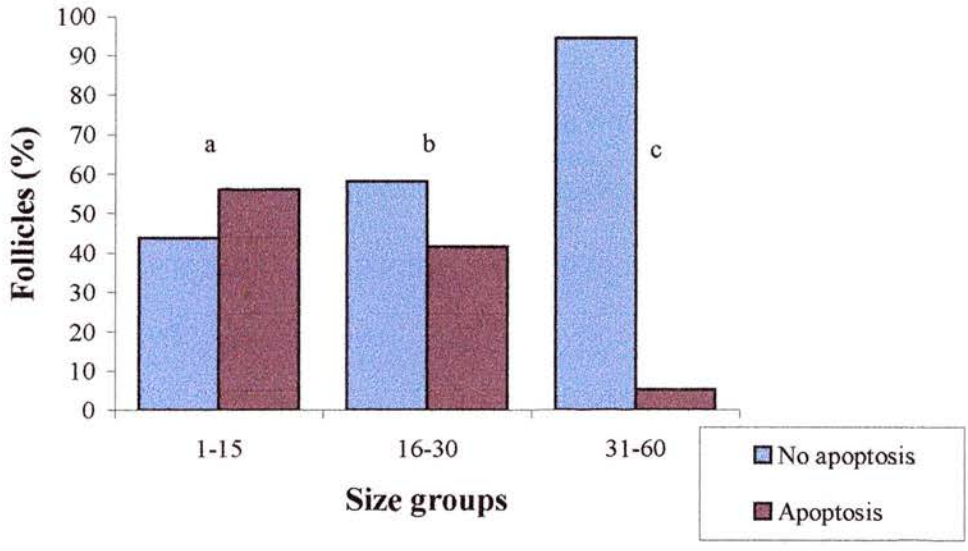


Figure 6.6. Distribution of apoptosis within size groups. There was a significant change in frequency of apoptosis in differently sized follicles. Letters a, b and c denote a difference. ab) $P = 0.09$, ac) $P < 0.001$, bc) $P < 0.01$.

6.4.5 Apoptosis in size groups on different days

In order to avoid bias from the effect of size on distribution of apoptosis, a specific size group's percent apoptosis was worked out over days to see changes within a size group between days (see Table 6-2). Unfortunately, splitting the data up in these subgroups, resulted in low numbers in some groups, so only frequencies are shown, but no analysis was performed. More data are needed to do a thorough analysis.

The 2-10 mm group had most apoptosis on day 6 and apoptosis gradually decreased through the cycle in this size group, perhaps as regressing follicles disappeared. During oestrus, more than 60% of the 2-10 mm follicles were non-apoptotic, and presumably growing. The 11-20 mm group were most healthy on day 15. The 21-30 mm group had fewer than 50% healthy follicles on days 11 and 15, but more than 60% healthy follicles on days 6 and during oestrus. There were so few follicles larger than 30 mm in other days than during oestrus, so it was not possible to compare the days.

Table 6-2 Apoptosis in size groups during the cycle.

	Day 6		Day 11		Day 15		Oestrus	
Size (mm)	n	Apoptosis (%)	n	Apoptosis (%)	n	Apoptosis (%)	n	Apoptosis (%)
2-5	27	63.0	21	61.9	15	60.0	7	28.6
6-10	23	73.9	16	50.0	20	50.0	25	40.0
11-15	1	100.0	12	83.3	11	36.4	20	60.0
16-20	1	0.0	7	28.6	11	45.5	8	37.5
21-30	3	33.3	5	60.0	9	55.6	11	36.4
31-60	0	-	2	0.0	1	0	16	6.3

6.4.6 Oocyte chromatin configuration during the cycle

The oocyte chromatin was classified as fluorescent nucleus (FN), loosely condensed chromatin (LCC), condensed chromatin (CC), diakinesis, metaphase, degenerated (Degen) and no chromatin. Oocyte chromatin configuration was significantly associated with day of the cycle as determined by distribution of FN, LCC and CC ($P < 0.001$) (Figure 6.7). The frequencies of the other groups were too low to also include them in the comparison between days. Day 6 and day 11 were similar in chromatin configuration distribution, but after day 11 the distribution changed. LCC and CC appeared to be in a reciprocal relationship with LCC being predominant in the beginning of the cycle and decreasing as CC increased as the cycle progressed. There were more degenerate forms as the cycle progressed, particularly on day 15, where 60% of the degenerate forms were of the no chromatin type. Degenerating

chromatin seemed to be a feature of follicles smaller than 20 mm. Only 2 out of 19 degenerated oocytes were from follicles larger than 20 mm. The 1 – 10 mm follicles during oestrus appeared to lose the suppressing capacity on meiotic maturation as only 20% (4/20 oocytes) were still in the immature state (FN + LCC), whereas 40% had progressed to CC and 40% showed either metaphase configuration or degeneration. This pattern was already evident on day 15. The figures for follicles larger than 20 mm were 14.3%, 61.95, 14.3% and 9.5% for FN + LCC, CC, metaphase and degenerated oocytes, respectively.

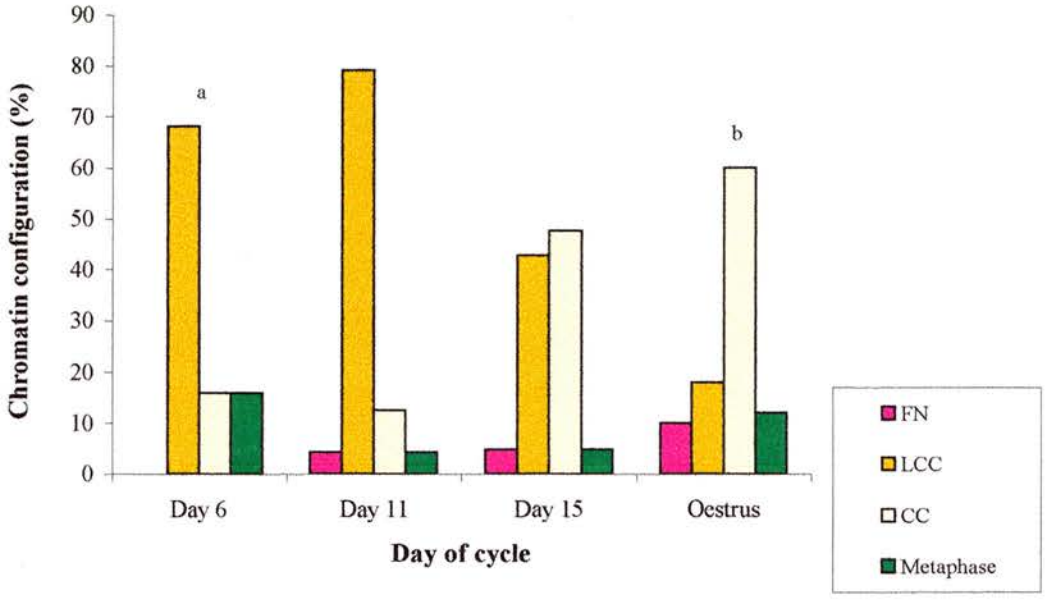


Figure 6.7. Chromatin configuration changes during the cycle. LCC decreased and CC increased as the cycle progressed. Numbers of oocytes on each day: Day 6 (n = 25), Day 11 (n = 26), Day 15 (n = 31), Oestrus (n = 57). Metaphase included diakinesis. Letters a and b denote a difference ($P < 0.001$).

6.4.7 Chromatin configuration in different sized follicles

There was a difference in chromatin distribution within the different size groups ($P < 0.05$) (Figure 6.8). The 1-15 mm follicles contained the highest proportion of degenerate and abnormal oocytes. When splitting the data into smaller size groups, the 1-5 mm follicles contained few oocytes (36%) with young configurations, but many with CC and metaphase configurations. The FN and LCC configurations frequencies increased with increasing follicle size, with maximum in the 16-20 mm group. After 20 mm, configurations changed toward more CC and metaphase, possibly due to normal progression of the meiotic process.

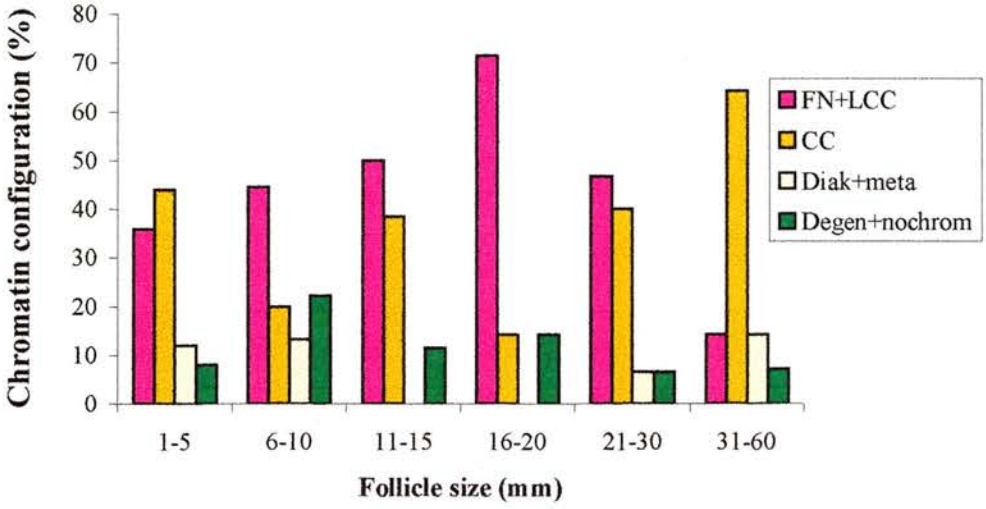


Figure 6.8. Chromatin configuration changes with different sizes of follicles. There was a difference in chromatin distribution within the different size groups ($P < 0.05$). More CC and less LCC was found in follicles larger than 20 mm. Diak, diakinesis; meta, metaphase; degen, degenerated chromatin; no chrom, no chromatin.

There was a significant difference in distribution of chromatin in the 21 – 60 mm group between day 6-15 and oestrus ($P < 0.05$). The data in days 6, 11 and 15 were added together because of low numbers of oocytes in the large size classes. The emerging picture was one of primarily young configurations (FN + LCC) early in the cycle where the follicles were not yet mature, and during oestrus the more mature configurations were predominant (CC + metaphase). None of the large follicles had entered metaphase before oestrus. See Figure 6.9.

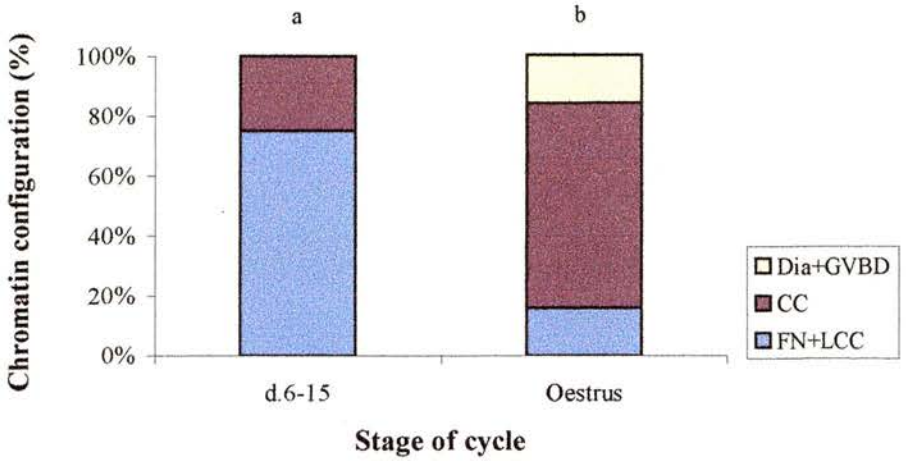


Figure 6.9. Changes of chromatin configuration in oocytes from follicles larger than 20 mm with stage of cycle. Follicles larger than 20 mm had more FN and LCC early in the cycle. Letters a and b denote a difference ($P < 0.05$).

6.4.8 Chromatin configuration in relation to follicular apoptosis

Follicles (n = 118) were examined for presence or absence of apoptosis and for oocyte chromatin configuration. A decision was made to divide the non-apoptotic follicles into non-atretic and atretic groups because histology in chapter 4 showed that the non-apoptotic group consisted of truly healthy follicles, but also very atretic follicles. Follicles were further divided into atresia grades based on microscopic appearance of the granulosa cells, relative DNA content, and oestradiol concentrations relative to size. If all 3 parameters pointed towards a healthy follicle, the follicle was labelled non-apoptotic, non-atretic grade 0, one parameter pointing towards decreased health (non-apoptotic, atresia grade 1), two parameters (non-apoptotic, atresia grade 2) and all 3 parameters (non-apoptotic, atresia grade 3). Non-apoptotic, atresia grade 0 and 1 were grouped together and follicles were assumed to be more healthy than not and therefore were labelled non-apoptotic and non-atretic, and atresia grade 2 and 3 were grouped together and assumed to be more atretic than not and therefore were labelled atretic. Apoptotic follicles were grouped as apoptosis grade 1 with presence of apoptosis and apoptosis grade 2 with distinct apoptosis.

The non-apoptotic, non-atretic group was the only group, which contained fluorescent nucleus oocytes, which is thought to be a young chromatin configuration. With increasing apoptosis grade, more oocytes contained chromatin consistent with meiosis-like alterations,

and metaphase stages, although this failed to reach significance (P = 0.08). The atretic follicles contained the least loosely condensed chromatin of all the groups, indicating that chromatin changes have progressed from immature oocytes towards configurations usually seen in maturing oocytes. See Figure 6.10.

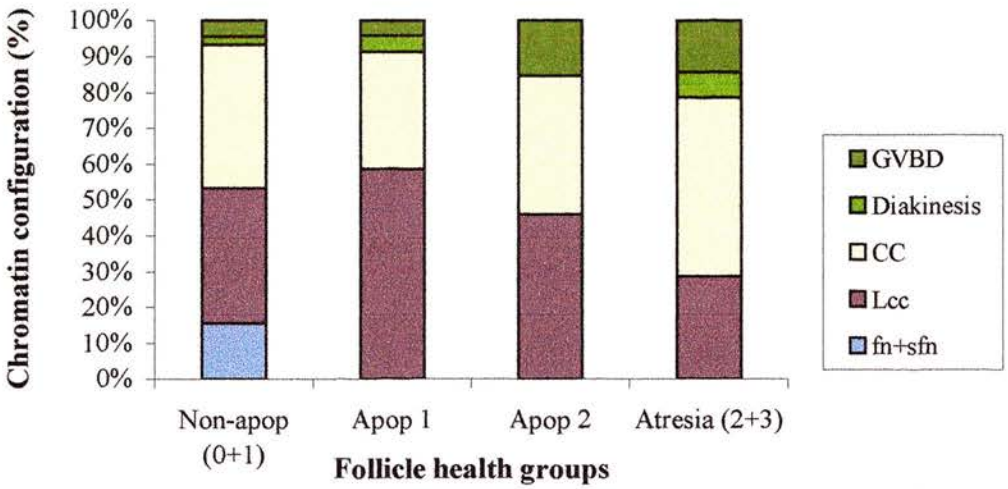


Figure 6.10. Chromatin configuration within follicles of varying health status. FN was only found in non-apoptotic and non-atretic follicles, and CC increased with increasing atresia grade. The two metaphase oocytes in non-apoptotic (0+1) follicles were from 45 mm follicles. Numbers of oocytes in each group: Non-apoptotic (0+1) (n = 45), Apoptosis 1 (n = 46), Apoptosis 2 (n = 13), Atresia (2+3) (n = 14).

6.4.9 Cumulus morphology during the cycle

A total of 174 cumuli oophori were recovered from follicles, resulting in a recovery rate of 59.4%. There was a trend of initially (on day 6) an equal number of follicles containing compact and expanded cumuli, followed by a maximum of compact cumuli on day 11, and a gradual decrease until oestrus during which the largest proportion of expanded cumuli were seen. Despite this apparent trend, it was not possible to obtain significance with a Chi-square analysis other than in cumulus morphology distribution between day 11 and oestrus, and day 15 and oestrus. The most denuded oocytes were found during oestrus. See Figure 6.11.

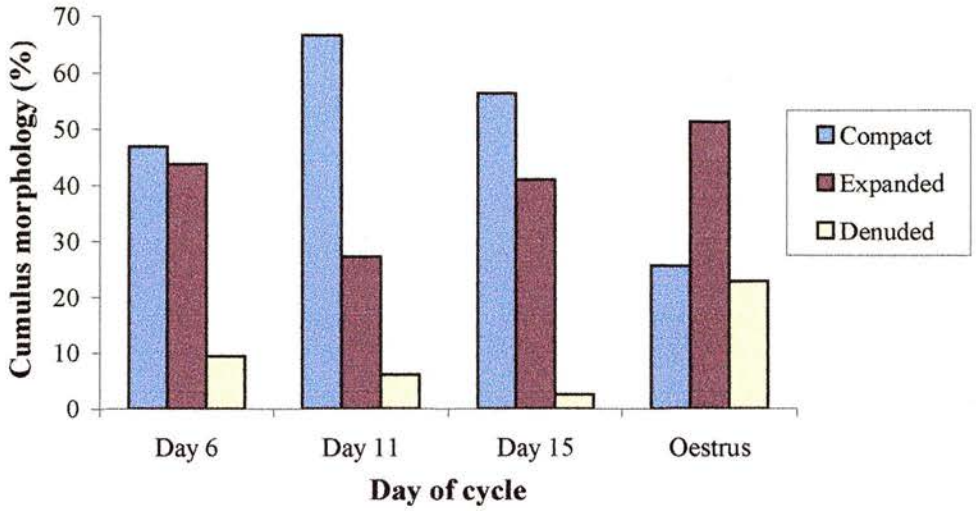


Figure 6.11. Cumulus morphology during the oestrous cycle. Cumulus morphology varied with stage of cycle. There was a significant difference between day 11 and oestrus ($P < 0.01$) and day 15 and oestrus ($P < 0.05$). Numbers of cumuli on each day: Day 6 ($n = 32$), Day 11 ($n = 33$), Day 15 ($n = 39$), Oestrus ($n = 70$).

6.4.10 Cumulus morphology in relation to follicle sizes

There was a difference in cumulus morphology between follicle sizes ($P < 0.001$) (Figure 6.12). Denuded oocytes were present in all size groups. Frequency of compact cumulus oocytes increased with increasing follicle size until 30 mm. Cumulus oocyte complexes from follicles larger than 30 mm started to expand, possibly due to natural maturation, whereas the smaller follicles' cumuli most likely were expanded due to a higher frequency atresia in the small follicles.

6.4.11 Chromatin configuration and cumulus morphology

Figure 6.13 shows the relationship between cumulus morphology and chromatin configuration. FN was only found in compact cumulus oocytes, and most LCC was present in this group. As the cumulus expanded, chromatin appeared to shift from the young configurations towards condensed chromatin and diakinesis to metaphase stages, which was most frequent in the fully expanded cumulus oocytes ($P < 0.001$).

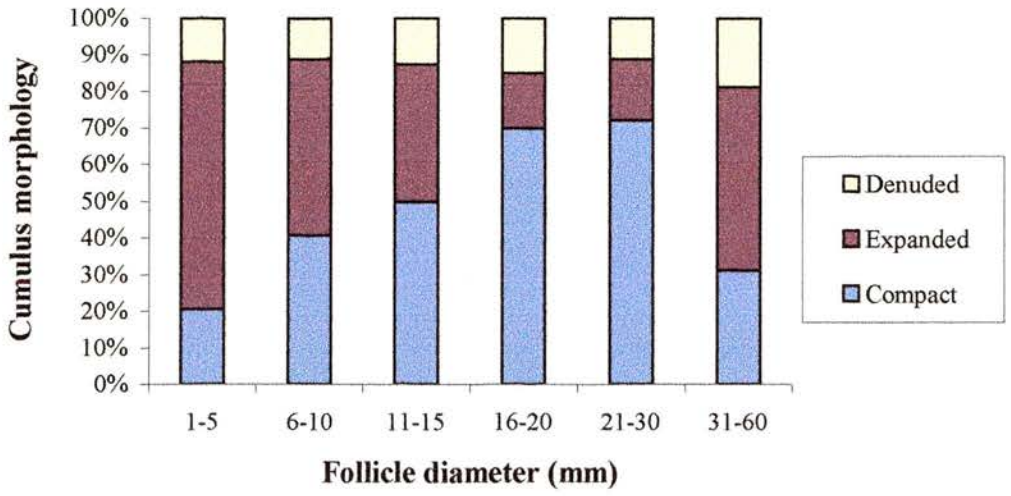


Figure 6.12. Cumulus morphology in relation to size groups. There was a significant difference between size groups ($P < 0.001$).

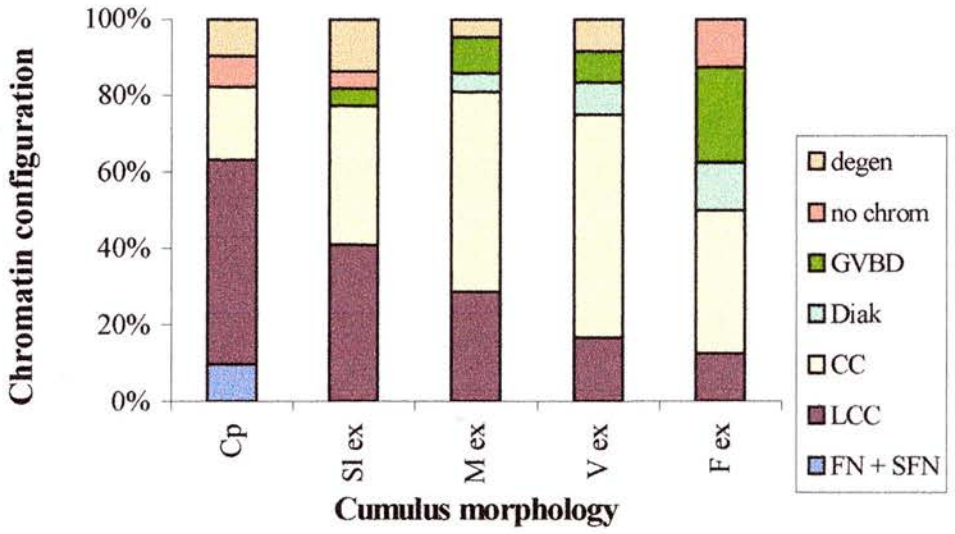


Figure 6.13. Chromatin distribution within cumulus groups. Chromatin configurations changed significantly with cumulus morphology ($P < 0.001$). Numbers of oocytes within each group: Cp (n = 62), Sl ex (n = 22), M ex (n = 21), V ex (n = 12), F ex (n = 8).

6.4.12 Apoptosis in relation to cumulus expansion

Follicles were grouped as healthy, apoptotic or atretic and the cumulus surrounding the oocyte within these follicles was grouped as compact or expanded in different degrees. Cumulus expansion was significantly correlated with atresia ($P < 0.001$). See Figure 6.14.

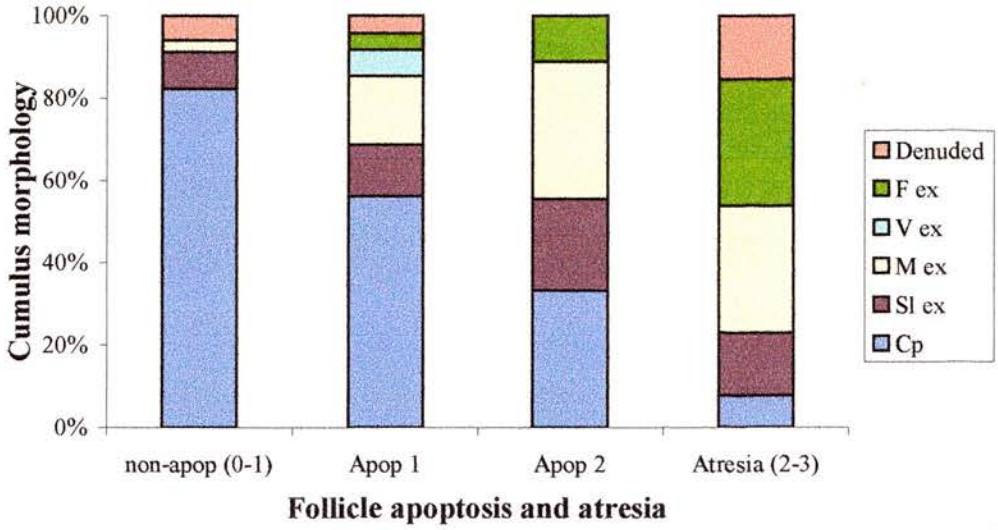


Figure 6.14. Cumulus morphology within follicle atresia group. Cumulus expansion was significantly correlated with apoptosis and atresia ($P < 0.001$). Number of cumuli in each group: No apoptosis (n = 34), Apoptosis 1 (n = 48), Apoptosis 2 (n = 9), Atresia (2-3) (n = 13).

6.4.13 Follicular fluid hormone levels during the cycle

Oestradiol concentrations in follicular fluid varied significantly with day of cycle, being least on day 6 and most during the follicular phase. Neither progesterone nor testosterone varied significantly with day of cycle, although progesterone tended to decrease as the cycle progressed. See Figure 6.15.

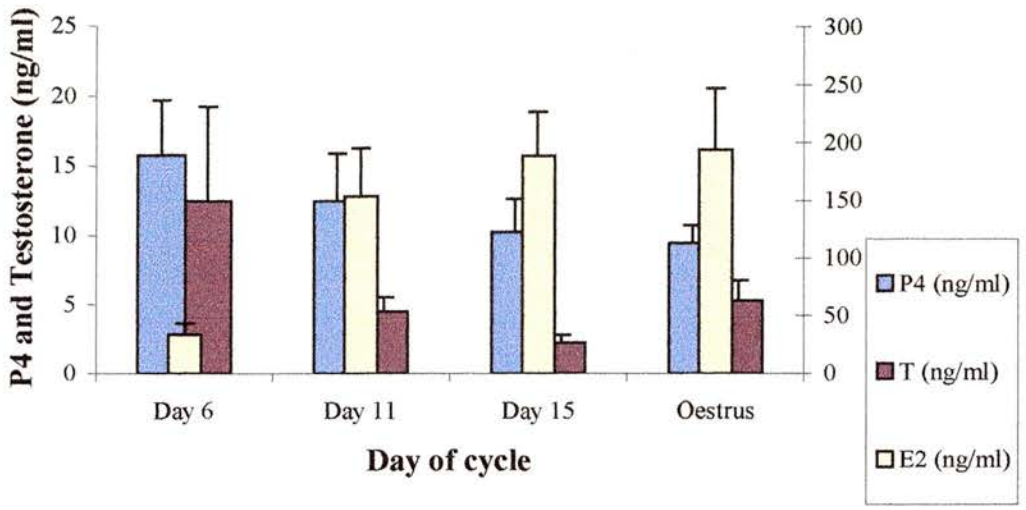


Figure 6.15. Steroids in follicular fluid during the cycle. There was a significant difference in hormone concentrations between day 6 and the following days ($p < 0.003$). No other significant difference was present due to the large variation of concentrations. The second y-axis is for oestradiol.

Mean follicle size on day 6, day 11, day 15 and oestrus was 6.9 ± 0.7 mm, 11.2 ± 1 mm, 12.4 ± 0.9 mm and 18.9 ± 1.5 mm respectively. When dividing oestrous follicles into follicles larger and smaller than 30 mm, the mean of follicles were 45.8 ± 1.7 mm and 12.7 ± 0.7 respectively. Day 11, day 15 and oestrus follicles less than 30 mm were all of a similar mean size. This means that when investigating differences in steroid content on day of cycle, there was a significant difference in follicle size between day 6 and the other days ($P < 0.001$), and between days 11 and 15 and oestrous follicles larger than 30 mm ($P < 0.001$). Thus size of follicle could be more important than actual stage of cycle. To investigate effect of size and effect of apoptosis on follicular fluid content, data were split up. Unfortunately, there was not enough data to do a split plot analysis with follicles divided into days, size groups and apoptosis groups, as too many groups would contain very few follicles.

6.4.14 Atresia and follicular fluid hormones during the cycle

There was no significant difference in oestradiol, progesterone or testosterone content of apoptotic versus non-apoptotic follicles within days other than in testosterone on day 15. There was no significant difference in oestradiol content in apoptotic versus non-apoptotic

follicles on any of the days, although concentrations tended to be lower in apoptotic follicles in all groups. Day had an effect on oestradiol concentration. See Table 6-3.

Table 6-3 Follicular fluid oestradiol concentrations during the cycle.

Oestradiol ± SEM (ng/ml)	Day 6	n	Day 11	n	Day 15	n	Oestrus, < 30 mm	n	Oestrus, > 30 mm	n
Non-apoptotic follicles	39.4 ± 23[a]	16	207 + 79[b]	26	210 + 67[b]	31	59.1 + 14[a]	39	945 ± 247[c]	15
Apoptotic follicles	29.6 + 10[a]	31	102 ± 36[b]	36	175 ± 42[b]	32	43 ± 19[a]	32	-	-

[abcd] Same letter in a row or in a column indicates no difference. [a-b]: $P < 0.05$. [a-c]: $P < 0.002$. [b-c]: $P < 0.01$

There was no significant difference in progesterone content in apoptotic versus non-apoptotic follicles on any of the days, although concentrations tended to be higher in apoptotic follicles in all groups apart from day 6. Day had no effect on progesterone concentration, but concentrations were significantly higher in preovulatory follicles. See Table 6-4.

Table 6-4 Follicular fluid progesterone concentrations during the cycle.

Progesterone ± SEM (ng/ml)	Day 6	n	Day 11	n	Day 15	n	Oestrus, < 30 mm	n	Oestrus, > 30 mm	n
Non-apoptotic follicles	17.1 ± 7.6[a]	6	8.1 ± 2.5[a]	20	6.6 ± 0.9[a]	27	5.0 ± 0.7[a]	32	25.7 ± 4[b]	15
Apoptotic follicles	14.7 ± 3.9[a]	7	16.6 ± 6[a]	22	14.3 ± 4.8[a]	25	6.4 ± 1.6[a]	29	-	-

[ab] Same letter in a row or in a column indicates no difference. [a-b]: $P < 0.001$

Apoptotic follicles tended to contain less testosterone than non-apoptotic follicles, but this only reached significance on day 15. Preovulatory follicles (always non-apoptotic) contained significantly less testosterone compared to other non-apoptotic follicles apart from day 15 (Table 6-5).

Table 6-5 Follicular fluid testosterone concentrations during the cycle.

Testosterone SEM (ng/ml)	±	Day 6	n	Day 11	n	Day 15	n	Oestrus, < 30 mm	n	Oestrus, > 30 mm	n
No apoptosis		14 ± 7.8[a]	6	5.3 ± 1.2[a]	10	2.6 ± 0.7[ac]	14	7.5 ± 3.1[a]	23	1.4 ± 0.4[bc]	7
Apoptosis		-	-	3.5 ± 1.8[ab]	8	1.1 ± 0.2[b]	5	5.1 ± 1.5[a]	13	-	-

[abc] Same letter in a row or in a column indicates no difference. Two letters in a cell means that there was no difference to e.g. neither cells with a, nor cells with b, although cells with either only a or only b differed from each other. [a-b]: $P < 0.05$. [a-c]: $P < 0.05$

6.4.15 Follicular fluid hormones in different size groups in relation to follicle health status

There was no significant difference in oestradiol, progesterone or testosterone content of apoptotic versus non-apoptotic follicles within size groups. In all size groups there was a tendency for oestradiol concentrations to be lower in apoptotic than non-apoptotic follicles, but this did not reach significance. There was increasingly higher oestradiol concentrations in larger follicles. See Table 6-6.

Table 6-6 Oestradiol in different follicle size groups.

E2 ± SEM (ng/ml)	2 - 10 mm	n	11 - 20 mm	n	21 - 30 mm	n	31 - 60 mm	n
Non-apoptotic follicles	53.3 ± 12[a]	61	139 ± 25[b]	34	282 ± 134[b]	14	948 ± 220[c]	18
Apoptotic follicles	42 ± 8[a]	80	120 ± 31[b]	37	245 ± 107[b]	13	-	

[abcd] Same letter in a row or in a column indicates no difference. [a-b]: $P < 0.05$. [a-c]: $P < 0.001$. [b-c]: $P < 0.01$

There was no difference in progesterone concentration between size groups other than in the follicles larger than 30 mm, which contained significantly more than the rest. Progesterone tended to be higher in apoptotic follicles, but this failed to be significant. See Table 6-7.

Table 6-7 Progesterone in different follicle size groups.

P4 ± SEM (ng/ml)	2 - 10 mm	n	11 - 20 mm	n	21 - 30 mm	n	31 - 60 mm	n
Non-apoptotic follicles	7.9 ± 2.1[a]	34	6.3 ± 0.6[a]	34	6.5 ± 1.3[a]	14	23.2 ± 3.7[b]	18
Apoptotic follicles	14.4 + 3.9[a]	34	9.7 ± 2.4[a]	35	13.2 + 8.3[a]	13	-	

[ab] Same letter in a row or in a column indicates no difference. [a-b]: $P < 0.001$

There was a non-significant tendency for testosterone to be lower in apoptotic versus non-apoptotic follicles, and a significant decrease in concentrations with increasing follicle size. See Table 6-8.

Table 6-8 Testosterone in different follicle size groups.

T ± SEM (ng/ml)	2 - 10 mm	n	11 - 20 mm	n	21 - 30 mm	n	31 - 60 mm	n
Non-apoptotic follicles	8.7 ± 3.3^{a}	15	5.3 ± 1.9^{b}	18	5.8 ± 4^{b}	10	1.9 ± 0.7^{c}	10
Apoptotic follicles	8.1 ± 4.2^{a}	4	3.7 ± 1.2^{b}	14	2.1 ± 0.6^{b}	9	-	

^{abcd} Same letter in a row or in a column indicates no difference. ^{ab}: $P > 0.1$. ^{ac}: $P < 0.05$. ^{bc}: $P > 0.1$

6.4.16 Steroid hormones in apoptotic versus non-apoptotic follicles

In the previous sections, I have referred to a tendency for apoptotic follicles to contain either less oestradiol and testosterone or more progesterone, but have not been able to show any significant difference due to large variation and few samples causing large standard deviations. In order to examine this relationship further, a regression analysis was performed on steroid concentration in non-apoptotic, apoptosis grade 1 and apoptosis grade 2 follicles.

In non-apoptotic follicles and apoptosis grade 1 follicles, oestradiol concentration was significantly correlated ($P < 0.001$) with follicle size, but this relationship was lost in apoptosis grade 2 follicles, signifying a break-down in oestradiol production (Figure 6.16). The fact that there was still a correlation between oestradiol and both non-apoptotic and apoptosis grade 1 follicles but not apoptosis grade 2 follicles, was either indicative of apoptosis occurring before any significant decrease in steroid production or alternatively that apoptosis grade 1 follicles were not necessarily destined to die, but perhaps could recover and grow on. The coefficient for the slope of the line of regression was highest for non-apoptotic follicles and lowest in apoptosis grade 2 follicles, indicating the presence of higher oestradiol concentration in healthy follicles than in apoptotic follicles of the same size.

In non-apoptotic follicles, progesterone concentration was significantly correlated ($P < 0.001$) with follicle size, but this relationship was lost in apoptosis grade 1 and 2 follicles. Progesterone increased with increasing size in non-apoptotic follicles. In apoptotic follicles, concentrations decreased with increasing size, but concentrations seemed to be higher in

apoptosis grade 1 follicles compared to both healthy and apoptosis grade 2 follicles in small follicles. See Figure 6.17.

Testosterone measurements varied greatly and there was no significant correlation with size. The coefficient for the regression line was negative, meaning that with increasing size, concentrations decreased (Figure 6.18). The decrease with size was more pronounced in the apoptotic than in the non-apoptotic follicles. There were only 6 measurements in the apoptosis grade 2 group, and this group was not analysed further.

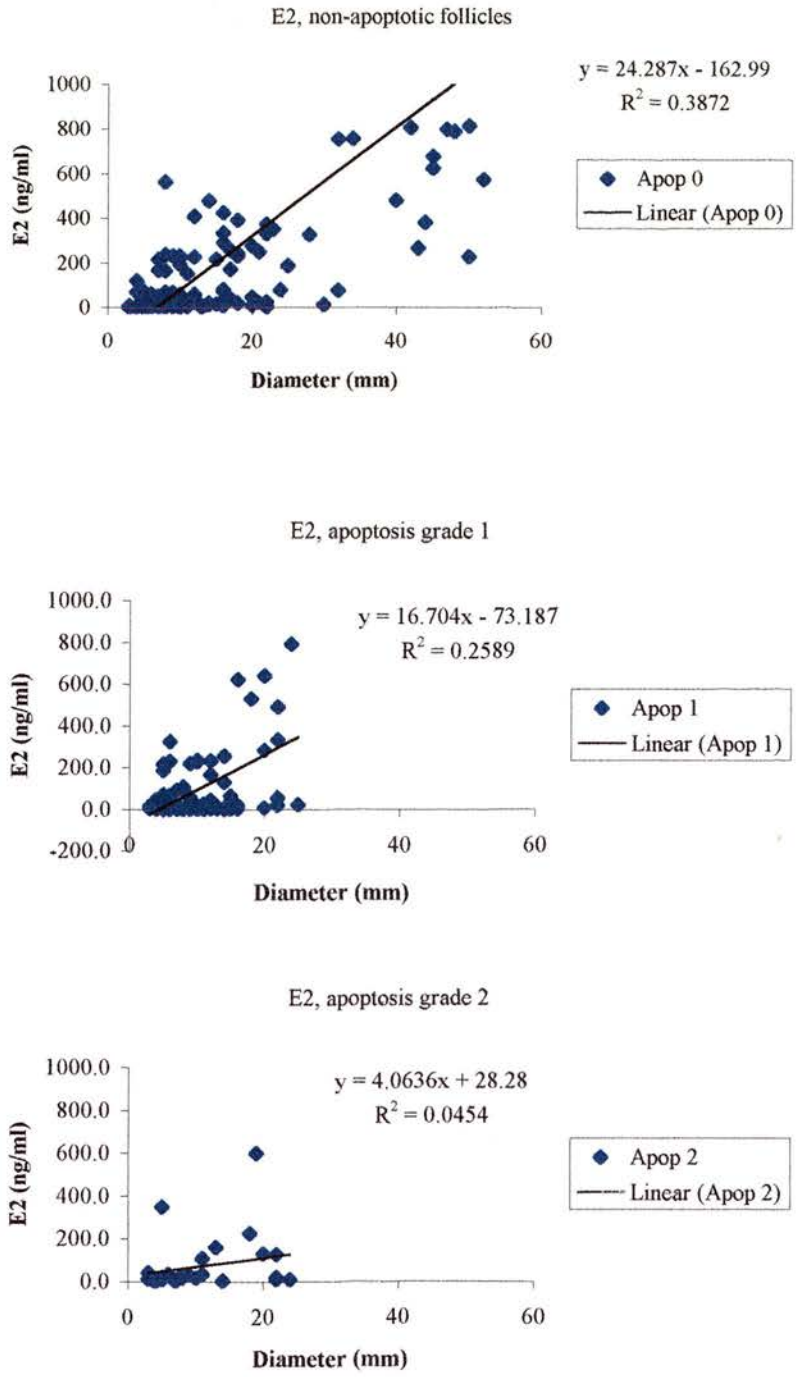


Figure 6.16. Oestradiol in non-apoptotic, apoptosis grade 1 and apoptosis grade 2 follicles.

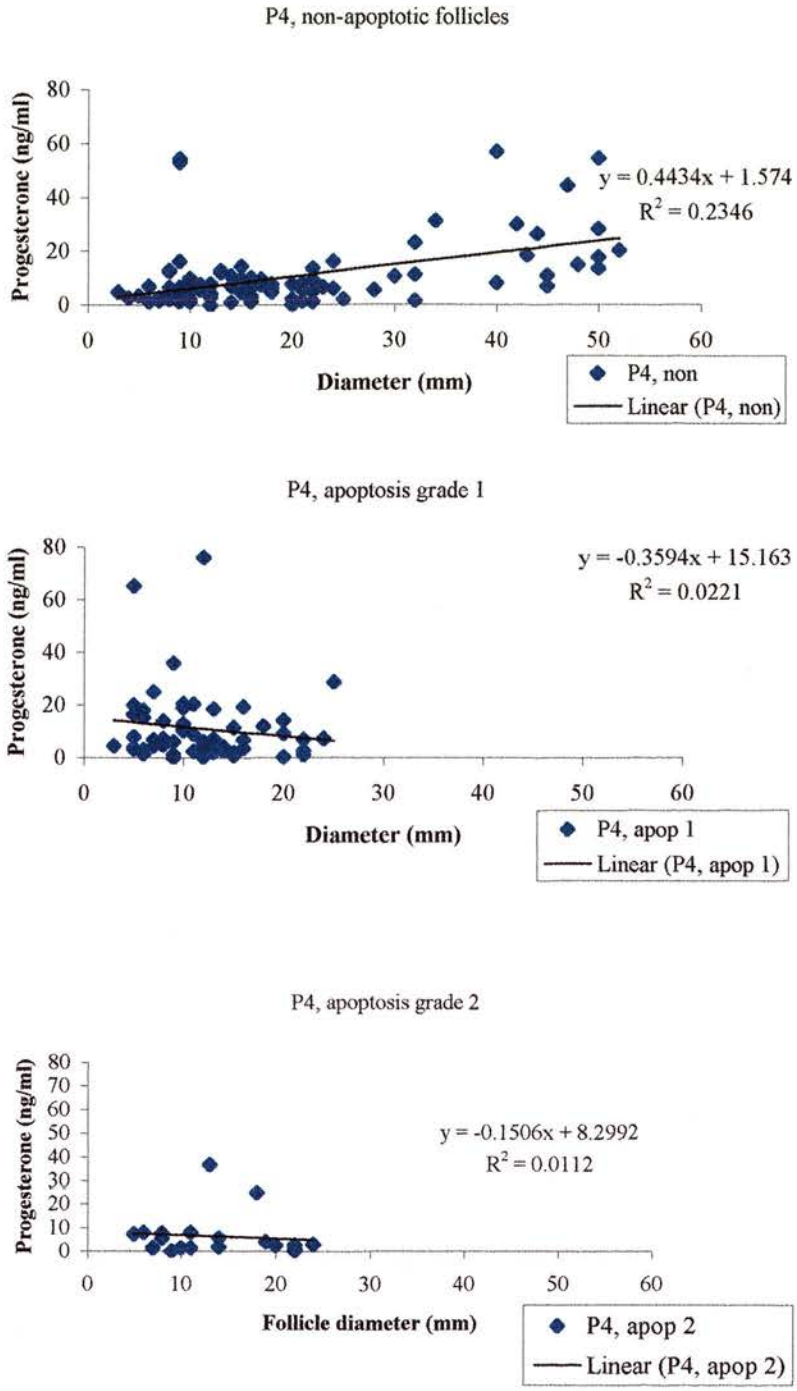


Figure 6.17. Progesterone in non-apoptotic, apoptosis grade 1 and apoptosis grade 2 follicles.

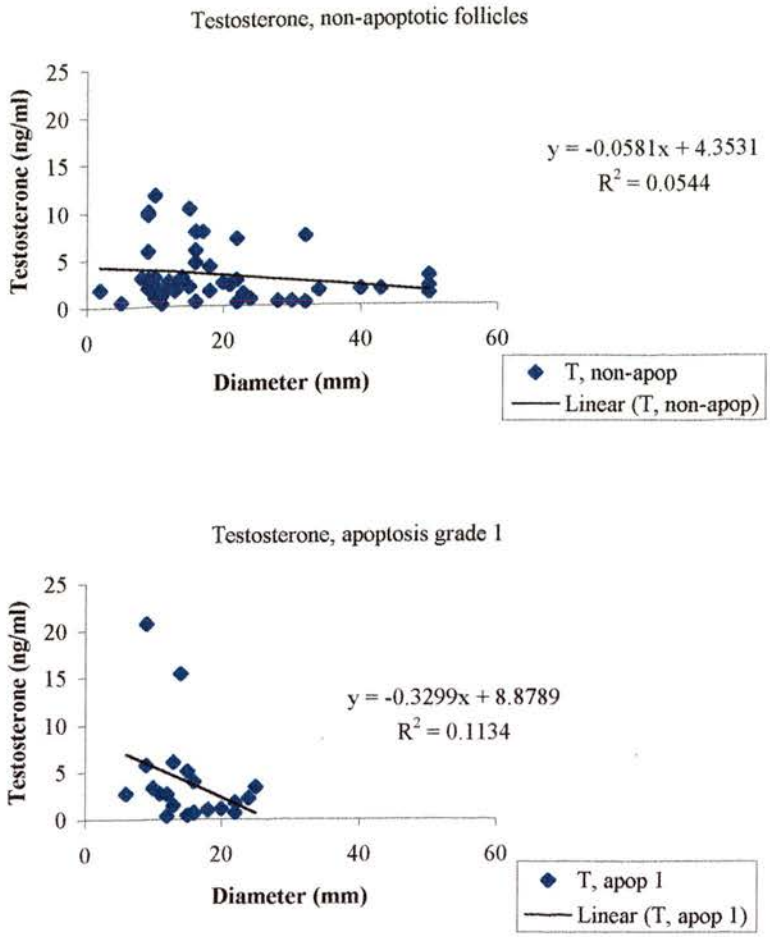


Figure 6.18 Testosterone in non-apoptotic and apoptotic follicles.

The above data should aid in illustrating that there may indeed be a tendency for apoptotic and non-apoptotic follicles to contain different concentrations of steroid hormones, although the present data contained too much variation to show statistically significant differences.

6.4.17 Determining health status in growing or regressing follicles

In the above, data were grouped together in various ways and analysed. In Appendix E is a description of findings in each individual animal on days 6 - 15, using every single parameter available to determine health status of the follicles. The appendix contains a follicle graph, a follicle map with colouring of the dominant, growing, intermediate, dying and atretic follicles on the day of ovariectomy as well as a commentary and summary table

for each individual animal. No statistical analysis is capable of incorporating all data available and therefore Appendix E is only meant to be a descriptive account of the different stages of the cycle. Figure 6.19 shows the data in Appendix E summarised.

Health status of the follicles at different days was determined based on granulosa cell apoptosis, oocyte chromatin configuration, cumulus morphology and follicular fluid steroid content in relation to the growth or regression of the follicle as detected with the use of follicle maps and in connection with determination of gonadotrophin levels. When deciding when a follicle was dominant, growing, intermediate (parameters contradicting each other), dying or atretic, most emphasis was put on presence of apoptosis and the appearance of the granulosa cells. Next came level of oestradiol and DNA content. These two parameters were reliable, but as both went through dilution steps when prepared, there was a risk of introducing error, and therefore they were less reliable than the presence of apoptosis and granulosa cell appearance, although these two parameters were not definite parameters of life and death either. The next parameters were progesterone concentration, cumulus appearance and follicular wall colour. Oocyte chromatin configuration was useful in the context of a non-dominant follicle showing meiotic changes, which was usually indicative of onset of atresia. No single parameter could be used on its own to determine life or death, but with the knowledge of the overall tendency of each parameter, used in conjunction they indicated the health status of a follicle.

Because the mares in the oestrous group were from a slaughterhouse, there was no data from the previous days about growth of the follicles, but the following is a brief resume of what was found. In the oestrous group, mares had been selected on the basis of having at least one follicle larger than 40 mm. All follicles larger than 40 were non-apoptotic, with healthy-looking granulosa cells, primarily chromatin in the CC configuration or in metaphase, elevated progesterone and relatively high levels of oestradiol. All parameters pointed towards healthy follicles. In the same horses, follicles between 20 - 30 mm all had relatively low levels of oestradiol, 3 out of 7 had started showing distinct apoptosis and CC configuration was predominant. These follicles probably had been selected against. Two follicles were between 32 – 34 mm and both appeared healthy with high levels of oestradiol. There is a possibility that these may have formed double ovulations as they appeared to have grown larger than the critical size in the twenties.

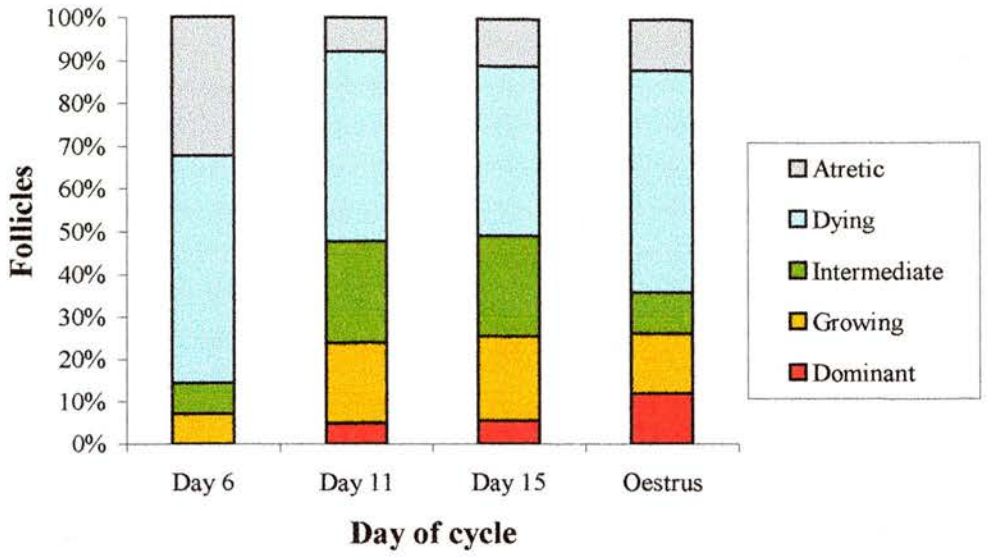


Figure 6.19. Growing and regressing follicles during the oestrous cycle. Health status of the follicles was based on presence of apoptosis, granulosa cell morphology, follicular fluid hormone content and chromatin configuration. The dominant follicle could in some cases be detected on day 11. Day 11 and 15 contained the least dying and atretic follicles, and day 6 contained the most. Follicles appeared to start dying between day 15 and oestrus, and progressively more dying follicles were present until between day 6 and day 11.

6.5 Discussion

The follicle size distribution varied significantly between days. The 2-5 mm group was largest on day 6, and gradually decreased through the oestrous cycle as more follicles either entered the larger size groups or degenerated. This was in accordance with other studies, which reported an increase in number of small follicles (2-10 mm) between oestrus and day 5 of dioestrus, followed by a decrease in number of small follicles on day 8 and day 11 (Pineda *et al.*, 1972a and 1973; Driancourt *et al.*, 1982a; Pierson *et al.*, 1987). In the present study, the 6-10 mm group decreased from day 6 to day 11, but then stayed steady throughout the rest of the cycle. Some follicles grew into this category and others probably entered after they had been larger and then underwent atresia and regressed. Pierson and Ginther (1987) found no significant day effect for the 6-10 mm and the 11-15 mm groups during the cycle, but an increase in numbers of 16-20 mm and follicles >20 mm between days 6 to 10, ending prior to ovulation. This is consistent with the present study, where the 16-30 mm group increased from day 6 to day 15 and decreased from day 15 to oestrus as the follicles either

entered the 31-60 mm group during oestrus or began to regress if not destined to ovulate. The 31-60 mm group contained most follicles during oestrus. Pierson and Ginther (1987) hypothesised that growth of follicles into the 2-5 mm pool, occurred at a constant rate, but that the increased number of 2-5 mm follicles in the postovulatory period was due to regression of larger follicles (Pierson *et al.*, 1987). Certainly that larger follicles regressed into the small size groups was also the conclusion of this study, but we could neither confirm nor negate the hypothesis about the constantly growing 2-5 mm follicles. One study found that the variations in the total number of follicles in the various size classes per day were overshadowed by the individual differences in the mares (Driancourt *et al.*, 1982a). The mares in this study varied widely in sizes of follicles even when ovariectomised on the same day in their cycle. Some mares had larger follicles on day 11 than mares on day 15. Length of cycle varied greatly with each animal as seen in chapter 5, which may account for some of the differences found. In summary, follicles enter and leave size groups as they either grow or regress during the cycle, leaving the ovary with a dynamic population of follicles, which changes all the time.

The highest proportion of oocytes displayed meiosis-like changes early in the cycle consistent with most follicles being atretic at this point. During oestrus the proportion of metaphase oocytes was also high, but some of the metaphase configurations were a sequel to normal meiotic maturation as these were found in follicles of 21 – 60 mm. The majority of the metaphase oocytes in oestrus was from follicles smaller than 10 mm, and thus likely was the form that happened in connection with atresia. This finding also suggested that many small follicles were atretic in oestrus. In a study comparing oocyte maturation rate in equine oocytes from normal follicles versus atretic follicles, full maturation of oocytes was obtained in 47% of normal follicles versus 76% of atretic follicles (Bezard *et al.*, 1997). Bovine oocytes from atretic follicles also showed better maturation rates than oocytes from non-atretic follicles (Blondin *et al.*, 1995). Supposedly the atretic follicles already have moved towards meiosis-like alterations, but it remains to be seen whether these oocytes actually can be fertilised and will grow normally or if they will stop their development. In other words: it is not yet known whether the oocytes from atretic follicles themselves are becoming atretic and thus will only develop to a certain point. Bovine oocytes from slightly atretic follicles were capable of reaching the 16-cell stage of the embryo in an equal frequency to oocytes from non-atretic follicles (Blondin *et al.*, 1995). Because most follicles were dying or atretic on day 6, it was surprising that the majority of oocytes were of the LCC configuration as LCC was associated with young, immature follicles. There are two possible explanations:

LCC was not a young configuration or the dying follicles lost the meiosis-suppressing activity at a late stage of atresia, when the follicles progressed beyond just apoptosis. The latter seemed most plausible, as LCC was associated with non-atretic rather than atretic follicles and associated with compact rather than expanded cumulus. Small follicles contained few oocytes with young configurations and many degenerated chromatin oocytes and oocytes with no chromatin, suggesting either that more small follicles underwent atresia than large follicles or perhaps rather that regressing follicles did not contain degenerate oocytes until they were below a certain size. The finding that small follicles contained fewer FN and LCC configurations than large follicles could be interpreted as these configurations not being young configurations. The large proportion of atretic, small follicles supported the theory that some small follicles were growing, but many were regressing, atretic and therefore contained degenerating oocytes, which emphasised the necessity of using several parameters when evaluating the follicles and oocytes. The diakinesis configuration was reported to be present in primarily compact cumulus oocytes from 1 – 5 mm follicles, suggesting that this was an immature configuration (Hinrichs *et al.*, 2000). The data in this study could not confirm this hypothesis, as the diakinesis configuration was found in small follicles with mainly expanded cumulus oocytes but also in large follicles. Perhaps the discrepancy was in the classification of the configuration, as diakinesis in the present study was a configuration where individual, condensed chromatin strands were seen, to which it may be argued that it may be more like prometaphase I. Again, the chromatin configurations are a continuum, and determining when a configuration changes to another may differ between workers.

From day 6 to day 11 configurations did not differ much, but a change was obvious between day 11 and day 15. As the cycle progressed, more CC configurations were found, perhaps due to a progression of maturation of the oocytes. Before oestrus, follicles larger than 20 mm contained oocytes with young or immature chromatin. During oestrus most large follicles contained oocytes with the more mature forms of chromatin (CC and metaphase), indicating that oocytes of follicles larger than 20 mm matured as the cycle progressed and that CC was a normal feature in this process. Also some of the apoptotic 21-30 mm follicles contained oocytes with the CC configuration. Either this configuration was part of the normal meiotic maturation, but also an end-stage for follicles that were not selected or alternatively, the apoptosis was only temporary and the follicles would continue to grow. The hormonal environment may have been more suitable for maturation during oestrus, which was supported by higher maturation rate of oocytes collected in the follicular phase than during

the luteal phase (Goudet, 1998b). Not only the preovulatory follicle's oocyte, but also oocytes in the subordinate follicles matured to a larger extent. In bovine oocytes there was no difference in maturation or fertilisation competence in relation to stage of cycle, but smaller follicles yielded oocytes with less capacity to produce blastocysts indicating that size was more important (Arlotto *et al.*, 1996; Jewgenow *et al.*, 1999). In equine oocytes the maturation rate was lower in 5-9 mm than in 10-19 mm and 20-27 mm follicles (45%, 69% and 67%) (Bezard *et al.*, 1997). A recent paper described that oocytes attained meiotic competence with increasing size, particularly so, when follicles were larger than 20 mm (Hinrichs *et al.*, 2000). In summary, size is probably the most important feature, but stage of cycle may aid both in that follicles are larger during the follicular phase, but also the hormonal environment is more suitable even for the smaller follicles.

As the follicles started to undergo apoptosis, more cumulus oophorus expansion was present. The factors preventing expansion seemed to disappear with reduction of normal function of the follicle in connection with atresia. More cumuli expanded and they expanded to a larger extent as seen by the progression from slightly and moderately expanded in apoptosis grade 1 to a large percentage of fully expanded cumuli in the atretic follicles. Other studies in horses also reported that cumulus expansion was significantly associated with histological atresia of the follicle (Hinrichs, 1991b and 1997). One study reported that of aspirated oocytes from follicles sized 5-27 mm, 5% had an expanded cumulus (Bezard *et al.*, 1997). This was in contrast to our study and Hinrichs *et al.*, (2000), where cumulus expansion decreased with increasing size, until reaching preovulatory stages, and at no point were there fewer than 15% expanded cumuli. Part of the discrepancy appeared to be due to the other workers' classification system, which was not based on viewing a complete cumulus, as much of this would have fallen off during aspiration.

There was a large variation in steroid hormone content in follicular fluid in the present study. The change, although non-significant, in all hormones in the apoptotic follicles suggested the possibility of decreased granulosa and theca cell function in connection with apoptosis.

Progesterone was independent of size until the follicles reached preovulatory sizes. The increase in progesterone concentration with increasing size in healthy follicles may have been related to a shift in favour of progesterone production in the preovulatory follicles in preparation for forming the corpus luteum. Grøndahl *et al.* (1995b) reported a 10-fold increase in progesterone concentrations during equine follicle maturation, particularly

associated with the progression from MI to MII stage oocytes. More progesterone was secreted by cultured granulosa cells from preovulatory follicles in late compared to early oestrus (Sirois *et al.*, 1991). Another study reported that progesterone and androstenedione showed no significant variation between follicle sizes or state of atresia in mares (Okolski *et al.*, 1991). In porcine follicles there was an increase in expression of 3βHSD with increasing size of the ovulatory follicle, and less 3βHSD expression in atretic follicles (Garrett & Guthrie, 1997). In the present study, the severely apoptotic follicles contained only little progesterone, possibly because granulosa cell disruption was now widespread, supported by the equivalent finding in oestradiol production.

In the present study, oestradiol increased with increasing follicle size. This was in agreement with other reports of equine follicle size being positively correlated with oestradiol and androstenedione concentrations in follicular fluid (Kenney *et al.*, 1979; Fay & Douglas, 1987; Meinecke *et al.*, 1987; Okolski *et al.*, 1991). The authors suggested that the increase in steroidogenic activity was a reflection of follicular maturation, perhaps due to increased LH and FSH receptors (Kenney *et al.*, 1979; Fay & Douglas, 1987). Also aromatase expression and aromatase mRNA increased with follicle size in mares (Boerbom *et al.*, 1999; Goudet *et al.*, 1999). Testosterone in the present study decreased significantly with increasing size. Testosterone concentrations varied greatly between follicles resulting in a large standard deviation and little significant difference partly due to the low numbers of follicles in each group. Apoptotic follicles tended to contain less testosterone than non-apoptotic follicles and the preovulatory follicles contained the least, perhaps a result of a high turnover of testosterone for oestradiol production. Another study found increasing testosterone concentration with increasing size contrasting the results of this study, but agreed on the finding that atresia reduced concentrations of testosterone (Kenney et al., 1979; Okolski *et al.*, 1991). In nonatretic porcine follicles, increased follicle size was associated with a decrease in androgen receptor expression (Garrett & Guthrie, 1997). Bovine follicles appeared to have limited expression of P450c17 in the granulosa cells during oestrus (Conley & Bird, 1997). In summary, steroid content was affected by follicle size.

There were no significant differences between follicular fluid steroid content on different days, which was not caused by a size difference in the follicles on these particular days. Size of follicle seemed to be the most important factor in steroid content. The mean follicle size was smaller on day 6 compared to the other days, which would have an effect on oestradiol content as seen in the difference to concentration on day 11 and day 15 where mean follicle

size was larger. Mean oestradiol concentration did not vary due to the stage of cycle but was a result directly of the size of the follicles as well as the production from the dominant follicle. A study in sheep supported the finding that size was the most important factor in oestradiol production, as the largest follicle was the one with the highest oestradiol content irrespective of cycle stage (Evans *et al.*, 2000). In sheep, the total steroid content of large non-atretic follicles exceeded that of large atretic follicles and also that of small non-atretic and atretic follicles (Moor *et al.*, 1978). Using a two-follicle model in mares, follicular fluid oestradiol concentrations in growing follicles were higher in the larger follicle than in the smaller follicle (Gastal *et al.*, 1999b). During oestrus in the present study, follicular fluid oestradiol content dropped to levels seen on day 6, despite the mean follicle sizes being equivalent to that of day 11 and day 15, suggesting a decreased function of the non-dominant follicles. Alternatively, the future ovulatory follicle on days 11 and 15 had increased mean E2 concentration single-handedly, and thus the difference during oestrus, where the dominant follicles were put in a group of their own, was removed. In summary, there was no effect of day of cycle on steroid content, which was not caused by the follicle size differences, except perhaps for oestradiol.

Overall there were a tendency for apoptotic follicles to contain different concentrations of steroid hormones than non-apoptotic follicles. Oestradiol and testosterone tended to be lower in apoptotic follicles, and progesterone tended to be higher. Severely atretic or apoptotic bovine follicles contained granulosa cells with a low capacity to metabolize testosterone to oestradiol, contained low concentrations of androstenedione and testosterone, but had high concentrations of progesterone (McNatty *et al.*, 1985; Jolly *et al.*, 1994b). Androgen receptor in granulosa cells and expression of P450 17 alpha-hydroxylase/C-17-20 lyase in theca cells were lower in atretic porcine follicles than in nonatretic follicles (Garrett & Guthrie, 1996). Most reports state that oestradiol decreased with atresia. In some studies progesterone decreased and androstenedione and testosterone increased, and some found the opposite to be the case, and yet others that atresia had no effect on follicular fluid steroid content. A study in the horse indicated lower levels of progesterone with the presence of atresia (Kenney *et al.*, 1979), which was contrary to the finding that progesterone tended to increase with presence of apoptosis in this study. Perhaps the degree of atresia has an influence, with late atretic follicles containing less progesterone as the granulosa cells and theca cells disappear. In the present study, progesterone concentration was higher in slightly apoptotic follicles as compared to non-apoptotic and severely apoptotic follicles. In the pig, follicular oestradiol concentration was lower in morphologically atretic follicles compared to

morphologically healthy follicles, but androstenedione and progesterone did not differ significantly (Guthrie *et al.*, 1994 and 1996). Aromatase cytochrome P450 was expressed in granulosa cells of nonatretic porcine follicles and was undetectable in atretic follicles (Garrett & Guthrie, 1996). An increase in spontaneous progesterone secretion and a reduction in follicular androgen and oestrogen production were observed in rat follicles after hypophysectomy (Braw *et al.*, 1981). Non-atretic and intermediately atretic bovine follicles had similar concentrations of oestradiol, progesterone, and testosterone and good capacities to bind gonadotrophins whereas strongly atretic follicles contained a higher concentration of progesterone, lower oestradiol, and a reduced capacity of granulosa cells to bind FSH (Grimes *et al.*, 1987). The basis for a shift in the oestrogen to progesterone ratio has been attributed to both a decrease in substrate for granulosa cell aromatisation and a loss of aromatase activity. The ability of atretic follicles to continue production of androgens and progesterone may be related to the retention of LH-specific binding in the theca cells and increased progesterone production in response to LH or may be a sign of accumulation due to decreased utilisation of substrate for oestradiol production.

It is not clear whether apoptosis occurred before any change in steroid production, but the tendency for the steroid hormones to be altered in the apoptotic follicles indicated that steroid production was altered either before or soon after the onset of detectable apoptosis. Several other studies have attempted to answer this question, but differing views exist. Small non-apoptotic porcine follicles had low oestradiol values similar in magnitude to most biochemically atretic follicles, making the authors suggest that they may have been in early atresia, with loss of aromatase activity preceding the fragmentation of DNA (Guthrie *et al.*, 1994). Loss of aromatase expression was an early event in atresia and was followed by decreased cell proliferation and decreased expression of P450c17α but unaffected 3βHSD expression in pigs (Garrett *et al.*, 1997). A study classifying equine follicles as healthy or atretic using histology found that follicle degeneration began when oestrogen levels were high (Kenney *et al.*, 1979). Histological signs of atresia preceded loss of steroidogenic function in bovine follicles (Grimes *et al.*, 1987). The follicles in the bovine study were only classified as healthy if they had at least 7 layers of healthy granulosa cells, which could potentially misclassify some of the smaller, healthy follicles if they had a thinner layer of granulosa cells thereby affecting the deduction that atresia appeared before alterations in steroid production. From the data in the present study, it would be tempting to conclude that the decreased oestradiol concentration during oestrus was a reflection of selection having taken place, but during oestrus there was very little apoptosis, and primarily growing

follicles according to chapter 5, where follicles did not start to decrease in size until after day 17. Perhaps oestradiol was decreased before apoptosis was detected, although this assumption is negated by the fact that there was no significant difference, only a tendency, for decreasing oestradiol concentration in apoptotic compared to non-apoptotic follicles. If we assume that the constant low level of E2 in the apoptotic follicles had indeed been significant, we could attempt to suggest that E2 may decrease before onset of apoptosis, based on the low level of E2 in follicles during oestrus, where we did not see much apoptosis, but decreased levels of E2, followed by much apoptosis and low E2 on day 6. One way of finding out whether onset of apoptosis precedes a decrease in steroid production, would be to induce apoptosis in the follicles, e.g. by reducing gonadotrophins by a GnRH antagonist, and then recover follicles at different time points following the treatment.

As a measure of health or atresia, (Condon *et al.*, 1979) suggested that oestrogen concentration less than 200 ng/ml, follicular fluid volumes less than 10 ml and little vascularity was indicative of atresia in equine follicles. The present study could not confirm such crude indicators of health, but certainly concentrations of oestradiol higher than 200 ng/ml were in agreement with our mean in non-apoptotic follicles. Large, apoptotic follicles could also contain this concentration. Fluid volumes less than 10 ml were found in follicles less than 27 mm. It is true that, follicles larger than 27 mm generally were healthy, but so were a lot of the small, growing follicles. Other workers have used oestradiol – progesterone ratio as a measure of health or atresia. In the present study, atretic follicles had a mean ratio of 2.9 ± 1.1 and follicles classified as growing had a mean ratio of 41.9 ± 2.8 of oestradiol to progesterone, so progesterone was relatively higher in atretic follicles. Goudet *et al.*, (1999) described a higher ratio of oestradiol to progesterone with increasing size, but did not examine effect of atresia. A ratio of progesterone to oestradiol in bovine follicular fluid greater than or equal to 10 indicated that a follicle was atretic (Grimes *et al.*, 1987). It was not realistic to set up a simple measure of health, and several parameters were needed to obtain a more precise picture.

In a study in mares, approximately 75% of follicles at a given time, regardless of size classification are undergoing atresia as determined by histology (Driancourt *et al.*, 1982b). In our study, frequency of apoptotic and atretic follicles ranged from 50.9 – 85.7%. The presence of many apoptotic and atretic follicles is probably a reflection of the slower regression rate compared to growth rate as seen in chapter 5, leaving the ovary with a larger population of dying than growing follicles. The frequency of atretic follicles changed with

day of cycle, indicating that there were periods of growth and periods of regression of follicles.

A large number of follicles were atretic or apoptotic on day 6, but frequency of growing follicles was high on day 11 and day 15 with the growing follicles primarily being in the medium-sized groups. This was in accordance with findings in another study where there was most atresia on day 6, but on day 14 there was a group of healthy follicles more than 10 mm (Driancourt *et al.*, 1982a). Up to 94% of human luteal phase follicles (greater than or equal to 1 mm diameter) were atretic as assessed by oocyte viability and granulosa cell number (McNatty *et al.*, 1983). The decreasing frequency of apoptosis in follicles as the cycle progressed was in excellent agreement with the follicle maps in chapter 5, which showed regression of follicles until day 6, followed by a wave of growth lasting until day 17-18. The oestrous mares had the least apoptosis but according to the follicle maps, the follicles started to regress midway through oestrus. The reason for the discrepancy in that there was least apoptosis at a point where follicles should start to regress could be that despite the follicles having stopped growing according to the follicle maps in chapter 5, apoptosis may not have been present yet or alternatively, regression may not initially be related to apoptosis. Perhaps apoptosis occurred at a later point as suggested by the large frequency of apoptotic follicles on day 6. Driancourt *et al.* (1982a) reported that between day 14 and day 17 there was a significant increase in number of follicles undergoing early atresia and that just before ovulation all follicles apart from the preovulatory follicle were atretic. It would appear that apoptosis may have been a later event than the start of the regression of follicles. Supporting this theory was a study where apoptotic porcine follicles were smaller than non-apoptotic follicles (Guthrie *et al.*, 1994), as we also found. In chapter 5, the regression of the largest subordinate follicle started from day 18 and the follicles could be followed until day 4 after ovulation, and on day 6, the frequency of apoptosis was largest. A study by Driancourt *et al* found more atretic than non-atretic follicles particularly in the early luteal phase (Driancourt *et al.*, 1982a and 1982b) which was in agreement with the slowly regressing follicles up to and after ovulation in this study.

Most morphologically atretic follicles were less than 10 mm and the majority of these were less than 5 mm, so perhaps follicles regressed and slowly became overtly atretic and contained degenerated oocytes surrounded by expanded cumuli. One study found that the mean total follicular population per mare consisted of 25 (ranging from 8-34) follicles larger than 1 mm of which 8 (32%) were classified as normal and 17 (68%) were atretic

(Driancourt *et al.*, 1982a). Two (12%) of the atretic follicles were in early atresia with only a few pyknotic nuclei per section and 15 (88%) were in advanced atresia with disorganisation of the granulosa cell layer (Driancourt *et al.*, 1982a). Hinrichs (1991b) also found a higher frequency of atresia in small follicles compared to larger follicles. In pigs, frequency of apoptosis was also largest in small follicles (Garrett *et al.*, 1997). It would be interesting to know if regressing large follicles do indeed regress all the way from for example 20 mm to 2 mm. During dissection, follicles surrounded by concentric rings of connective tissue were thought to be large, regressed follicles as they always were degenerated. Collapsed follicles were only noted during microscopy of sections of follicles, i.e. only when the follicles were smaller than what could be distinguished with the naked eye, did they collapse. The only trace left was a hyaline membrane, which was previously the basement membrane. The histological atresia discussed in chapter 4, revealed that there was 26.1 % of the non-atretic follicles, which were larger than 20 mm, but no follicles larger than 20 mm were in atresia state 2 or 3. This meant that the very degenerated follicles always were smaller than 20 mm, and most were less than 10 mm (71%). Opposing our findings was a report which stated that the percentage of atresia was positively correlated with follicle size: 17% atretic granulosa cells in follicles less than 10 mm, 41% in follicles 10-19 mm, and 72% in 20-27 mm follicles (Bezard *et al.*, 1997). The granulosa cells were determined to be atretic by the use of Feulgen stain alone. The Feulgen stain is a specific DNA stain. Either the follicles were indeed regressing because they were aspirated just before ovulation and perhaps at a later time than our oestrous follicles, or their method of determining presence of atresia by staining aspirated cells with Feulgen was not accurate to determine the health status of the follicle.

Frequency of apoptosis decreased with increasing follicle size suggesting that large follicles do not undergo apoptosis until they decrease in size. Generally no follicles larger than 30 mm were apoptotic. Of 47 follicles larger than 30 mm collected over the period of the whole PhD study, 5 were apoptotic. Three of these came from pregnant mares around day 30 of pregnancy. These follicles were not destined to ovulate an oocyte, which would be fertilised, but more likely just luteinise to form secondary corpora luteae. Of the remaining two follicles, one had been held in the ovary for 4-6 hours and apoptosis could have been induced artificially, and the last was a 33 mm follicle, which would be expected to be in good health. In the present study apoptosis appeared to be a feature of smaller rather than larger follicles. In a study of 50 preovulatory equine follicles, 88% were classified as normal and 12% as atretic as determined by Feulgen staining of granulosa cells (Bezard *et al.*, 1997). Preovulatory sheep follicles have been shown to contain apoptotic granulosa cells. In preovulatory ovine follicles, as ovulation approached, there was a progressive increase in

apoptotic cells within the ovarian surface epithelium, tunica albuginea, and in the area of the granulosa cells where the follicle was expected to rupture. Granulosa cells in the basal region of preovulatory follicles were not associated with apoptosis (Murdoch, 1995). In rats, gonadotrophin withdrawal in vivo induced apoptosis in small- to medium-sized antral follicles. Some large follicles also were affected, but the occurrence of apoptosis was less frequent. Although atresia can occur anytime during follicular development, atresia in rats is most commonly observed in smaller follicles beginning the process of antrum formation and not in large preovulatory follicles (Boone *et al.*, 1997b). Also avian granulosa cells from slow growing, small (atresia-prone) follicles were found to undergo rapid and progressively extensive apoptosis after incubation for 6-24 hours, whereas cells from the largest preovulatory follicle showed less apoptosis (Johnson *et al.*, 1996). The reason for preovulatory follicles not undergoing apoptosis to the same extent as smaller follicles may be that expression of death-suppressing genes are capable of making cells resistant to apoptosis (Johnson *et al.*, 1996). Elevated expression of bcl-xlong in preovulatory follicles was correlated with increased resistance to the process of apoptosis in vitro, and the virtual absence of follicle atresia at this stage of development in vivo.

There seems to be evidence that apoptosis is not necessarily related to death of the follicle, because some apparently dominant follicles contained apoptotic granulosa cells. In ewes, the presence of apoptotic granulosa cells in follicles considered to be healthy before their commitment to preovulatory enlargement and ovulation, suggested that apoptosis may be a physiological process in developing follicles or a very early event in atresia (Jolly *et al.*, 1997b). In the present study, chromatin configurations of oocytes in non-atretic + non-apoptotic follicles were very similar to oocytes from apoptosis grade 1 follicles. Once the apoptosis was more pronounced as in apoptosis grade 2, chromatin configurations moved towards those seen in non-apoptotic but atretic follicles' oocytes. The only other equine study which has attempted to use apoptosis as a measure for follicle atresia, considered follicles apoptotic if there were more than 50% apoptotic bodies in the granulosa cells and DNA laddering was present. They found no significant difference between maturation rate or success of ICSI in apoptotic and non-apoptotic follicles supporting the theory that apoptosis may not mean that the follicle is necessarily dying (Oberhammer *et al.*, 1994). Perhaps it is an equilibrium between healthy and apoptotic granulosa cells in a follicle, which can either shift towards mitotic processes in the favour of healthy follicles, and the follicle keeps growing, or if the environment is not supportive of growth, the shift is towards apoptosis, and finally death. The presence of fluorescent nucleus oocytes only in non-apoptotic + non-

atretic follicles, suggested that there was some disruption to the follicles, which did contain apoptotic granulosa cells. This was also a reflection of the oocyte chromatin configuration reacting to changes at a slower pace than cumulus. The cumulus reflected the changes with apoptosis much more than the chromatin, and an immediate effect on cumulus was seen in apoptosis grade 1 follicles. There was a tendency, although not significant, for follicles with apoptosis grade 1 to have higher concentrations of E2, P4 and T than follicles with apoptosis grade 2, indicating that distinctly apoptotic follicles are less steroidogenic. This supported the theory of apoptosis grade 1 follicles perhaps being capable of recovering but apoptosis grade 2 follicles being on the verge of becoming atretic. Quantifying numbers of apoptotic granulosa cells by flow cytometry could be useful for obtaining a measure of how much of the follicle is affected before other parameters change.

When studying the individual mares, the exact stage where a follicle was non-functional was not possible to determine. It appeared to be a gradual death, where it was difficult to determine whether the follicle can recover from e.g. apoptosis and keep on growing or if it would truly die. It appeared that oestradiol decreased at an early point, irrespective of presence apoptosis. The fact that apoptosis appeared to be present in the possible dominant follicle, suggested that it can be present without necessarily resulting in death. Perhaps function of the follicle was most clearly reflected in production of steroid, and that apoptosis could be present in truly dying and dead follicles, but also in follicles which were growing. Perhaps it was a result of changing gonadotrophin concentrations, and the follicle which could adapt before dying, would survive if the follicle was not at a critical stage in its development. Manipulation of hormone levels at different stages of the cycle may help in determining if this indeed could be an explanation.

When determining health status of the follicles of individual mares on different days, findings revealed that on day 6 most follicles appeared to be regressing, with occasional growing follicles. This was in excellent agreement with the follicle maps in chapter 5, which showed that a wave of growth did not occur until after day 6. One study found that histologically all follicles larger than 10 mm were atretic on day 6 (Driancourt *et al.*, 1982a). In the present study, only 5 out of 60 follicles were larger than 10 mm on day 6 and 2 of these were apoptotic. On day 11 one follicle appeared to start to establish dominance but others were still growing or becoming static and starting to show signs of impending death. Few follicles were atretic. One day after wave emergence, before selection, the largest follicles of a wave were histomorphologically indistinguishable in cows (Singh *et al.*, 2000).

This was similar to some of the mares in this study, where a group of follicles all appeared to be growing but when using more parameters it was possible to estimate which follicle was or would become dominant. On day 11, 3 out of 4 mares had a follicle, which appeared to have established dominance. Day 11 apparently was close to the time of selection for dominance. This finding was in agreement with the follicle maps in chapter 5, where there was a size difference between the dominant follicle and the subordinates, which kept growing. On day 15, 4 out of 4 mares had a follicle, which had established dominance, and selection had taken place. Otherwise the two days were rather similar, and many subordinate follicles would still be growing or show only slight signs of pending death. The subordinate follicles presumably became overtly atretic between day 15 and day 6. In a histological study all follicles larger than 10 mm apart from the preovulatory follicle, were atretic just before ovulation (Driancourt *et al.*, 1982a). In summary, there were more established dominant follicles as the cycle progressed. There were least growing follicles on day 6, but most on day 11 and 15, and growth decreased again during oestrus after selection had happened. Most follicles were dying or atretic during day 6 and oestrus, although not apoptotic during oestrus. Several follicles were showing signs of dying during day 11 and 15, possibly due to selection. This method of determining life or death was not definitive and was to a certain extent subjective, as there was no specific cut off point for when a follicle was alive or dead. Ideally, we would make a graph for e.g. oestradiol, which would show cut-off points of concentration between health and atresia, but sizes would also affect concentrations, thus making it impossible. Using apoptosis alone to determine life or death was shown in this chapter not to be a straightforward measure of death, as even dominant follicles would appear to contain apoptotic granulosa cells at some point. Hence there are some discrepancies between the parameters of health during the stages of cycle.

Earlier in the chapter it was reported that apoptosis decreased as the cycle progressed, and that this was in agreement with the growing follicles as seen in chapter 5. But using all the parameters as shown in the above section, we now see that maybe the follicles later in the cycle were growing and maybe they were not apoptotic, but health was none the less decreasing gradually. As some follicles were atretic but not apoptotic, it raised the question whether follicles can die without becoming apoptotic at first. Do they undergo apoptosis and then the fragments disappear the more progressed the atresia is, or do only some selected follicles undergo apoptosis and if so, why? Could mode of death depend on whether death is induced by paracrine factors from the dominant follicle or by lack of optimal gonadotrophin environment?

6.6 Conclusion

The aim of this chapter was to evaluate how a viable follicle differs biochemically from an atretic follicle and to determine at which stage of cycle the dominant follicle was selected for survival and when the remaining follicles started to undergo atresia and regress. We developed some indicators of follicle health and atresia, but because the death of follicles appeared to happen gradually, it was not possible to set out definite points for when this happened. Therefore the conclusion is that selection for dominance is a gradual process, where one follicle seemed to have an advantage over the competitors. The competitors started to regress between day 15 and oestrus and continued until day 6. At all time points were follicles atretic or dying, suggesting that the process of degeneration occurred over a significant period of time.

7 General discussion

The present study investigated indicators of growth, selection and regression in the mare. The aim was to determine when selection of the dominant follicle occurs and how this was reflected in the dominant and the subordinate follicles. The mare is unique in that the follicles are so large that a complete set of information can be gathered from the same follicle, and furthermore, the same follicles' growth and regression can be monitored ultrasonically in the days prior to excision. The methods used allowed for collection of oocytes, follicular fluid and granulosa cells within the same follicle as well as obtaining information about the stage of cycle, hormonal environment and follicle growth prior to collection. This provided information about follicles from the gonadotrophin independent stage to follicles at the preovulatory stage.

At first each parameter was investigated in detail at a microscopic level: oocyte chromatin configuration, cumulus morphology, follicle wall atresia, granulosa cell apoptosis. Then the study followed a whole animal approach with monitoring of follicular growth via ultrasonography. Finally all of the information was put together when studying ovaries recovered at different stages of the cycle in an attempt to reveal when selection took place and how this was reflected in the parameters of the individual follicle.

Oocyte chromatin configuration was found to change from even distribution within the germinal vesicle, through different degrees of condensation and organization to metaphase stages with increasing apoptosis or atresia of the follicle. The condensed chromatin configuration may be part of the normal development but may also be an indication of atresia as this configuration was correlated with atresia in the follicle. A hypothesis was developed as to how the chromatin configurations change, which was different to a previous hypothesis (Hinrichs *et al.*, 1993a). In the present study, the LCC configuration was primarily associated with healthy follicles and proposed to be an early and immature form, whereas the previous study suggested that LCC was a configuration found after CC and just before metaphase. A study published earlier this year by the same author was in better agreement with the present study, now also suggesting that the LCC is a younger configuration than CC (Hinrichs *et al.*, 2000).

The relationship between cumulus morphology and chromatin configuration was described in more detail than previous studies (Hinrichs *et al.*, 1993b, 1997, 2000). Compact cumulus

morphology was associated with young oocyte chromatin configurations and fully expanded cumuli were correlated with metaphase chromatin as found in the previous studies, but the stage of chromatin configuration was directly a reflection of the degree of cumulus expansion. Each sequential step of cumulus expansion represented a further step towards acquisition of meiotic maturation.

Chromatin configuration and cumulus morphology was affected by health status of the follicle as well as by size of the follicle. There was a population of small, atretic follicles with expanded cumuli and oocytes with chromatin progressed towards meiosis, and a population of large, healthy follicles, with young oocyte configurations and no cumulus expansion, and finally a population of follicles larger than 30 mm, which were healthy, but had started to move towards maturation in vivo, i.e. getting ready for ovulation. Size of the follicle appeared to be more important than stage of cycle for predicting chromatin configuration, cumulus morphology and atresia and apoptosis, but size of follicle was correlated with stage of cycle.

The present study provides an explanation as to why increased in vitro maturation rates are correlated with cumulus expansion and condensed chromatin (Hinrichs, 1991b, 1997, 2000). Cumulus expansion is correlated with oocyte chromatin progression towards meiotic readiness and condensed chromatin is correlated with cumulus expansion, atresia of the follicle and the size of the follicle, suggesting that oocytes from follicles with expanded cumulus and condensed chromatin have started to move towards meiosis in vivo, be it via atresia or via the follicle becoming preovulatory. However, it is not known whether in vitro maturation of equine oocytes from degenerating follicles will actually lead to a fertilizable oocyte or whether the changes are only degeneration and thus useless in terms of in vitro fertilization. Bovine oocytes from slightly atretic follicles were capable of reaching the 16-cell stage of the embryo in an equal frequency to oocytes from non-atretic follicles (Blondin *et al.*, 1995). The next step would be to perform ICSI on the oocytes from atretic equine follicles and compare them with in vivo matured oocytes from preovulatory follicles to establish whether they are capable of development. ICSI is an emerging technique in horses (Grondahl *et al.*, 1997; Dellaquila *et al.*, 1997; Guignot *et al.*, 1998) and should provide some answers to this question soon.

When used in combination with plasma hormone levels, daily mapping of follicles made it possible to detect days of changes in follicular growth both of the individual large follicle

and smaller follicles in groups in relation to hormone changes. It appeared that the growth of the largest follicle to a point where it was capable of producing oestradiol caused decreasing levels of FSH, which in turn caused the smaller follicles to regress. The oestradiol increased LH production as well as initiating the process of luteolysis, with resulting further increased levels of LH due to lack of negative feed back from progesterone. When the inhibiting effect of oestradiol disappeared just before ovulation, FSH started to increase, resulting in growth of a new wave of follicles. Divergence in growth rates happened early in the cycle, when FSH levels were high, LH was low and progesterone was still produced. The cause of deviation in this study remains unknown. The follicle maps were found to be a reliable method of determining growth and regression, when accepting that there are influences from regressing, atretic follicles during periods of general growth and that it is not possible to monitor the individual small follicle.

The decreasing frequency of apoptosis in follicles as the cycle progressed was in excellent agreement with the follicle maps, which showed regression of follicles until day 6, followed by a wave of growth lasting until day 17-18. Also data on chromatin and cumulus supported the presence of more atretic follicles early in the cycle. The frequency of apoptotic follicles was lowest in the oestrous mares but according to the follicle maps, the follicles started to regress midway through oestrus. The reason for the discrepancy in that there was least apoptosis at a point where follicles should start to regress could be that despite the follicles having stopped growing according to the follicle maps, apoptosis may not have been present yet or alternatively, regression may not initially be related to apoptosis. The data from the individual mares on day 6, 11, 15 and oestrus certainly indicated that many follicles were atretic or dying during oestrus, although not necessarily as determined by presence of apoptosis.

An extensive evaluation of follicle health based on granulosa cell morphology, follicular wall atresia and apoptosis showed that each of the methods to detect atresia and apoptosis had advantages and limitations and that it was necessary to use the methods in conjunction because they supplemented each other. Apoptosis was not a definite measure of death, because growing follicles, as determined by ultrasonography, could be apoptotic and have no other indications of pending death of the follicle, although apoptosis primarily was associated with atresia of the follicle. Also presumably healthy bovine follicles contained apoptotic granulosa cells with no other sign of atresia (Jolly *et al.*, 1994b). Non-apoptotic and non-atretic follicles contained the same chromatin configuration frequencies as apoptosis

grade 1 follicles, apart from the FN configuration, which was missing in the apoptotic follicles. This supported the finding that presumably growing follicles could contain apoptotic granulosa cells. The presence of apoptosis in otherwise healthy follicles may indicate that either apoptosis occurred to a limited extent at different times during the normal follicle development or alternatively, that apoptosis was present before any other signs of degeneration. In ewes, the presence of apoptotic granulosa cells in follicles considered to be healthy before their commitment to preovulatory enlargement and ovulation, suggested that apoptosis may be a physiological process in developing follicles or a very early event in atresia (Jolly *et al.*, 1997b). Atretic follicles with or without apoptosis on the other hand had more parameters consistent with death of the follicle, and atresia was primarily seen in smaller follicles. Size of the follicles appeared to be a better indicator of follicle health than stage of cycle. A study in pigs also concluded that the proportion of atretic follicles was unaffected by day but was related to size of the follicle (Garrett *et al.*, 1997). Larger follicles had more parameters consistent with growth than smaller follicles. This led to the theory that despite the larger follicles having stopped growing according to the follicle maps made from ultrasound scans, regression may not initially be related to apoptosis, and that apoptosis may be a later event as suggested by the large frequency of apoptotic follicles on day 6. Apoptotic porcine follicles were smaller than the non-apoptotic follicles (Guthrie *et al.*, 1994). The finding that most atretic follicles were small in the present study suggested that follicles regressed and slowly became overtly atretic and contained low levels of oestradiol and degenerate oocytes surrounded by expanded cumuli.

Some data suggested that apoptosis may be present early in the atretic process before other parameters had changed, and other data indicated that apoptosis was not present at the time when these parameters were consistent with decreased follicular function. More information is needed on the subject of when apoptosis and atresia occur, and if apoptotic follicles do indeed become atretic after a period of time or if they can recover. Undoubtedly apoptosis is related to follicle death, but whether apoptosis alone is reliable as an indicator of follicle health is questionable according to the findings in this study. Induction of atresia or apoptosis in a healthy population of follicles, perhaps by administration of a GnRH antagonist, would allow for ultrasound monitoring of the follicles in order to detect any growth or regression. Combining this with ovariectomy of the follicles at different time points after induction of apoptosis or atresia, may aid in answering the question. Also the use of a model where only few, growing follicles were present on the ovary, such as the 2-follicle model described by (Gastal *et al.*, 1997b), would be useful in combination with

ultrasound scanning and detection of atresia and apoptosis, because there would be no interference from a pool of slowly regressing follicles.

When studying the individual mares, selection of the dominant follicle was completed on day 11 in the majority of the mares. This was in excellent agreement with the follicle maps, which showed deviation in size and growth of the dominant follicle starting on day 9. The largest subordinate follicles varied in state of health. Some were apoptotic and dying, and others were intermediate, meaning that they were not apoptotic, but showed other signs of decreased function. Very few subordinate follicles of a similar size to the dominant follicle were growing. The exact stage when a follicle was non-functional was not possible to determine. Death appeared to be gradual, and it was difficult to determine whether the follicle could recover from apoptosis and could keep on growing or if it would truly die. It appeared that oestradiol decreased at an early point, irrespective of presence apoptosis. The fact that apoptosis appeared to be present in the possible dominant follicle suggested that apoptosis could be present without necessarily resulting in degeneration of the follicle. Perhaps function of the follicle was most clearly reflected by production of steroid, and that apoptosis could be present in truly dying and dead follicles, but also in follicles which were growing. Maybe selection for death was a result of changing endocrine environment, and the follicle which could adapt before dying, would survive. Manipulation of hormone levels at different stages of the cycle may help in determining if this indeed could be an explanation.

7.1 Conclusion

The overall aim of this study was to determine at which stage of cycle the dominant follicle was selected for survival and when the remaining follicles started to undergo atresia and regress as reflected by follicle maps, apoptosis in granulosa cells, steroid hormone content in follicular fluid and state of oocyte health. The conclusion of this thesis was that oocyte chromatin configuration, cumulus morphology, follicular fluid steroid concentration and granulosa cell health all are changing parameters with changes in follicle health, and that ultrasound scanning is an important and necessary tool to use in conjunction when studying follicle growth, selection and regression. As the death of follicles appeared to happen gradually, it was not possible to set out definite time points as to when this happened. Therefore the conclusion is that selection for dominance is a gradual process, where one follicle seemed to gain advantage over the competitors, resulting in dominance at an early stage during the cycle.

7.2 Future plans

In order to determine whether condensed chromatin is a normal configuration in equine oocytes rather than a sign of degeneration, oocytes could be cultured following induction of the condensed chromatin configuration by keeping ovaries at 5 °C. If the oocytes progressed to metaphase stages, it is likely that it is a normal configuration. An even better way would be to stain equine oocytes with the Hoechst stain before and after different lengths of culture. In general, culture of oocytes should be performed with the additional information about the effect of apoptosis on initial oocyte chromatin configuration.

To further study the follicles' dependency on LH and FSH, a GnRH antagonist or down-regulation via a GnRH agonist would provide information in an area, which has virtually not been studied in mares. The follicle growth released following down-regulation of hormones and thereby reduced follicular growth, would be a useful model for studying selection for dominance as few regressing follicles would be present on the ovary to interfere with the mapping of the growth of the follicles.

More work needs to be performed on equine granulosa cell apoptosis. Some specific questions were raised: can follicles degenerate without becoming apoptotic at first? Do they undergo apoptosis followed by disappearance of the fragments as the atresia progress, or do only some selected follicles undergo apoptosis? Could mode of death depend on whether death is induced by paracrine factors from the dominant follicle or by lack of optimal gonadotrophin environment? Induction of apoptosis in granulosa cells of growing follicles by administration of a GnRH antagonist, followed by retrieval of ovaries at different time points would provide some information on timing of onset of apoptosis, histological and biochemical characteristics of the apoptotic granulosa cells as well changes in the oocyte chromatin configuration and follicular fluid composition. Alternatively, granulosa cells and follicular fluid could be obtained by transvaginal aspiration of the same follicle at different time points after induction of apoptosis to follow the change within the same follicle.

The present study provided useful information and showed trends, but there is still a lot more to be learned from future experiments and the specific questions arising from this thesis need to be addressed.

8 Appendices

8.1 APPENDIX A

8.1.1 Evaluation of gel grading

Apoptosis was graded with two different grading systems, both of which were subjective. The grading system, which used apoptosis grades 0, 1 and 2 was less detailed than the grading system using grades 10 to 70. See chapter 2 for a detailed description of the grading systems. Originally the detailed gel grading was an expression solely for the type of DNA distribution within a lane. Based on the information from histology and gels, granulosa cell morphology and gels and the time study, the following interpretation of the different grades was made (Figure A1).

Apoptosis grade 30 appeared to contain most follicles with normal granulosa cells (49.5% granulosa cell grade 0), to be present in most histologically non-atretic follicles (83.3%), and to represent most follicles at time point 0 of the time study. This suggested that apoptosis grade 30 was consistent with non-apoptotic follicles. The presence of a wide size range of DNA seen in this group, was initially thought to represent necrosis, but may instead reflect a normal appearance of the extracted non-apoptotic DNA. One report suggested that smearing of DNA could depend on method of extraction (Herrmann *et al.*, 1994). This may indicate that our method of storage, handling and extraction might have led to random fragmentation of the DNA.

Apoptosis grade 20 contained fewer follicles with morphologically normal granulosa cells (40.6%), but otherwise appeared to be very similar to apoptosis grade 30. Perhaps it represented the same group of follicles as apoptosis grade 30 or maybe it represented the step before apoptosis became evident.

Apoptosis grade 50 contained more follicles with a mixed population of granulosa cells (granulosa cell grades 1.5, 2 and 3), and fewer granulosa cell grade 0, 0.5 and 1 than did apoptosis grade 30 (46.2% versus 29.1% and 53.9% versus 70.9%). This suggested that this grade had follicles containing increasing numbers of degenerating granulosa cells. Apoptosis grade 50 was found only in histologically atretic follicles, and it was the apoptotic score found after 5 hours of granulosa cell incubation. This suggested that apoptosis grade 50 is the grade showing the beginning of apoptosis in a follicle, or rather that apoptosis is present in enough cells to cause DNA laddering.

Apoptosis grades 60 and 70 contained progressively fewer apparently normal looking granulosa cells, and more granulosa cell grade 2 instead. Grade 70 designated distinct apoptosis and it seemed to be consistent with advanced atresia.

In the time study, apoptosis grade 50 arose earlier than the other apoptotic grades (60 and 70) and may indicate a state where enough cells were apoptotic to cause laddering. At the same time many cells could be normal and therefore appear like a grade 30, obscuring the ladder with a "veil" of DNA of varying sizes. As apoptosis became prevalent and the oligonucleosomes accumulated, laddering became the main feature, and fewer cells caused the non-apoptotic "veil" (grade 30). This hypothesis was supported by (Wyllie, 1980) where thymocytes incubated with dexamethasone showed stronger laddering at 5 hours than at 3 hours. Care needs to be taken when assessing degree of laddering. The amount of degraded, low molecular weight DNA, which can be extracted from a single cell, varies depending on the stage of apoptosis. Early during apoptosis only a small DNA fraction is degraded but when apoptosis is more advanced nearly all DNA is fragmented (Darzynkiewicz *et al.*, 1998). Theoretically the DNA fragments in this study could stem from few, very apoptotic cells, but the use of more than 90% of granulosa cells from the follicles will diminish chances of only a few cells being very apoptotic and the remainder not apoptotic at all.

Apoptosis grade 10 is rare, and appeared to reflect a mixed group of follicles, perhaps because it is a product of too little DNA in the lane due to spillage at the time of loading the well with DNA and buffer solution or an error at measuring DNA content. Forty four out of 787 (5.6%) of follicles were graded as 10, and because of the suspicion of too little DNA in the well, this group was excluded from analysis.

The apoptosis grade 40 contained 83.3% granulosa cell grade 0, 0.5 and 1, which suggested primarily normal granulosa cells. This may indicate an intermediate state between apoptosis grade 30 and 50, where the large percentage of granulosa cell grade 1 would suggest an increase in degenerating granulosa cells. Opposing this theory is the information that apoptosis grade 50 contained more granulosa cell grade 0 than the apoptosis grade 40 did. To support the placement between apoptosis grade 30 and 50, the time study showed that apoptosis grade 40 appeared more often as time went on. The strong smearing certainly would obscure underlying faint laddering. There is only one follicle with apoptosis grade 40, which has been sectioned for histology (atresia grade 0), so in conclusion there is too little information on this group to place in context with the other groups yet.

The grading system developed was based solely on a subjective evaluation, and could be further developed by doing quantitative studies on apoptotic follicles to confirm the

suspicion that intensity of laddering may indeed be correlated with the degree of oligonucleosome formation.

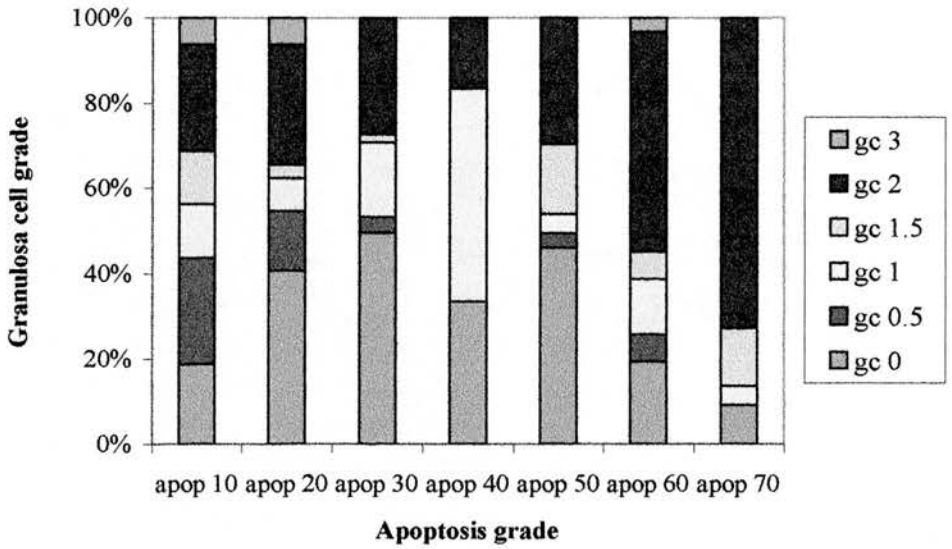


Figure A1. Granulosa cell morphology within apoptosis groups.

8.2 APPENDIX B

8.2.1 Annexin V and Hoechst

In this preliminary study, granulosa cells were stained simultaneously with Annexin V, propidium iodide and Hoechst, but due to the time limits of this project, these techniques were not perfected and a full study carried out. The following observations were made for future use.

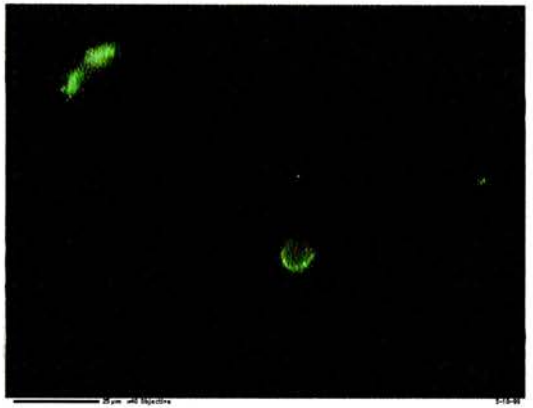
Detection of translocation of phosphatidylserine to the surface of cell membranes with Annexin V staining of fresh granulosa cells specifically detects apoptotic cells. Phosphatidylserine is located on the inner leaflet of the cell plasma membrane in normal cells. In apoptotic cells the distribution of phospholipids is changed and they also appear on the outside of the plasma membrane. Simultaneous staining with propidium iodide, which crosses the damaged plasma membrane of necrotic cells, detects necrosis or pyknosis. Staining granulosa cells with Hoechst 33258 visualises the DNA in non-viable cells, but not in viable cells by becoming highly fluorescent after binding to DNA. This is a way of morphologically evaluating the nuclei for apoptosis.

Live, healthy granulosa cells were not stained at all. Some cells were only stained by Annexin V indicating early apoptosis, and some stained up with all the stains as a sign of the plasma membrane being damaged as in necrosis or advanced apoptosis. The Hoechst stain was much better than propidium iodide when evaluating the shape of the nucleus (stronger fluorescence), and therefore was useful in detection of apoptotic fragments at later stages of apoptosis. In one follicle, morphologically healthy granulosa cells as seen by stereo microscopy all showed staining of both the cell membranes and nuclei after centrifugation to remove superfluous flush medium. The centrifugation obviously damaged the cell membrane. This technique necessitates gentle treatment of the cells so as to not damage the cell membrane: gentle scraping of the follicular wall, as little handling as possible, holding in medium at 37 °C, and evaluation under fluorescence microscope within a short time after retrieval of the cells. The major advantage of using Annexin V is that presence or absence of apoptosis in the granulosa cells is revealed immediately. This will be of importance when collecting oocytes for maturation. More work needs to be done to correlate findings of Annexin V staining with ethidium bromide staining and state of the oocyte before it can be used to give information about the time needed for the oocyte to mature. Also Hoechst

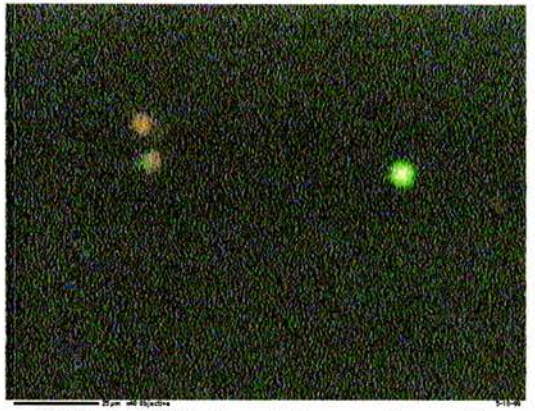
33258 should be investigated further as it can distinguish between live and dead follicles, and it will stain up the chromatin very well, allowing for assessment of presence of apoptosis, although maybe not the earliest form. Hoechst 33258 is cheap and easy to use.

Ideally the stain will give immediate information about the state of the follicle and indirectly inform about the nuclear morphology of the oocyte, which can then be used to estimate necessary time for the oocyte to be incubated before reaching MII. Hinrichs & Williams (1997) found that oocytes from atretic follicles matured quicker than from non-atretic follicles and therefore immediate and accurate information about the state of the follicle is desirable.

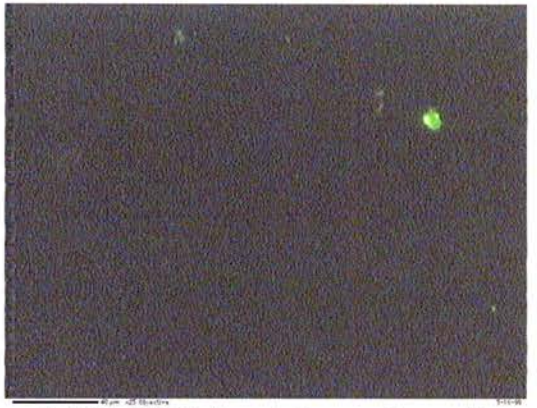
The following pictures show the same granulosa cells stained with Annexin V, propidium iodide (top row) and Hoechst (bottom row).



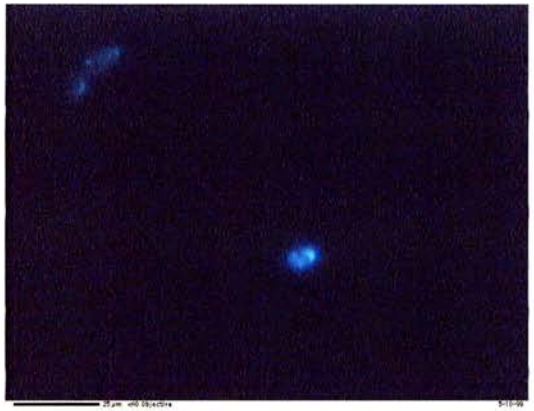
Follicle 959,1: Apoptotic granulosa cell stained by Annexin V.



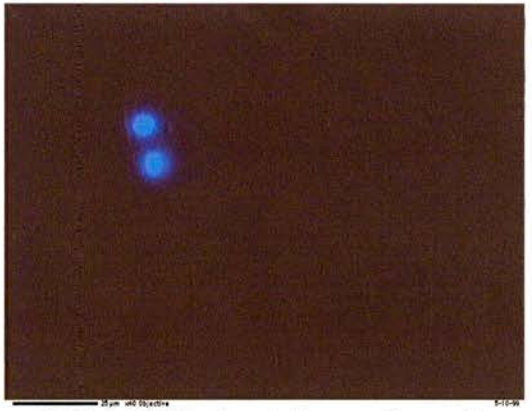
Follicle 959,2: Apoptotic granulosa cells stained by Annexin V. The two cells to the left are also stained by propidium iodide.



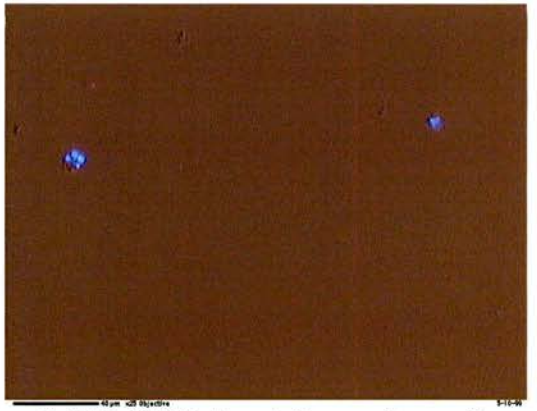
Follicle 959,3: Apoptotic granulosa cells stained by Annexin V.



Follicle 959,1: Apoptotic granulosa cell stained by Hoechst. Note fragmentation of the nucleus.



Follicle 959,2: Apoptotic granulosa cells stained by Hoechst. Note the apoptotic cell to the right was not stained by Hoechst.

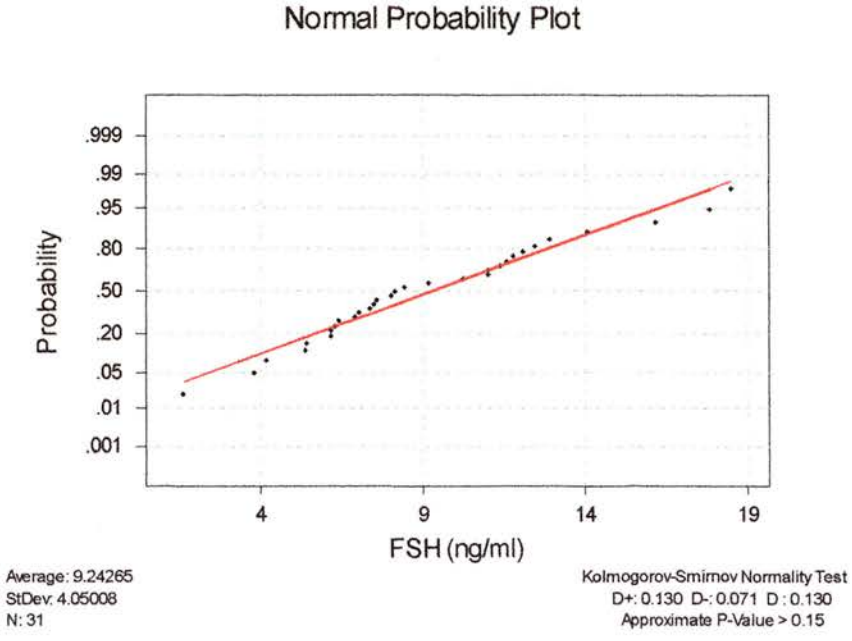


Follicle 959,3: Apoptotic granulosa cells stained by Hoechst. The fragmentated cell to the left was not stained by Annexin V.

8.3 APPENDIX C

8.3.1 Normality test

Statistical tests based on the parametric methods require that the data is normally distributed.



Test for normality with a Kolmogorov-Smirnov normality test. The individual hormone data are plotted as the x-values and the calculated probabilities are the y-values plotted on a log scale. The least-squares line is an estimate of the cumulative distribution function for the population, and the plotted data should fit well around the line if the data are normally distributed. A P-value indicates if the null hypothesis (H_0: the data follow a normal distribution) should be accepted or rejected. In the example, FSH data are plotted evenly around the least-squares line, the data apparently follow a normal distribution. The null-hypothesis is accepted at the 95% level.

8.4 APPENDIX D

8.4.1 Follicle maps

Mares were scanned daily to create follicle maps. Figure 1 shows pictures of scans with different ovarian characteristics. Follicle maps were drawn for each day. Figure 2 shows a follicle map of raw data from one pony's full cycle. Figure 3 demonstrates the principle of the procedure of grouping the follicles (see Chapter 4 for a detailed description). Figure 4 and 5 show the same follicle map before and after grouping of the follicles, respectively.

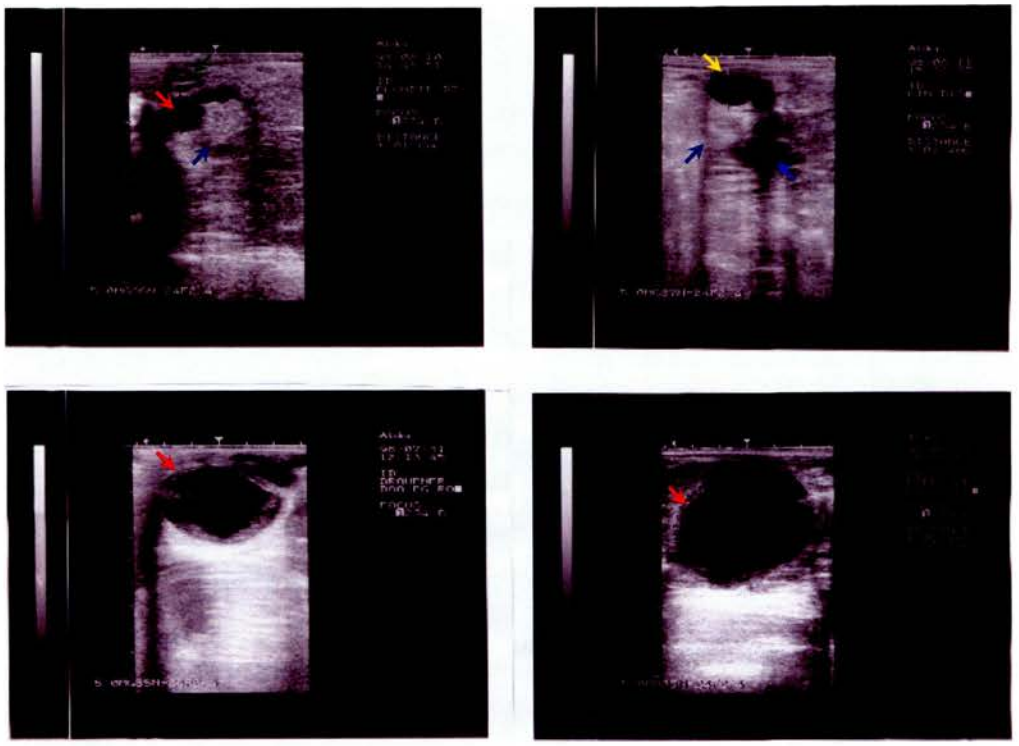


Figure 1. Scanning pictures of follicles. Clockwise from top left corner: Ovary day 7 after ovulation. A 16 mm follicle (→), 4 mm follicles (→) and a corpus luteum (→). Top right corner: Day 13 after ovulation. 14 (→) and 12 mm (→) follicles. A corpus luteum (→) is present underneath the 14 mm follicle. Bottom left corner: Day 8 after prostaglandin. 40x30 mm follicle (→) with a rim of granulosa cells. Bottom right corner: 54 mm preovulatory follicle (→) with a thick rim of granulosa cells.

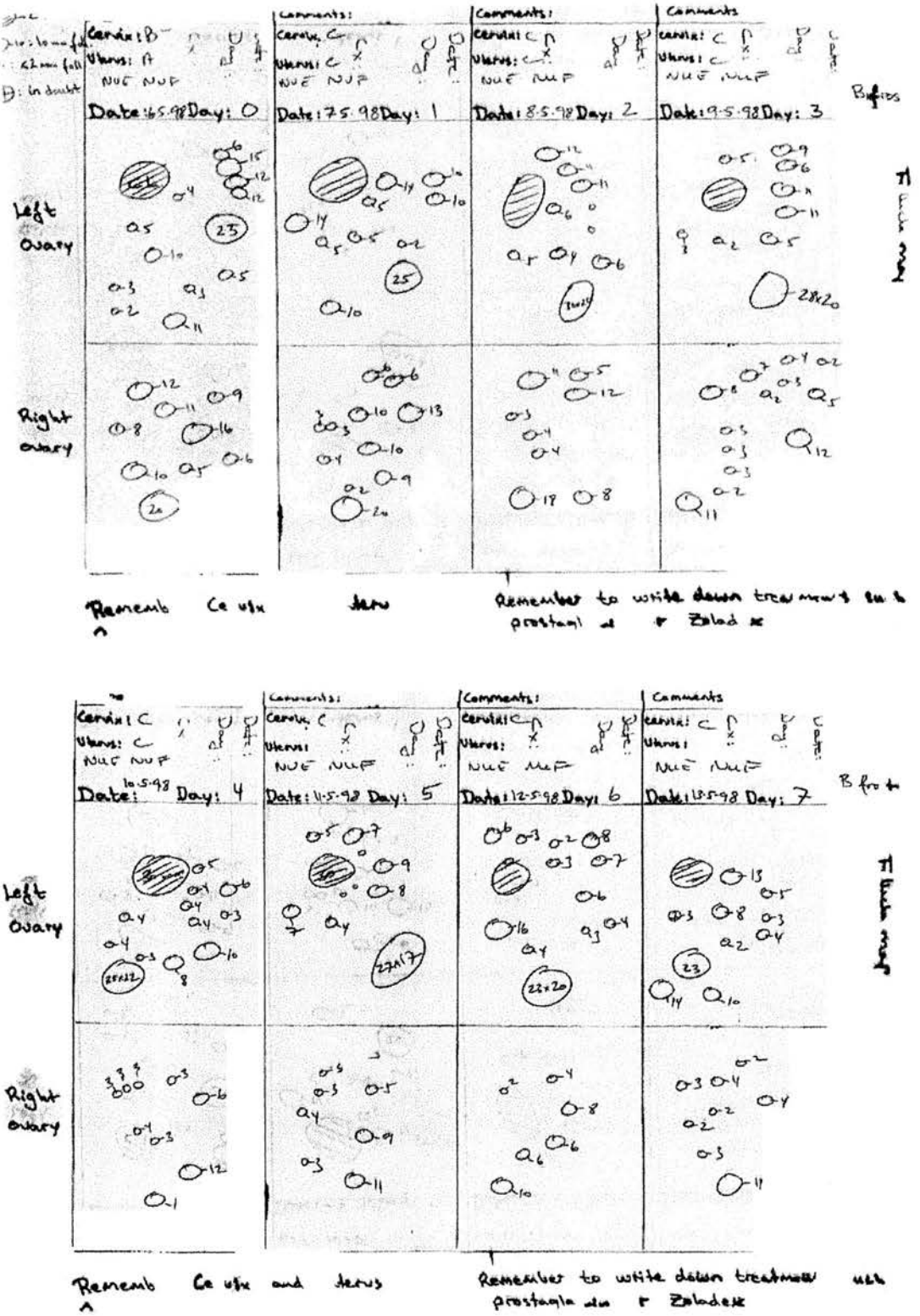


Figure 2a. Follicle map. Circle = follicle, hatched circle = corpus luteum.

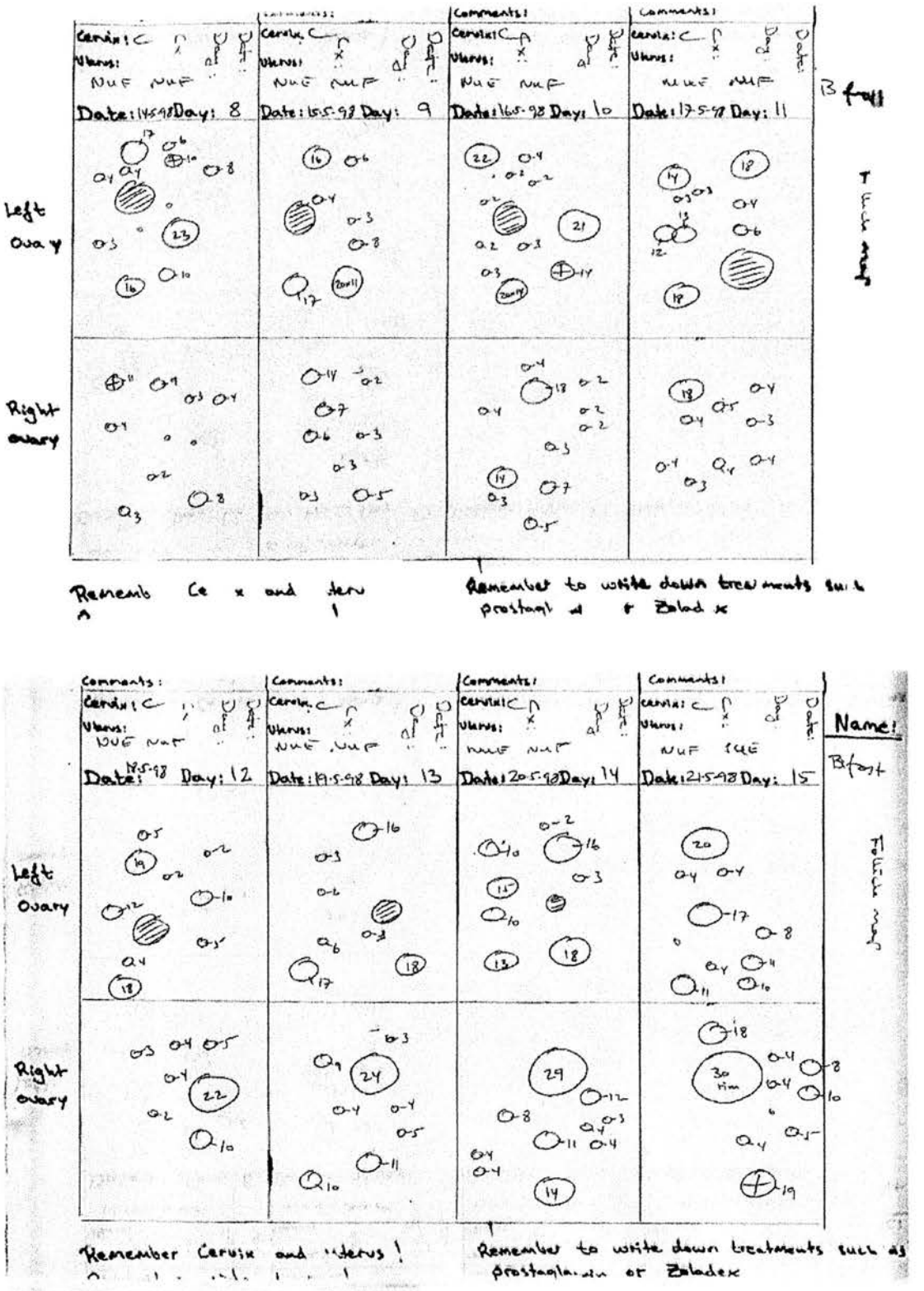


Figure 2b. Follicle map. Circle = follicle, hatched circle = corpus luteum.

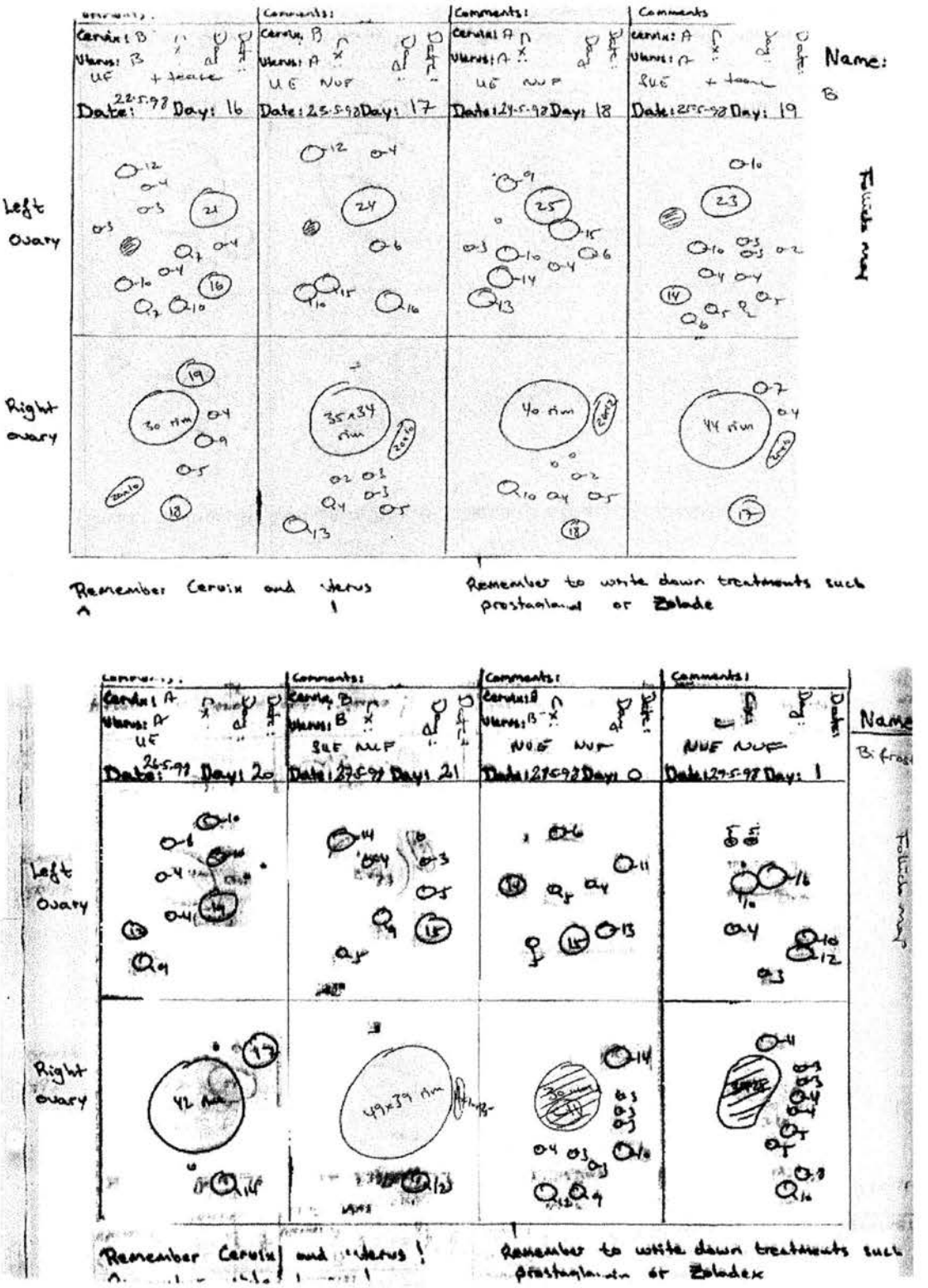


Figure 2c. Follicle map. Circle = follicle, hatched circle = corpus luteum.

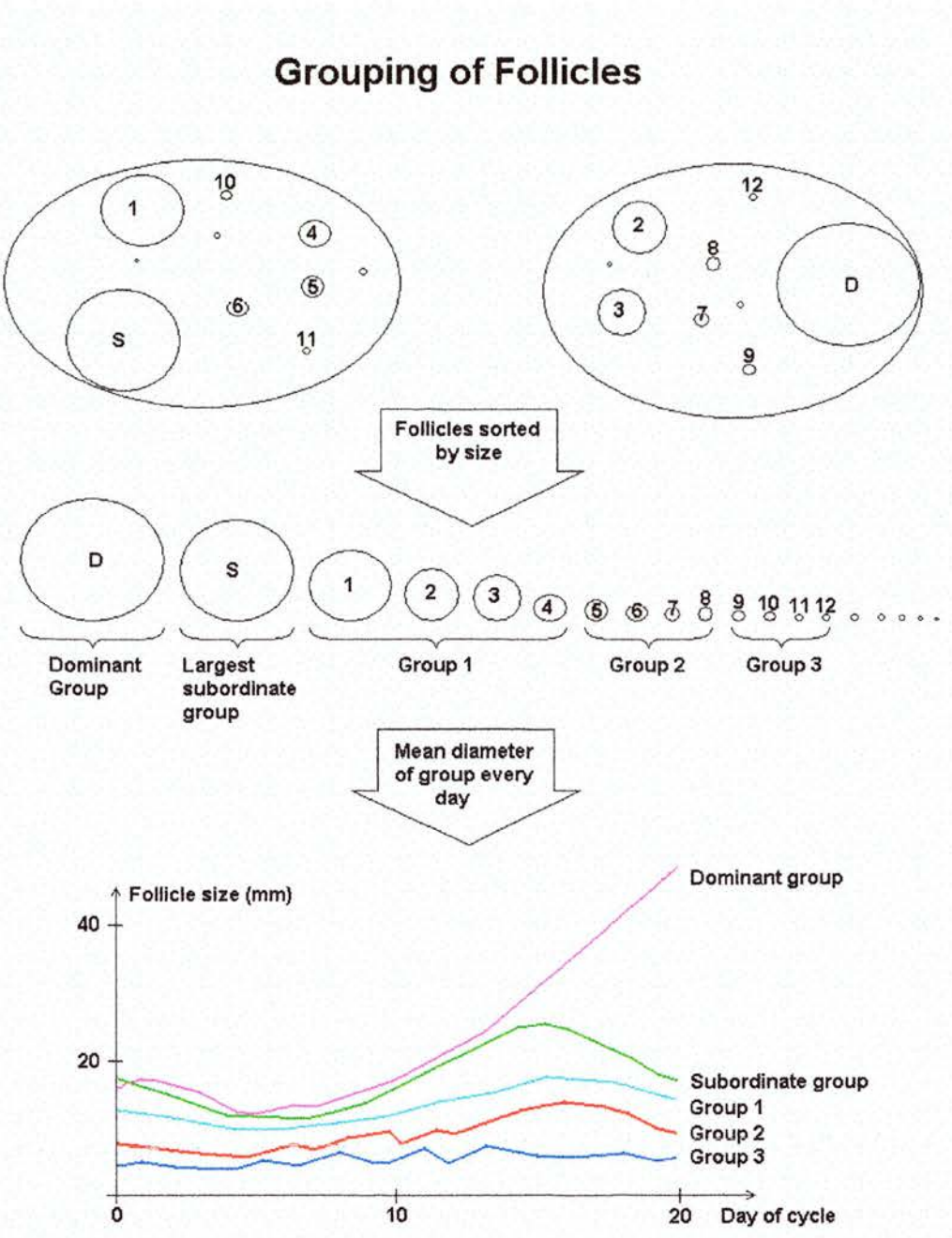


Figure 3

The dominant and the largest subordinate follicle were kept separate each day. The remaining follicles were combined for both ovaries and ranked from largest to smallest without regard to day to day identity of the individual follicles. The ranked follicles were divided into groups with the 4 largest follicles in group 1, the 4 next largest in group 2 until all follicles had been designated. The results from each day were plotted in a graph and depicted the waves of growth during the cycle.

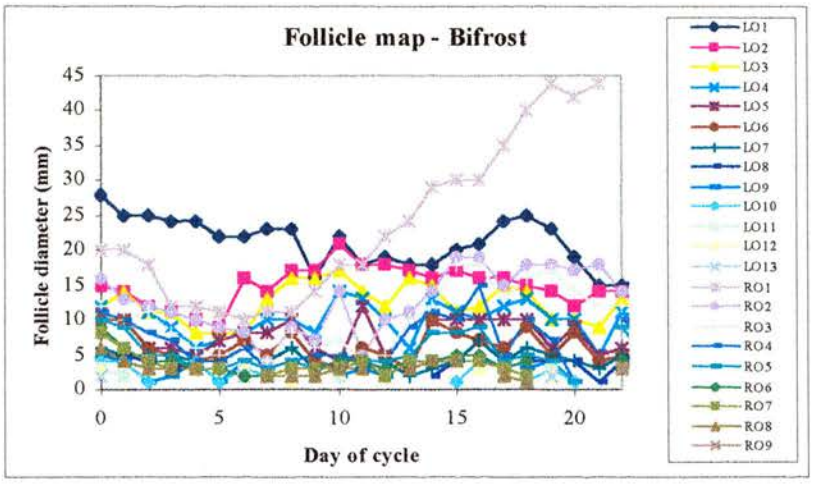


Figure 4. Data from follicle map. Follicle sizes were ranked from largest to smallest on each ovary for each day and plotted against time. No grouping of follicles was done in this graph.

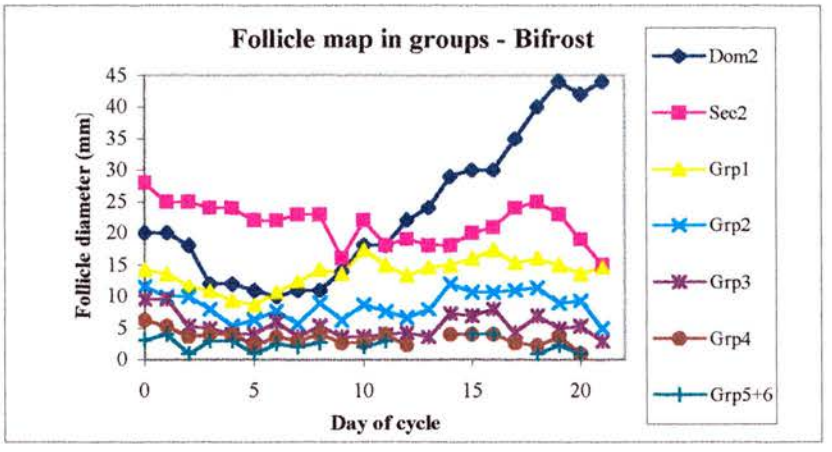


Figure 5. Data from follicle map after grouping the follicles. The dominant and the largest subordinate follicles are individual follicles. The remaining follicles were ranked from largest to smallest, divided into groups with 3 follicles in each and a mean was obtained. Each group's mean was plotted against time.

8.5 APPENDIX E

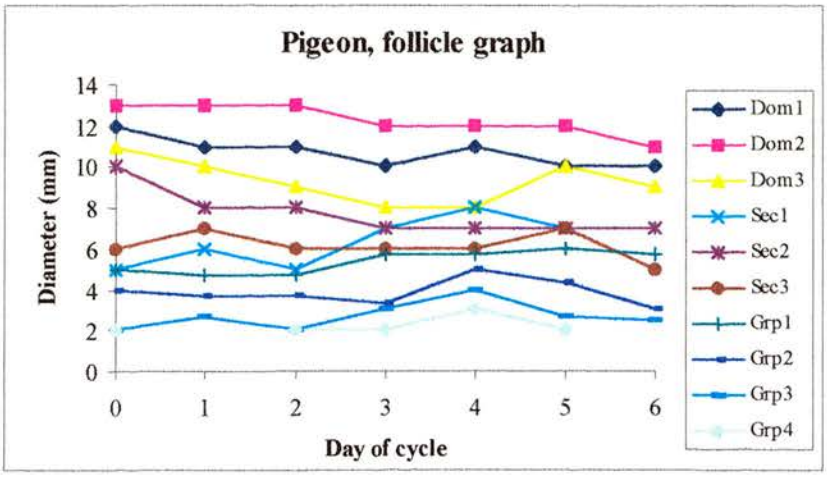
8.5.1 Follicle graph and follicle map for each mare on day 6, 11 and 15

The present appendix contains the follicle graph and the follicle map with colouring of the dominant, growing, intermediate, dying and atretic follicles on the day of ovariectomy as well as a commentary and summary table for each individual animal from days 6, 11 and 15.

Health status of the follicles at different days was determined based on granulosa cell apoptosis, oocyte chromatin configuration, cumulus morphology and follicular fluid steroid content in relation to the growth or regression of the follicle as detected with the use of follicle maps and in connection with determination of gonadotrophin levels as described in chapters 3, 4 and 5. When deciding when a follicle was dominant, growing, intermediate (parameters contradicting each other), dying or atretic, most emphasis was put on presence of apoptosis and the appearance of the granulosa cells. Next came level of oestradiol and DNA content. These two parameters were reliable, but as both went through dilution steps when prepared, there was a risk of introducing error, and therefore they were less reliable than the presence of apoptosis and granulosa cell appearance, although these two parameters were not definite parameters of life and death either. The next parameters were progesterone concentration, cumulus appearance and follicular wall colour. Oocyte chromatin configuration was useful in the context of a non-dominant follicle showing meiotic changes, which was usually indicative of onset of atresia. No single parameter could be used on its own to determine life or death, but with the knowledge of the overall tendency of each parameter, used in conjunction they indicated the health status of a follicle.

The follicle graphs showed the follicles of any one day ranked from largest to smallest. The follicle maps of the last 4 days before ovariectomy allowed for detection of growing or regressing follicles. This appendix contains the follicle graph and the follicle map with colouring of the dominant (red), growing (orange), intermediate (green), dying (blue) and atretic (grey) follicles on the day of ovariectomy for each mare. The data are summarised in a table for each mare. The follicle graph shows whether a follicle was growing at the time of ovariectomy, and the map informs about the morphological and biochemical status. The follicle graph shows the six largest follicles individually, and follicles smaller than these were grouped together as described in chapter 5.

8.5.1.1 Day 6



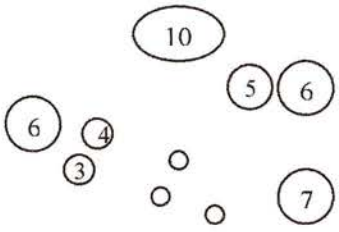
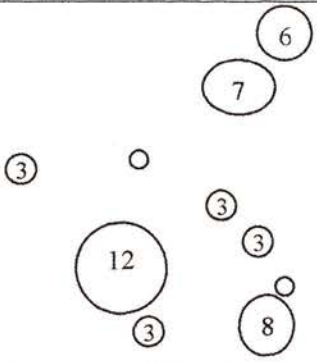
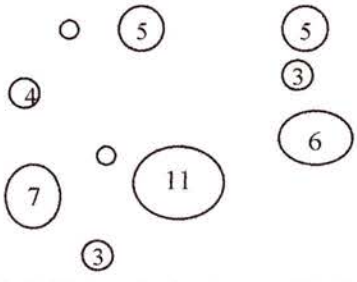
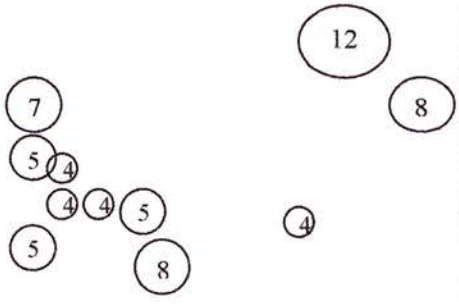
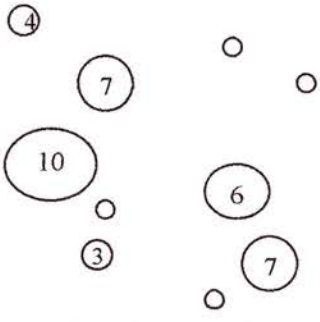
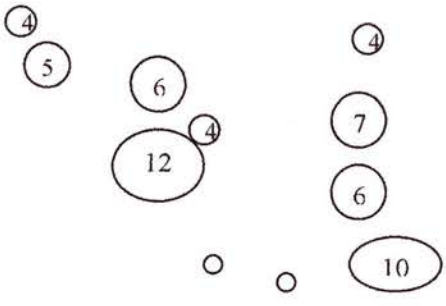
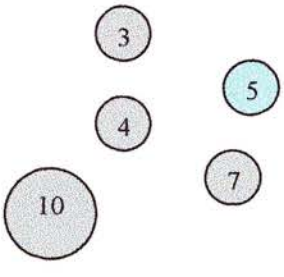
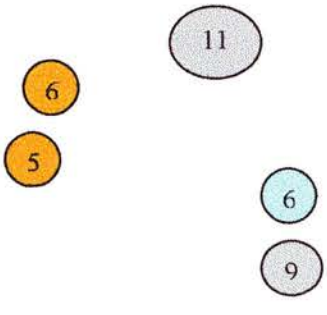
8.5.1.1.1 Pigeon

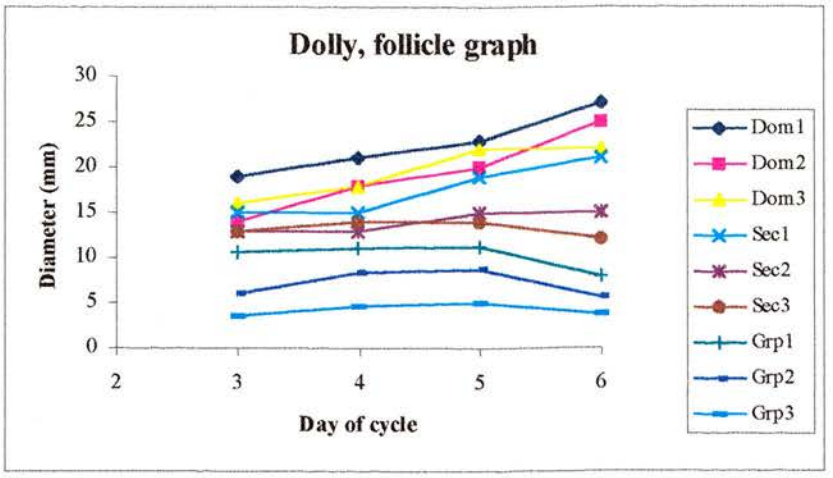
Follicle maps: follicles were regressing or static.

Follicle parameter interpretation: Two 4-5 mm follicles were growing. The rest were atretic or dying. The two growing follicles may have been part of a new wave starting around this time.

Table 8.1. Pigeon

	Growing	Intermediate	Dying	Atretic	Total
n	2	0	2	6	10
%	20	0	20	60	100

Pigeon	Left ovary	Right ovary
Day 3		
Day 4		
Day 5		
Day 6		



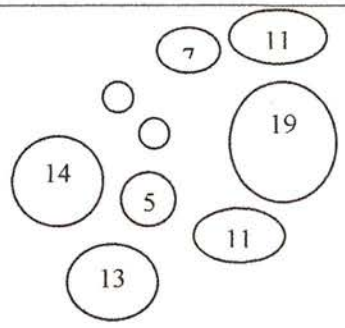
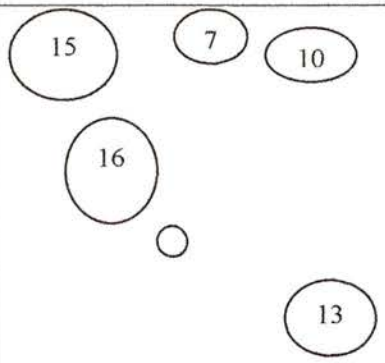
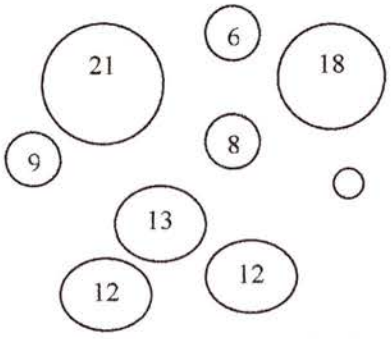
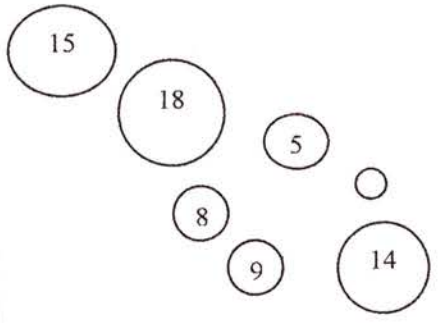
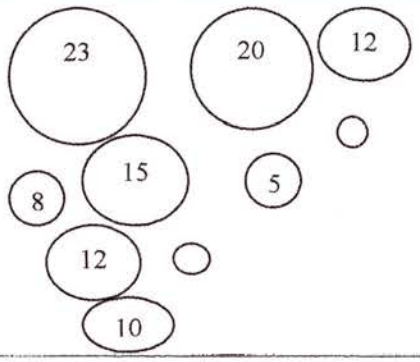
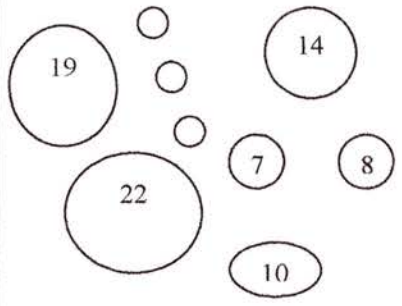
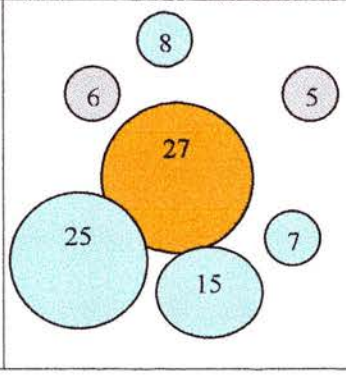
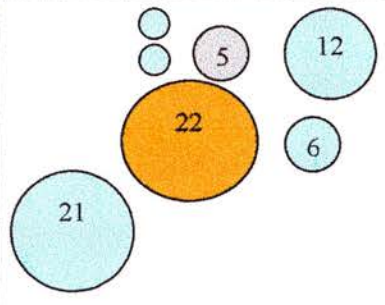
8.5.1.1.2 Dolly

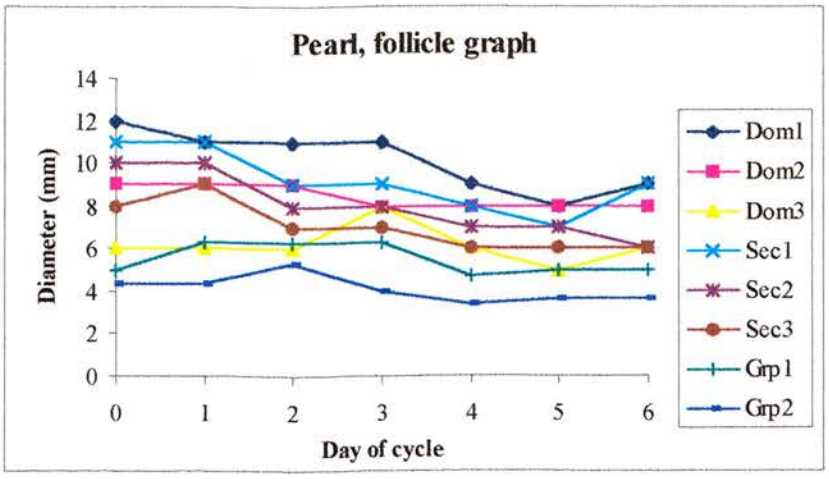
Follicle maps: the largest follicle on each ovary was growing, the remainder static or atretic.

Follicle parameter interpretation: The two largest follicles were growing. The rest were dying or atretic. It is rather puzzling why there were two large and presumably growing follicles at this stage.

Table 8.2. Dolly

	Growing	Intermediate	Dying	Atretic	Total
n	2	0	8	3	13
%	15.38	0	61.5	23.1	100

Dolly	Left ovary	Right ovary
Day 3		
Day 4		
Day 5		
Day 6		



8.5.1.1.3 Pearl

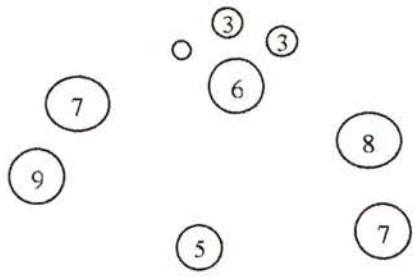
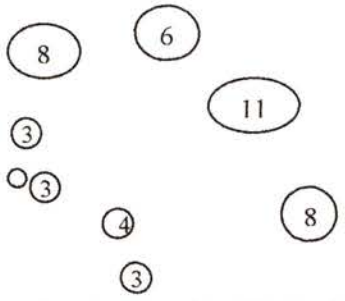
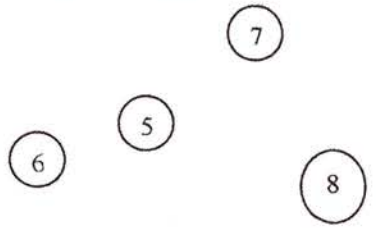
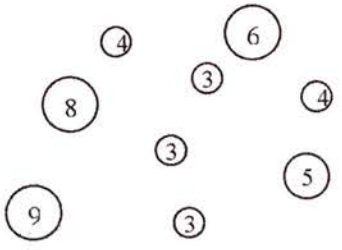
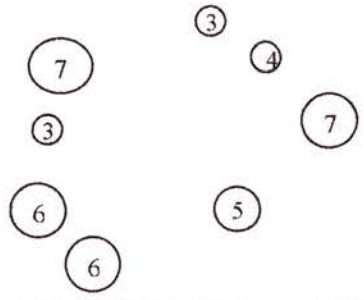
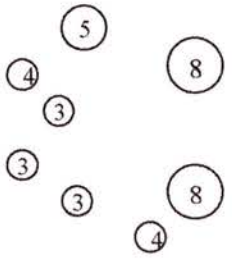
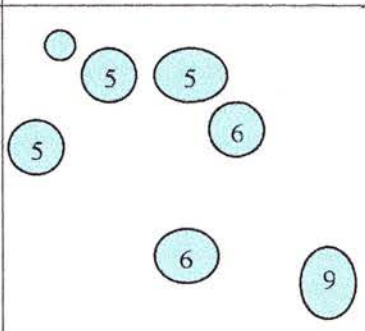
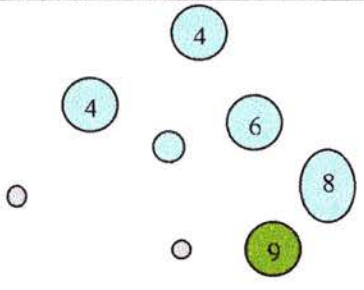
Follicle maps: follicles were static or regressing perhaps apart from one follicle, which appeared to have increased in size from the previous day from no larger than 7 mm to 9 mm.

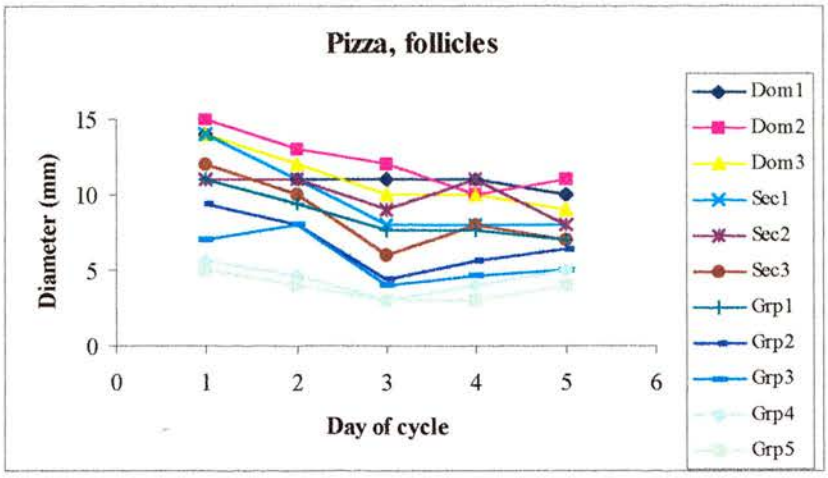
Follicle parameter interpretation: All follicles are undergoing atresia, perhaps bar one.

There were no waves of follicles underway yet, unless some of the small follicles undergoing apoptosis could actually recover. Three follicles had large DNA content despite the apoptosis.

Table 8.3. Pearl

	Growing	Intermediate	Dying	Atretic	Total
n	0	1	12	2	15
%	0	6.7	80	13.3	100

Pearl	Left ovary	Right ovary
Day 3		
Day 4		
Day 5		
Day 6		



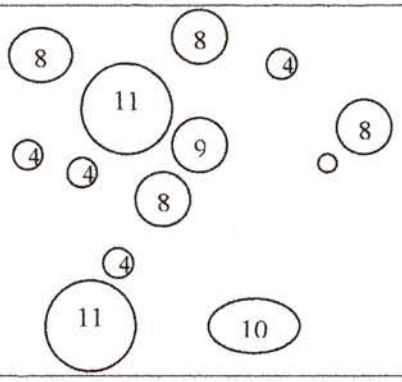
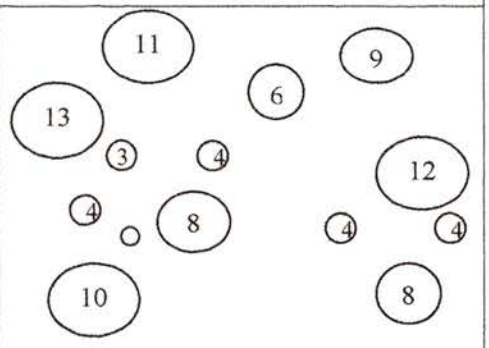
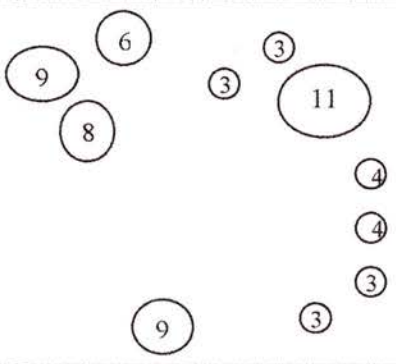
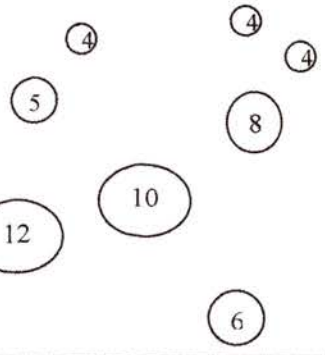
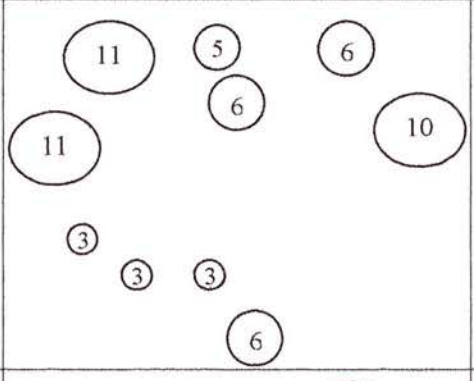
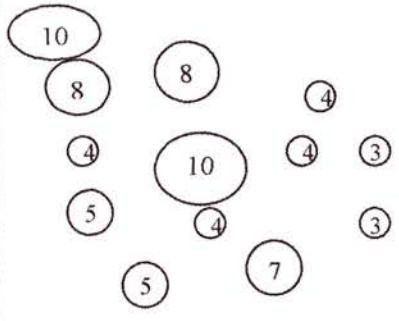
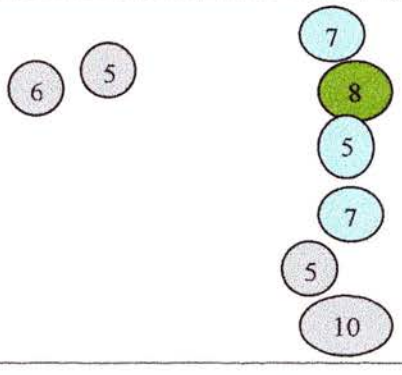
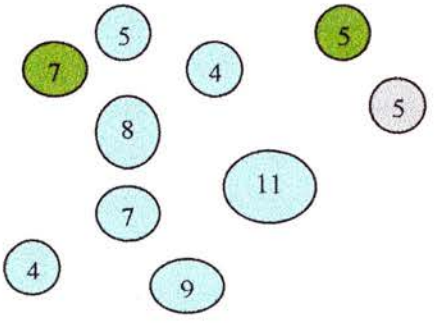
8.5.1.1.4 Pizza

Follicle maps: Most follicles were either regressing or static, but a group of 4-6 mm follicles appeared to increase slightly in size.

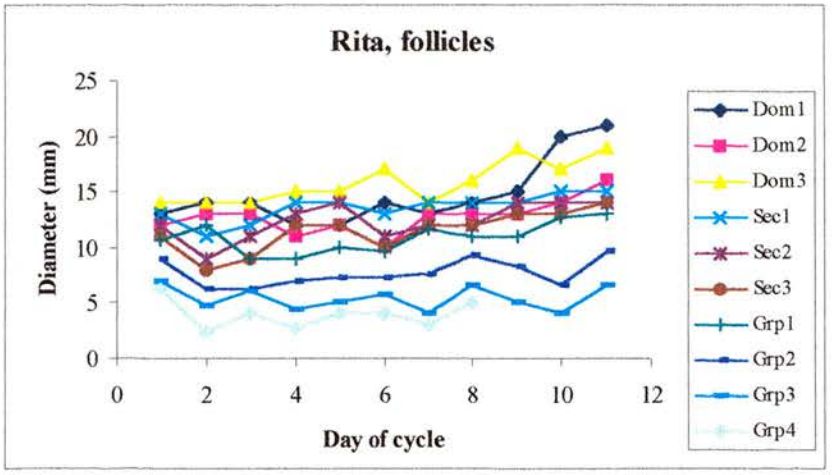
Follicle parameter interpretation: follicles were dying, and there was not yet any sign of a new wave of growth unless two of the larger, apoptotic follicles could recover from the apoptosis.

Table 8.4. Pizza

	Growing	Intermediate	Dying	Atretic	Total
n	0	3	9	6	18
%	0	16.7	50	33.3	100

Pizza	Left ovary	Right ovary
Day 2		
Day 3		
Day 4		
Day 5		

8.5.1.2 Day 11



8.5.1.2.1 Rita

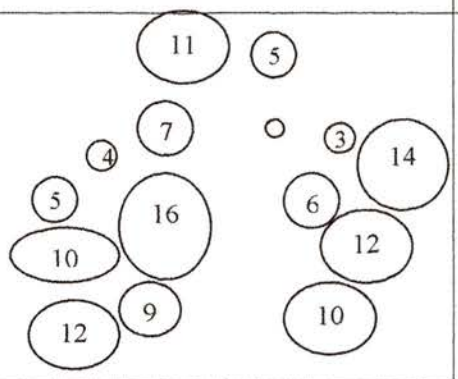
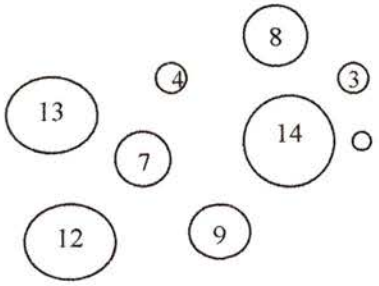
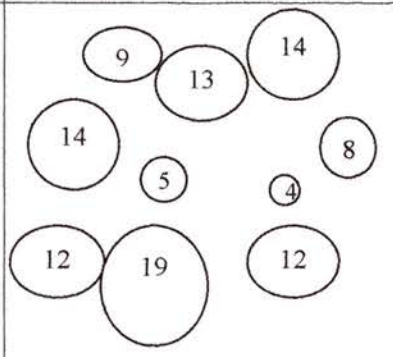
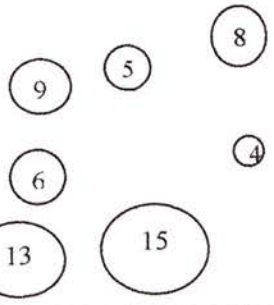
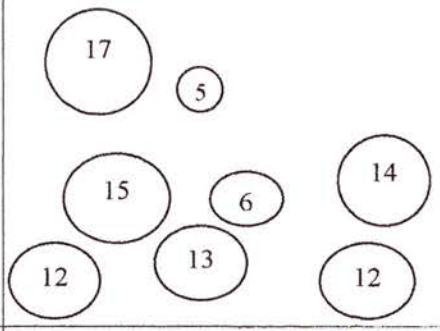
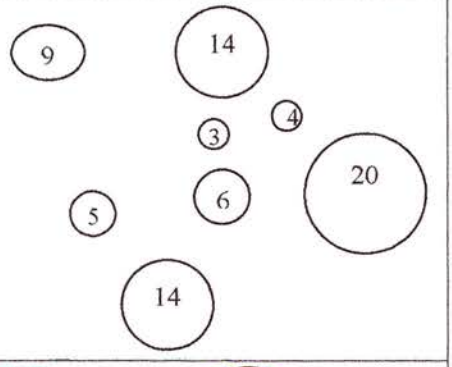
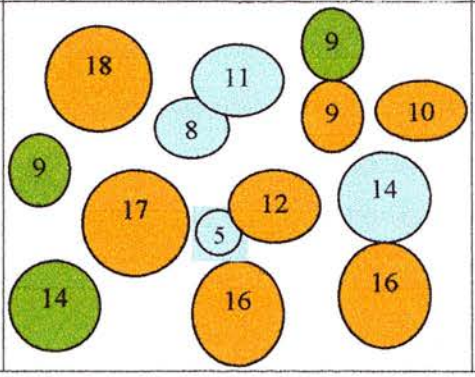
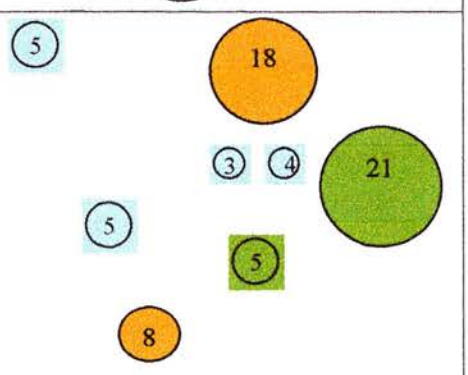
Follicle map: most follicles appeared to increase in size, although there were some static follicles. One follicle was larger than the rest and seemed to grow at a faster pace.

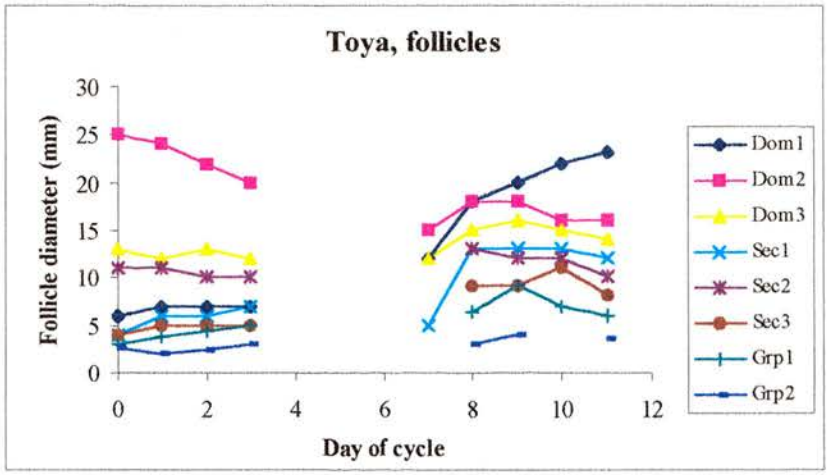
Follicle parameter interpretation: the largest follicle was apoptotic, but all other parameters indicated growth.

The majority of follicles appeared to be in good health. Some were dying, but none were overtly atretic.

Table 8.5. Rita

	Growing	Intermediate	Dying	Atretic	Total
n	9	5	8	0	22
%	40.9	22.7	36.4	0.0	100

Rita	Left ovary	Right ovary
Day 8		
Day 9		
Day 10		
Day 11		



Data were missing due to a problem with the video recorder.

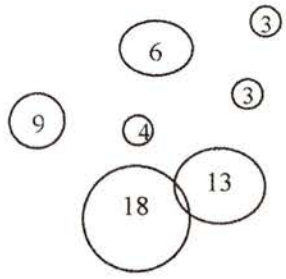
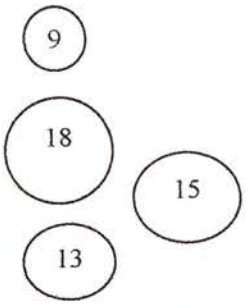
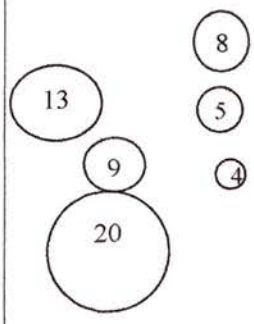
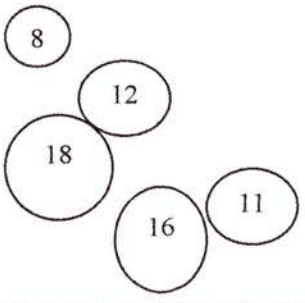
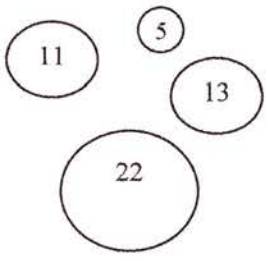
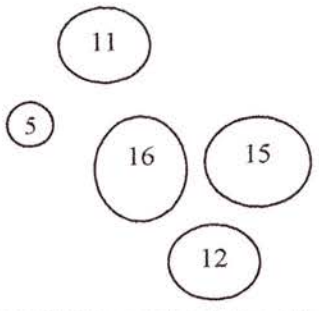
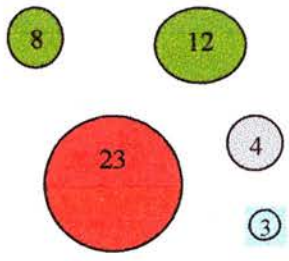
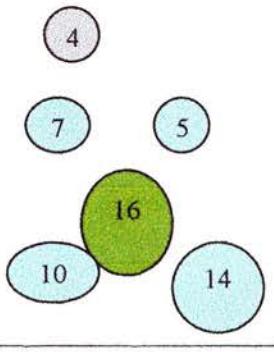
8.5.1.2.2 Toya

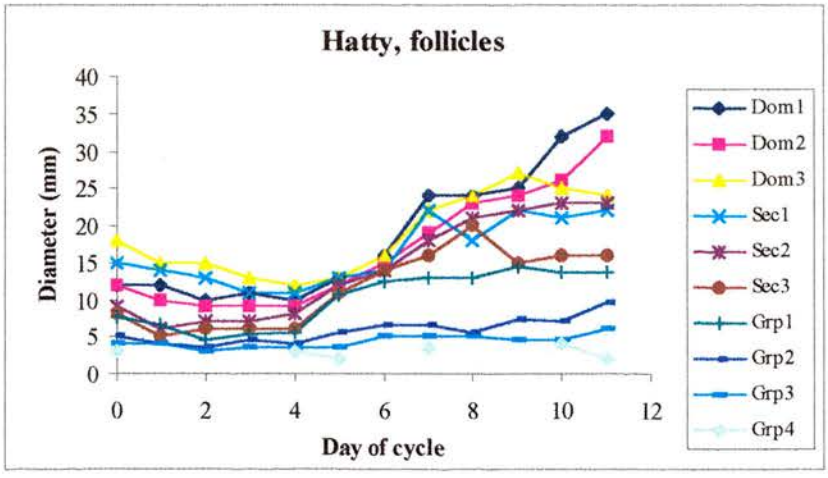
Follicle map: the largest follicle increased in size and had a size advantage from day 9. From day 10 it appeared to be the only follicle increasing in size whereas the others decreased or were static. This follicle was 12 mm on day 7 and grew to 23 mm on day 11. The follicle closest in size appeared to have been static for a few days.

Follicle parameter interpretation: One follicle appeared to be dominant although it did contain apoptotic cells. Another follicle was close in size, and was classified as intermediate with apoptosis as the only sign of pending death. This would correspond with this follicle not having grown during the previous days and possibly facing death. Most follicles were too small to compete for dominance, and some looked reasonably healthy still, despite signs of apoptosis, but the majority were dying or were atretic.

Table 8.6. Toya

	Growing	Intermediate	Dying	Atretic	Total
n	1	3	5	2	11
%	9.1	27.3	45.5	18.2	100

Toya	Left ovary	Right ovary
Day 8		
Day 9		
Day 10		
Day 11		



8.5.1.2.3 Hatty

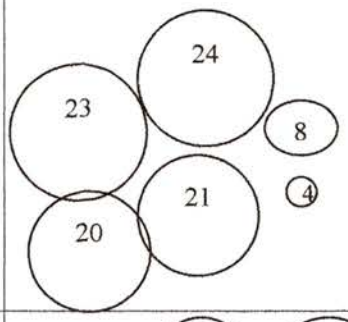
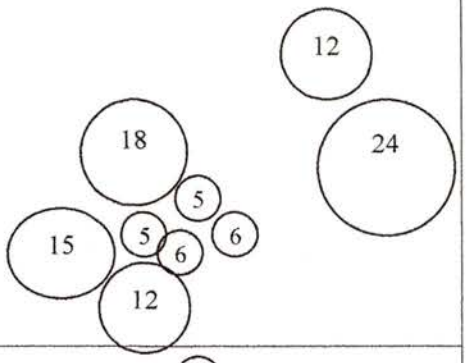
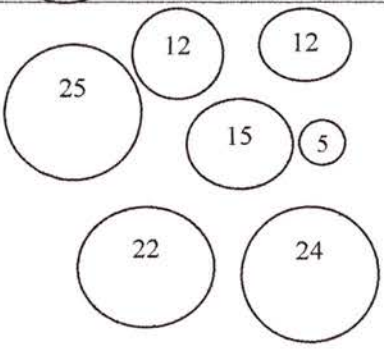
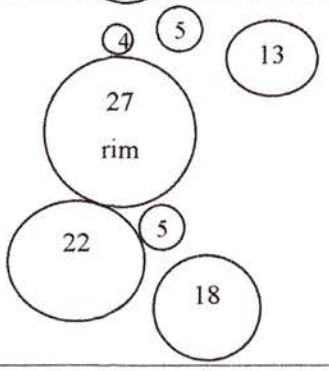
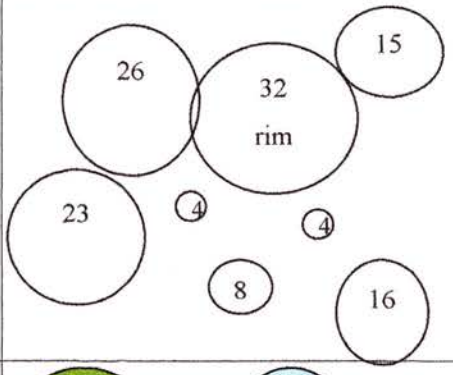
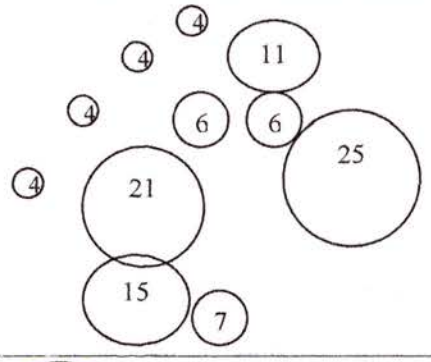
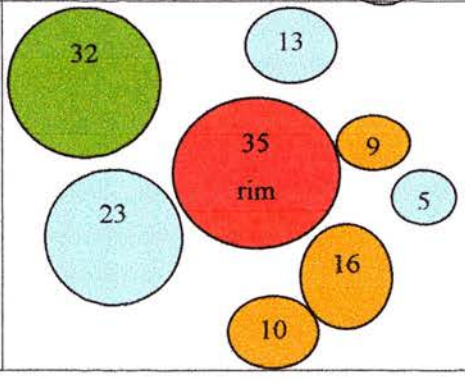
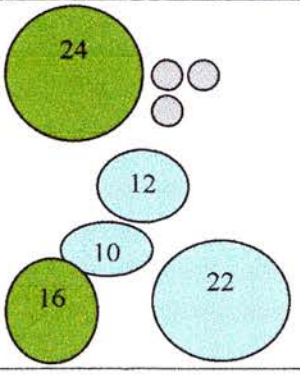
Follicle map: Two follicles were growing quickly, most follicles increased slowly, and a few appeared to be static or regressing slowly.

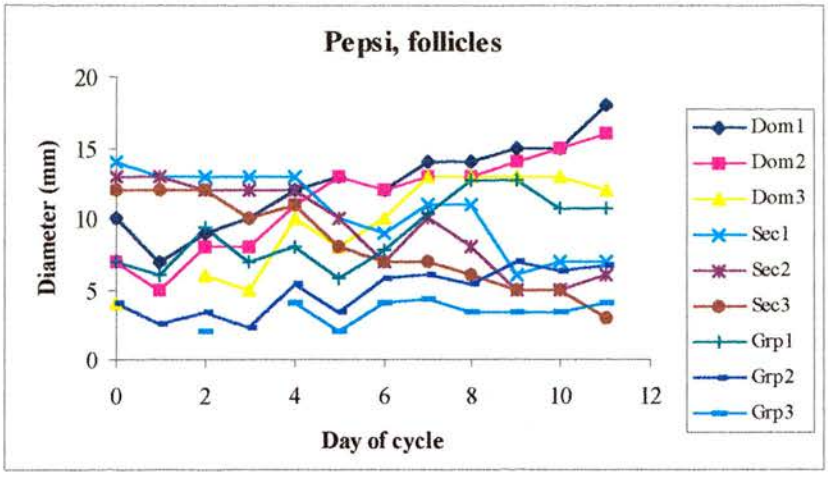
Follicle parameter interpretation: the largest follicle was active, but the second largest follicle, which was also growing, contained less oestradiol than would be expected and was classified as intermediate. This could indicate that the largest follicle had obtained dominance. The other smaller follicles appeared to have indicators of decreasing function, particularly decreased oestradiol production and in some cases apoptosis.

It seemed that around this time, the dominant follicle was starting to take over even though the other follicles were not yet dying. Selection had taken place and dominance was being established, but some of the competitors were still viable and growing.

Table 8.7. Hatty

	Growing	Intermediate	Dying	Atretic	Total
n	4	3	6	3	16
%	25.0	18.8	37.5	18.8	100

Hatty	Left ovary	Right ovary
Day 8		
Day 9		
Day 10		
Day 11		



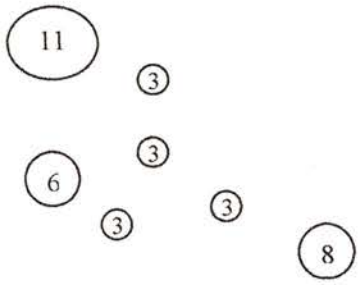
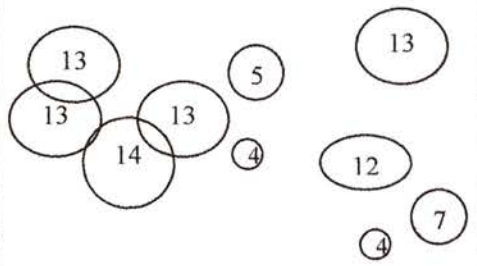
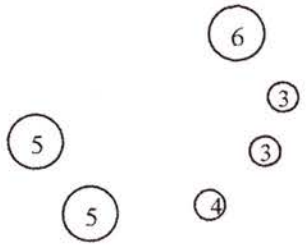
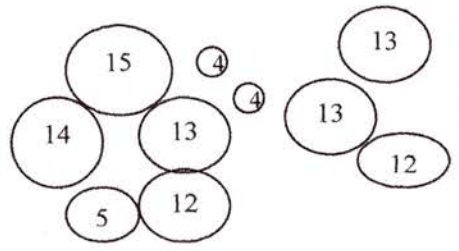
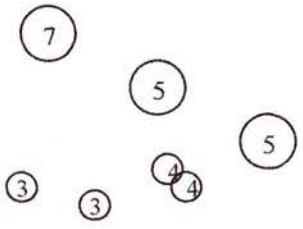
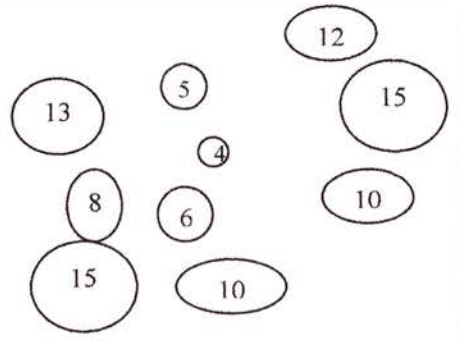
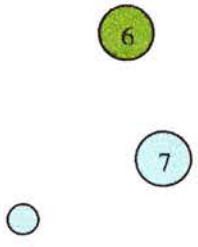
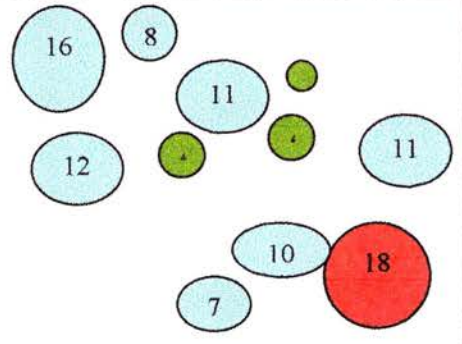
8.5.1.2.4 Pepsi

Follicle map: Two follicles were growing, and the remaining follicles appeared to be static or regressing.

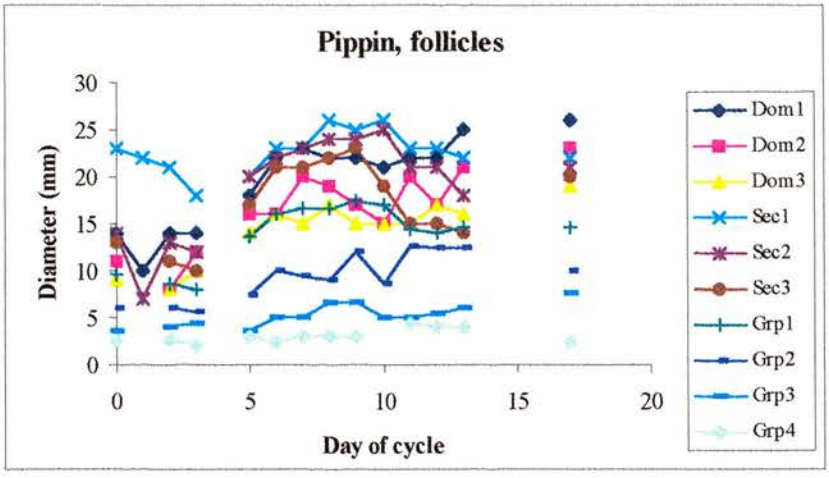
Follicle parameter interpretation: No follicle appeared to be dominant, but perhaps the most likely contender for dominance was the largest follicle, despite the apoptosis and reasonably low oestradiol concentration. The follicles closest in size appeared to be dying, and so was the follicle, which on the follicle map seemed to have increased in size. No follicle was yet atretic, so most seemed to just have started to die, which was in agreement with the follicle map.

Table 8.8. Pepsi

	Growing	Intermediate	Dying	Atretic	Total
n	1	4	9	0	14
%	7.1	28.6	64.3	0.0	100

Pepsi	Left ovary	Right ovary
Day 8		
Day 9		
Day 10		
Day 11		

8.5.1.3 Day 15



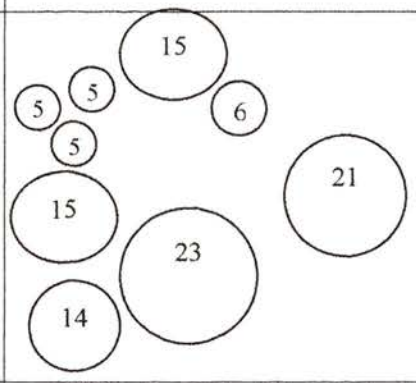
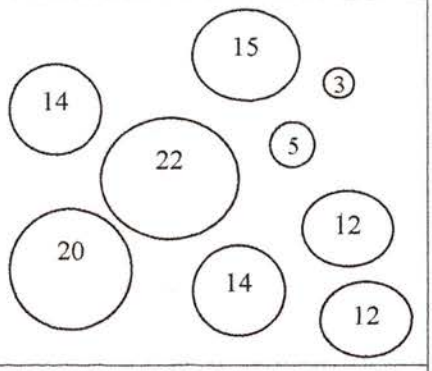
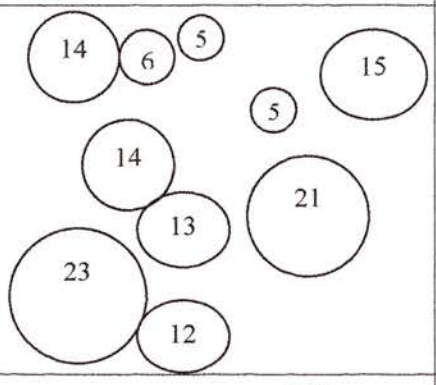
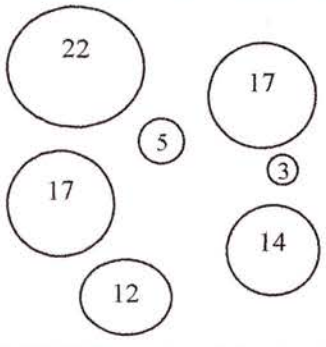
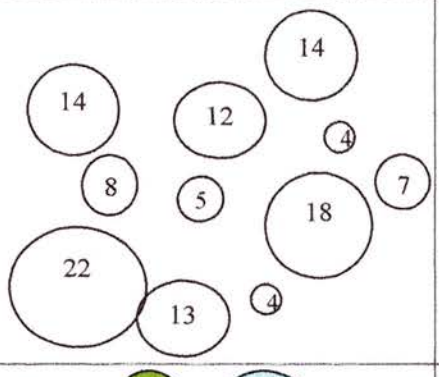
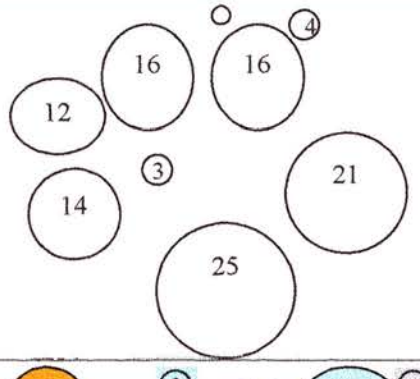
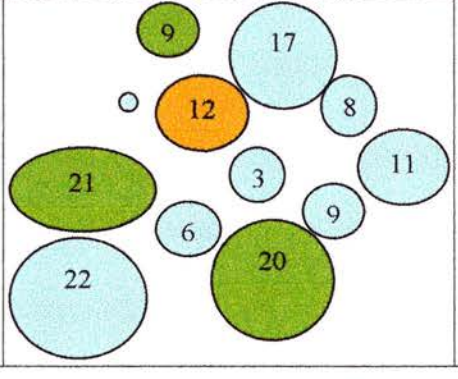
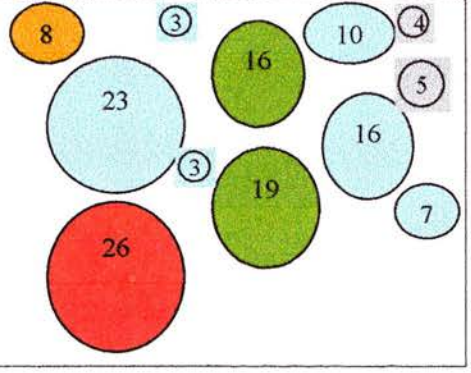
8.5.1.3.1 Pippin

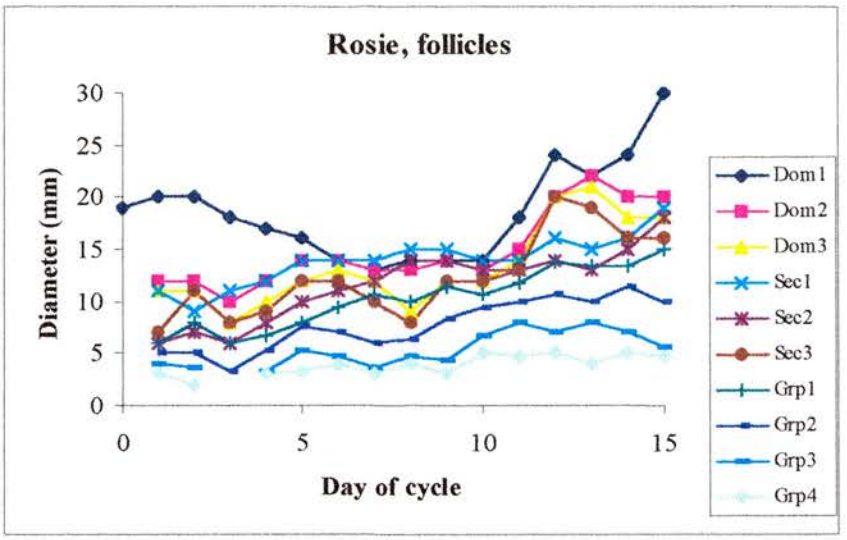
Follicle map: some follicles seemed to be growing and some were regressing.

Follicle parameter interpretation: The largest follicle most likely had been selected, as this was the only follicle of any considerable size, which is growing. All follicles close in size were apoptotic and most likely were in the process of dying.

Table 8.9. Pippin

	Growing	Intermediate	Dying	Atretic	Total
n	3	5	14	2	24
%	12.5	20.8	58.3	8.3	100

Pippin	Left ovary	Right ovary
Day 11		
Day 12		
Day 13		
Day 17		



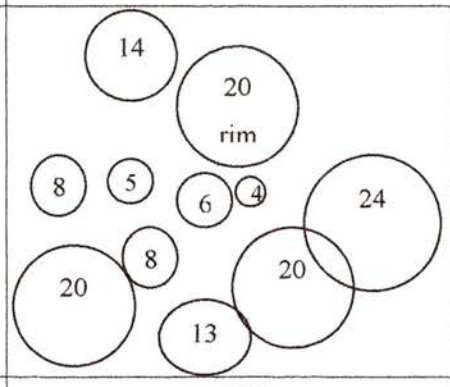
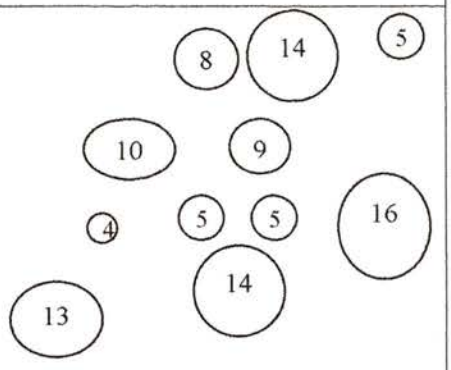
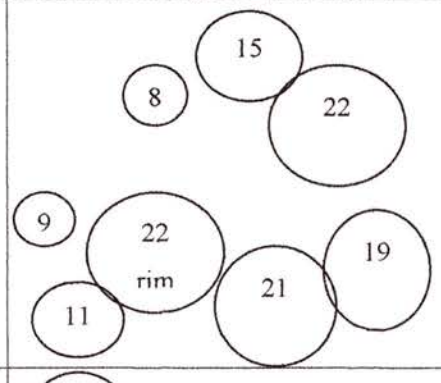
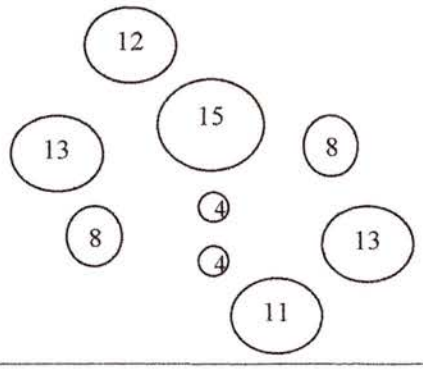
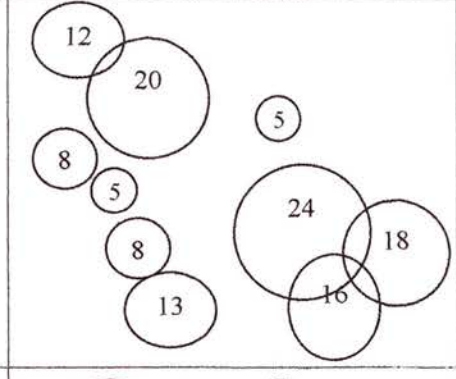
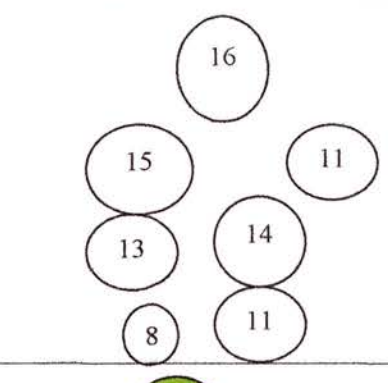
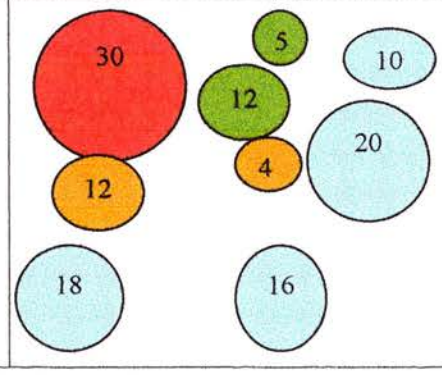
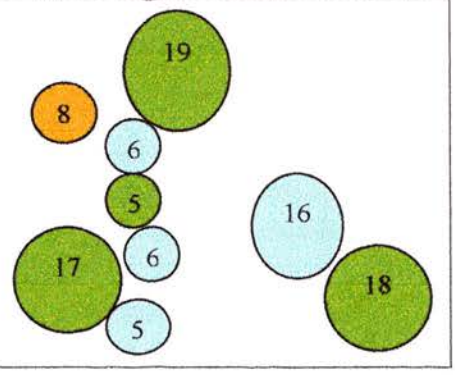
8.5.1.3.2 Rosie

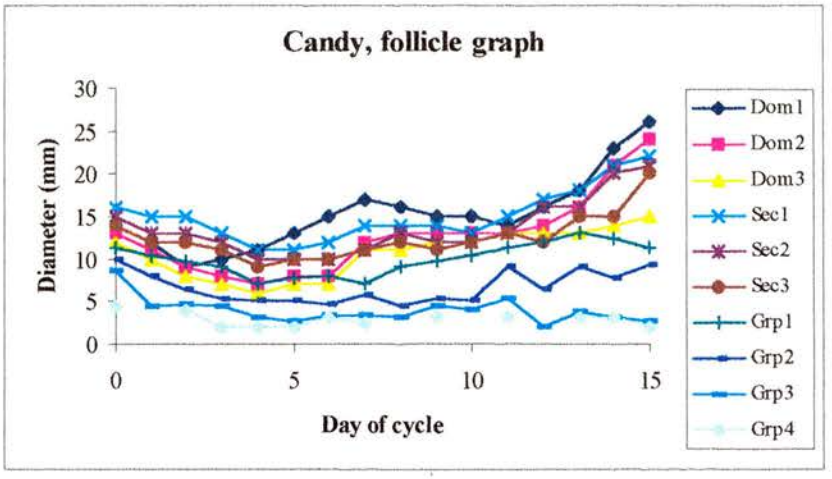
Follicle map: One follicle was growing fast, and follicles, which had been similar in size to the largest follicle (15 – 20 mm) on the previous days were regressing. Some of the smaller (13-18 mm) follicles were growing, whereas the size groups less than 10 mm were static or regressing.

Follicle parameter interpretation: The largest follicle appeared to have been selected for dominance. Of the 15-18 mm follicles, 4 were dying and 3 were intermediate. The intermediate follicles may be the ones that were increasing in size in the follicle map.

Table 8.10. Rosie

	Growing	Intermediate	Dying	Atretic	Total
n	4	6	8	0	18
%	22.2	33.3	44.4	0.0	100

Rosie	Left ovary	Right ovary
Day 12		
Day 13		
Day 14		
Day 15		



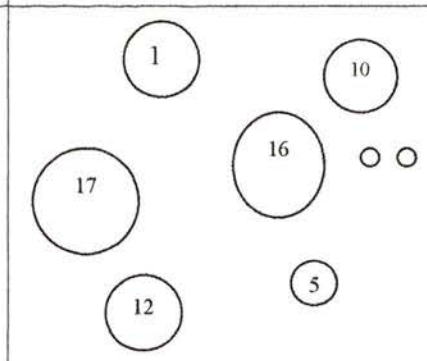
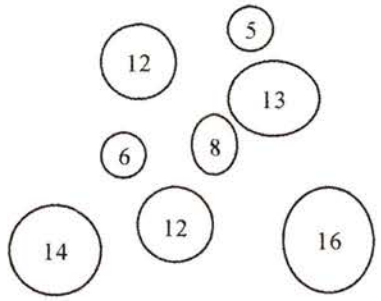
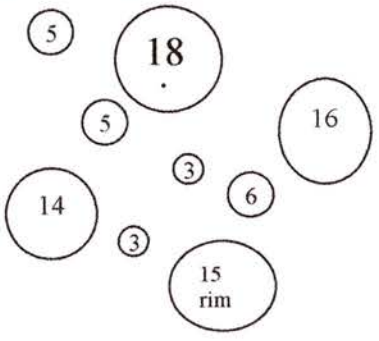
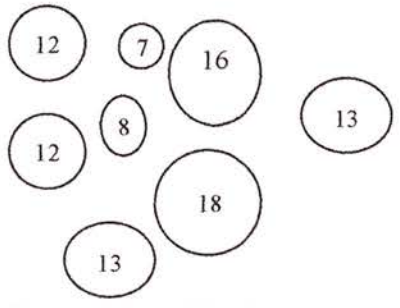
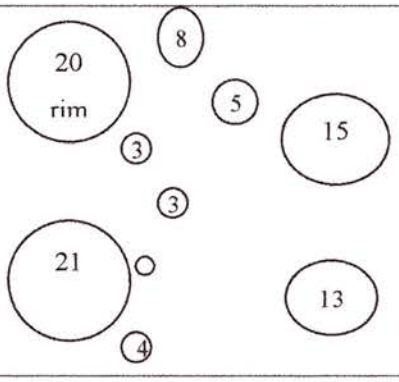
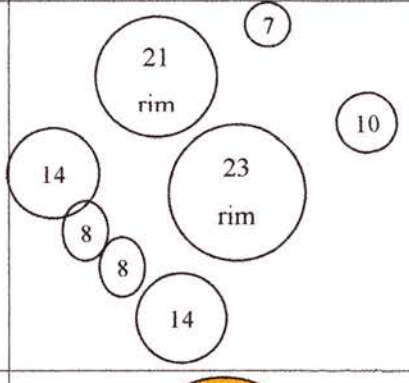
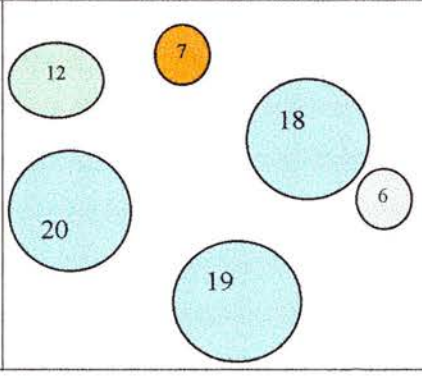
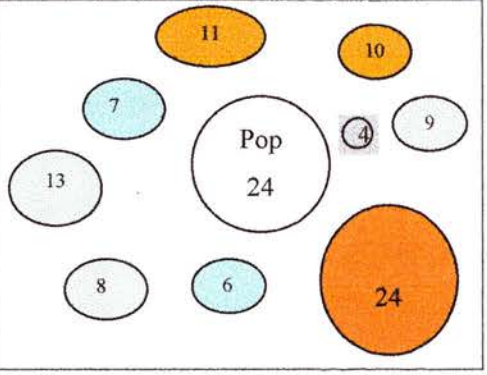
8.5.1.3.3 Candy

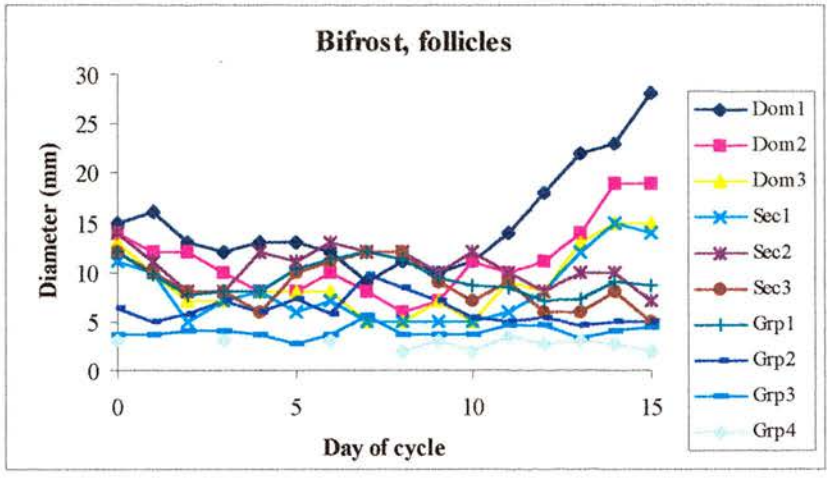
Follicle map: Follicles larger than 15 mm were all growing, and follicles smaller than 15 mm appeared to be static or regressing.

Follicle parameter interpretation: Selection had taken place and the dominant follicle was largest. All parameters in this follicle indicated growth, whereas the competing follicles had started undergoing apoptosis, although the remaining parameters suggested health. The follicle map indicated that these were growing, but they appeared to have been selected against. Several follicles were atretic.

Table 8.11. Candy

	Growing	Intermediate	Dying	Atretic	Total
n	4	4	2	5	15
%	26.67	26.7	13.3	33.33	100

Candy	Left ovary	Right ovary
Day 12		
Day 13		
Day 14		
Day 15		



8.5.1.3.4 Bifrost

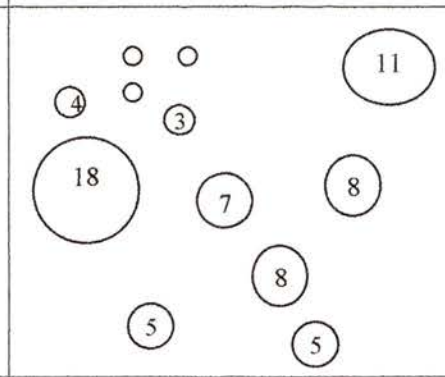
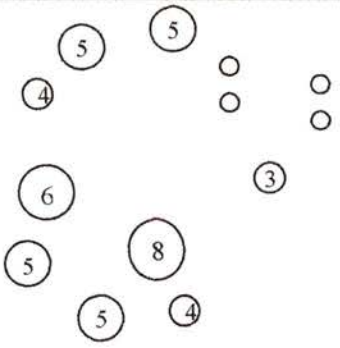
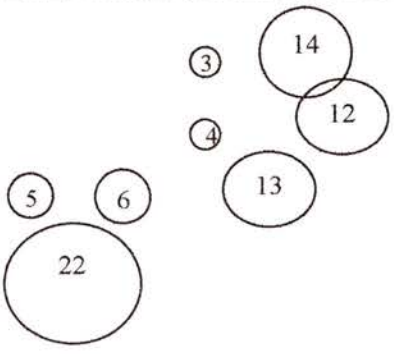
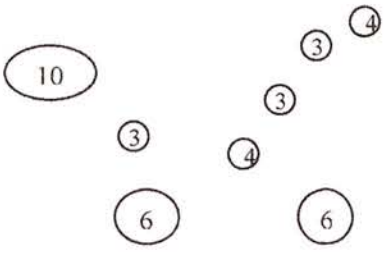
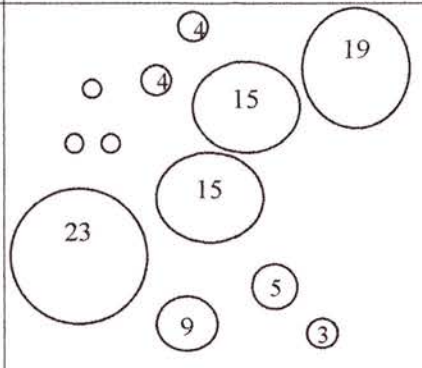
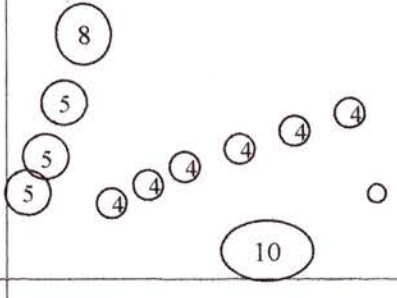
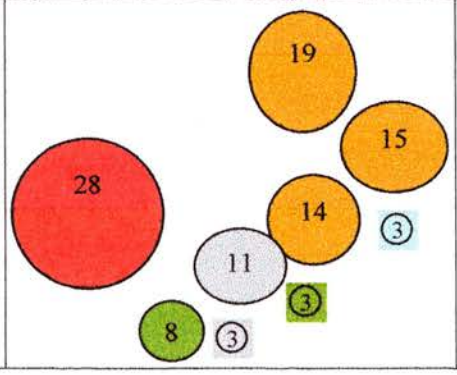
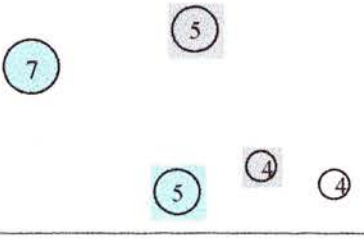
Follicle map: One follicle was clearly dominant, but 3 other follicles had grown until the day before ovariectomy. Follicles less than 10 mm were regressing or static.

Follicle parameter interpretation: all large follicles seemed to be alive and only the small follicles were atretic.

Selection had taken place, but none of the other growing follicles had started dying yet. This was in good agreement with the follicle map.

Table 8.12. Bifrost

	Growing	Intermediate	Dying	Atretic	Total
n	4	2	3	4	13
%	30.8	15.4	23.1	30.8	100

Bifrost	Left ovary	Right ovary
Day 12		
Day 13		
Day 14		
Day 15		

9 Bibliography

Aaltonen J, Laitinen MP, Vuojolainen K, Jaatinen R, Horelli-Kuitunen N, Seppa L, Louhio H, Tuuri T, Sjoberg J, Butzow R, Hovata O, Dale L, Ritvos O. Human growth differentiation factor 9 (GDF-9) and its novel homolog GDF-9B are expressed in oocytes during early folliculogenesis. *J.Clin.Endocrinol.Metab.* 1999; 84: 2744-2750.

Abdennebi L, Couture L, Grebert D, Pajot E, Salesse R, Remy JJ. Generating FSH antagonists and agonists through immunization against FSH receptor N-terminal decapeptides. *J.Mol.Endocrinol.* 1999; 22:151-159.

Abeydeera LR, Wang WH, Cantley TC, Rieke A, Prather RS, Day BN. Presence of epidermal growth factor during in vitro maturation of pig oocytes and embryo culture can modulate blastocyst development after in vitro fertilization. *Mol.Reprod.Dev.* 1998; 51:395-401.

Ackerman RC, Murdoch WJ. Prostaglandin-induced apoptosis of ovarian surface epithelial- cells. *Prostaglandins* 1993; 45:475-485.

Adams GP, Matteri RL, Kastelic JP, Ko JC, Ginther OJ. Association between surges of follicle-stimulating hormone and the emergence of follicular waves in heifers. *J.Reprod.Fertil.* 1992; 94:177-188.

Adams TE, Horton MB, Watson JG, Adams BM. Biological activity of luteinizing hormone (LH) during the estrous cycle of the mare. *Domestic Animal Endocrinology* 1986; 3:69-77.

Afanas'ev VN, Korol BA, Mantsygin YuA, Nelipovich PA, Pechatnikov VA, Umansky SR. Flow cytometry and biochemical analysis of DNA degradation characteristic of two types of cell death. *FEBS Lett.* 1986; 194:347-350.

Ainsworth L, Tsang BK, Downey BR, Marcus GJ, Armstrong DT. Interrelationships between follicular fluid steroid levels, gonadotropic stimuli, and oocyte maturation during preovulatory development of porcine follicles. *Biol.Reprod.* 1980; 23:621-627.

Aitken WA. Some observations on the oestous cycle and reproductive phenomena of the mare. *J Am Vet Med Assoc* 1926; 481-491.

Albertini DF. Novel morphological approaches for the study of oocyte maturation. *Biol.Reprod.* 1984; 30:13-28.

Alexander S, Irvine CH. Radioimmunoassay and in-vitro bioassay of serum LH throughout the equine oestrous cycle. *J.Reprod.Fertil.Suppl.* 1982; 32:253-260.

Alexander SL, Irvine CH, Turner JE. Comparison by three different radioimmunoassay systems of the polymorphism of plasma FSH in mares in various reproductive states. *J.Reprod.Fertil.Suppl.* 1987a; 35:9-18.

Alexander SL, Irvine CH. Secretion rates and short-term patterns of gonadotrophin-releasing hormone, FSH and LH throughout the periovulatory period in the mare. *J.Endocrinol.* 1987b; 114:351-362.

Alm H, Hinrichs K. Effect of cycloheximide on nuclear maturation of horse oocytes and its relation to initial cumulus morphology. *J.Reprod.Fertil.* 1996; 107:215-220.

Aman RR, Parks JE. Effects of cooling and rewarming on the meiotic spindle and chromosomes of in vitro-matured bovine oocytes. *Biol.Reprod.* 1994; 50:103-110.

Ansari B, Coates PJ, Greenstein BD, Hall PA. In situ end-labelling detects DNA strand breaks in apoptosis and other physiological and pathological states. *J.Pathol.* 1993; 170:1-8.

Arlotto T, Schwartz JL, First NL, Leibfriedrutledge ML. Aspects of follicle and oocyte stage that affect in vitro maturation and development of bovine oocytes. *Theriogenology* 1996; 45:943-956.

Avery B, Hay-Schmidt A, Hyttel P, Greve T. Embryo development, oocyte morphology, and kinetics of meiotic maturation in bovine oocytes exposed to 6-dimethylaminopurine prior to in vitro maturation. *Mol.Reprod.Dev.* 1998; 50:334-344.

Azambuja RM, Kraemer DC, Westhusin ME. Effect of low temperatures on in-vitro matured bovine oocytes. *Theriogenology.* 1998; 49: 1155-1164.

Back DG, Pickett BW, Voss JL, Seidel GE. Observations on the sexual behavior of nonlactating mares. *J. Amer. Vet. Med. Assoc.* 1974; 165: 717-720.

Baird DT, Campbell BK, Mann GE, McNeilly AS. Inhibin and estradiol in the control of fsh-secretion in the sheep. *J Reprod Fert* 1991; 125-138.

Baird DT, Campbell BK. Follicle selection in sheep with breed differences in ovulation rate. *Mol.Cell Endocrinol.* 1998; 145:89-95.

Bartlewski PM, Beard AP, Cook SJ, Chandolia RK, Honaramooz A, Rawlings NC. Ovarian antral follicular dynamics and their relationships with endocrine variables throughout the oestrous cycle in breeds of sheep differing in prolificacy. *J.Reprod.Fertil.* 1999; 115:111-124.

Behrman HR, Aten RF, Ireland JJ, Milvae RA. Characteristics of an antigonadotrophic GnRH-like protein in the ovaries of diverse mammals. *J.Reprod.Fertil.Suppl.* 1989; 37:189-94:189-194.

Bergfelt DR, Ginther OJ. Delayed follicular development and ovulation following inhibition of FSH with equine follicular fluid in the mare. *Theriogenology* 1985; 24:99-108.

Bergfelt DR, Mann BG, Schwartz NB, Ginther OJ. Circulating concentrations of immunoreactive inhibin and fsh during the estrous-cycle of mares. *Journal of Equine Veterinary Science* 1991; 11:319-322.

Bergfelt DR, Ginther OJ. Relationships between circulating concentrations of fsh and follicular waves during early-pregnancy in mares. *Journal of Equine Veterinary Science* 1992; 12:274-279.

Bergfelt DR, Ginther OJ. Relationships between FSH surges and follicular waves during the estrous cycle in mares. *Theriogenology* 1993; 39:781-796.

Bergfelt DR, Ginther OJ. Ovarian, uterine and embryo dynamics in horses versus ponies. *Journal of Equine Veterinary Science* 1996; 16:66-72.

Bezard J, Mekarska A, Goudet G, Duchamp G, Palmer E. Meiotic stage of the preovulatory equine oocyte at collection and competence of immature oocytes for in vitro maturation: effect of interval from induction of ovulation to follicle puncture. *Theriogenology* 1997; 47:386-386.

Billig H, Chun SY, Eisenhauer K, Hsueh AJ. Gonadal cell apoptosis: hormone-regulated cell demise. *Hum.Reprod.Update.* 1996; 2:103-117.

Blondin P, Sirard MA. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. *Mol.Reprod.Dev.* 1995; 41:54-62.

Blondin P, Dufour M, Sirard MA. Analysis of atresia in bovine follicles using different methods: flow cytometry, enzyme-linked immunosorbent assay, and classic histology. *Biol.Reprod.* 1996; 54:631-637.

Bodensteiner KJ, Wiltbank MC, Bergfelt DR, Ginther OJ. Alterations in follicular estradiol and gonadotropin receptors during development of bovine antral follicles. *Theriogenology* 1996; 45:499-512.

Boerboom D, Kerban A, Sirois J. Dual regulation of promoter II- and promoter 1f-derived cytochrome P450 aromatase transcripts in equine granulosa cells during human chorionic gonadotropin-induced ovulation: a novel model for the study of aromatase promoter switching. *Endocrinol.* 1999; 140: 4133-4141.

Boone DL, Tsang BK. Identification and localization of deoxyribonuclease I in the rat ovary. *Biol.Reprod.* 1997a; 57:813-821.

Boone DL, Carnegie JA, Rippstein PU, Tsang BK. Induction of apoptosis in equine chorionic gonadotropin (eCG)- primed rat ovaries by anti-eCG antibody. *Biol.Reprod.* 1997b; 57:420-427.

Bouniol-Baly C, Hamraoui L , Guibert J, Beaujean N, Szollosi MS, Debey P. Differential transcriptional activity associated with chromatin configuration in fully grown mouse germinal vesicle oocytes. *Biol.Reprod.* 1999; 60:580-587.

Braw RH, Bar-Ami S, Tsafriri A. Effect of hypophysectomy on atresia of rat preovulatory follicles. *Biol.Reprod.* 1981; 25:989-996.

Bruck I, Greve T, Hyttel P. Morphology of the oocyte-follicular connection in the mare. *Anat.Embryol.(Berl)* 1999; 199:21-28.

Buccione R, Vanderhyden BC , Caron PJ, Eppig JJ. FSH-induced expansion of the mouse cumulus oophorus in vitro is dependent upon a specific factor(s) secreted by the oocyte. *Dev.Biol.* 1990; 138:16-25.

Burns PJ and Douglas RH. Effects of daily administration of oestradiol-17b on follicular growth, ovulation, and plasma hormones in mares. *Biol. Reprod.* 1981; 24: 1026-1031.

Byskov AG. Cell kinetic studies of follicular atresia in the mouse ovary. *J.Reprod.Fertil.* 1974; 37:277-285.

Byskov AG. Atresia. In: Midgley AR, Sadler WA, eds. *Ovarian follicular development and function*. New York, Raven Press, 1979:41-57.

Byskov AG, Andersen CY, Nordholm L, Thogersen H, Xia G, Wassmann O, Andersen JV, Guddal E, Roed T. Chemical structure of sterols that activate oocyte meiosis. *Nature* 1995; 374:559-562.

Byskov AG, Yding Andersen C, Hossaini A, Guoliang X . Cumulus cells of oocyte-cumulus complexes secrete a meiosis- activating substance when stimulated with FSH. *Mol.Reprod.Dev.* 1997; 46:296-305.

Campbell BK, Dobson H, Baird DT, Scaramuzzi RJ. Examination of the relative role of FSH and LH in the mechanism of ovulatory follicle selection in sheep. *J.Reprod.Fertil.* 1999; 117:355-367.

Channing CP, Pomerantz SH, Bae IH, Evans VW, Atlas SJ. Actions of hormones and other factors upon oocyte maturation. *Adv.Exp.Med.Biol.* 1982; 147:189-210:189-210.

Chen L, Wert SE, Hendrix EM, Russell PT, Cannon M, Larsen WJ. Hyaluronic acid synthesis and gap junction endocytosis are necessary for normal expansion of the cumulus mass. *Mol.Reprod.Dev.* 1990; 26:236-247.

Chun SY, Eisenhauer KM, Kubo M, Hsueh AJ. Interleukin-1 beta suppresses apoptosis in rat ovarian follicles by increasing nitric oxide production. *Endocrinology* 1995; 136:3120-3127.

Clarke HJ, Masui Y. The induction of reversible and irreversible chromosome decondensation by protein synthesis inhibition during meiotic maturation of mouse oocytes. *Dev.Biol.* 1983; 97:291-301.

Cochran, R., Meintjes, M., Reggio, B., Hylan, D., Pinto, C., Carter, J., Paccamonti, D., and Godke, R. A. In vitro development and transfer of in vitro derived embryos produced from sperm-injected oocytes harvested from pregnant mares. 135-136. 1998. University of Pretoria, 7th International symposium on equine reproduction.

Cohen JJ, Duke RC. Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J.Immunol.* 1984; 132:38-42.

Colbern GT, Reagan WJ. Ovariectomy by colpotomy in mares. *Compendium on Continuing Education for the Practicing Veterinarian* 1987; 9:1035-1038.

Condon WA, Ganjam VK, Kenney RM, Channing CP. Follicular atresia in the mare. In: Midgley AR, Sadler WA, eds. *Ovarian follicular development and function*. New York, Raven Press, 1979:75-77.

Conley AJ, Bird IM. The role of cytochrome p450 17 alpha-hydroxylase and 3 beta-hydroxysteroid dehydrogenase in the integration of gonadal and adrenal steroidogenesis via the delta 5 and delta 4 pathways of steroidogenesis in mammals. *Biol Reprod* 1997; 56:789-799.

Cooke DJ, Crowe MA, Roche JF. Circulating FSH isoform patterns during recurrent increases in FSH throughout the oestrous cycle of heifers. *J.Reprod.Fertil.* 1997; 110:339-345.

Corrie JET, Hunter WM, Macpherson JS. A strategy for radioimmunoassay of plasma progesterone with the use of a homologous site ^{125}I-labelled radioligand. *Clinical Chemistry* 1981; 27:594-599.

Cran DG. Qualitative and quantitative structural changes during pig oocyte maturation. *J.Reprod.Fertil.* 1985; 74:237-245.

Crosby IM, Osborn JC, Moor RM. Follicle cell regulation of protein synthesis and developmental competence in sheep oocytes. *J.Reprod.Fertil.* 1981; 62:575-582.

Crosby IM, Moor RM, Heslop JP, Osborn JC. cAMP in ovine oocytes: localization of synthesis and its action on protein synthesis, phosphorylation, and meiosis. *J.Exp.Zool.* 1985; 234:307-318.

Crozet N. Nucleolar structure and RNA synthesis in mammalian oocytes. *J Reprod Fert* 1989; Supplement 38:9-16.

Daels PF, Ammon DC, Stabenfeldt GH, Liu IKM, Hughes JP, Lasley BL. Urinary and plasma estrogen conjugates, estradiol and estrone concentrations in nonpregnant and early pregnant mares. *Theriogenology* 1991; 35:1001-1017.

Darzynkiewicz Z, Bedner E, Traganos F, Murakami T. Critical aspects in the analysis of apoptosis and necrosis. *Hum.Cell* 1998; 11:3-12.

Deanesly R. Germ cell development and the meiotic prophase in the fetal horse ovary. *J.Reprod.Fertil.Suppl.* 1975; 547-552.

Deanesly R. Germ cell proliferations in the fetal horse ovary. *Cell Tissue Res.* 1977; 185:361-371.

Dekel N, Kraicer PF. Induction in vitro of mucification of rat cumulus oophorus by gonadotrophins and adenosine 3',5'-monophosphate. *Endocrinology* 1978; 102:1797-1802.

Delcampo MR, Delcampo CH, Mapletoft RJ, Ginther OJ. Morphology and location of attached follicular cumulus oocyte complexes in horses, cattle and llamas. *Theriogenology* 1995a; 43:533-542.

Delcampo MR, Donoso X, Parrish JJ, Ginther OJ. Selection of follicles, preculture oocyte evaluation, and duration of culture for in-vitro maturation of equine oocytes. *Theriogenology* 1995b; 43:1141-1153.

Dellaquila ME, Cho YS, Minoia P, Traina V, Fusco S, Lacalandra GM, Maritato F. Intracytoplasmic sperm injection (icsi) versus conventional ivf on abattoir-derived and in vitro-matured equine oocytes. *Theriogenology* 1997; 47:1139-1156.

Didion BA, Pomp D, Martin MJ, Homanics GE, Markert CL. Observations on the cooling and cryopreservation of pig oocytes at the germinal vesicle stage. *J.Anim.Sci.* 1990; 68:2803-2810.

Dobson H. Effects of chronic treatment with a gnrh agonist on estrous behavior and on the secretion of lh and progesterone in the ewe. *Theriogenology* 1985; 24:1-11.

Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM. Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* 1996; 383: 531-535.

Driancourt MA. Follicular kinetics in the mare ovary. *Ann Biol anim Bioch Biophys* 1979; 19:1443-1453.

Driancourt MA, Mariana JC, Palmer E. Effect of the stage of the estrous-cycle on the follicular population in pony mares. *Reproduction Nutrition Developpement* 1982a; 22:803-812.

Driancourt MA, Paris A, Roux C, Mariana JC, Palmer E. Ovarian follicular populations in pony and saddle-type mares. *Reproduction Nutrition Developpement* 1982b; 22:1035-1047.

Driancourt MA, Prunier A, Palmer E, Mariana JC. Seasonal effects on ovarian follicular development in pony mares. *Reproduction Nutrition Developpement* 1983; 23:207-215.

Driancourt MA, Palmer E. Time of ovarian follicular recruitment in cyclic pony mares . *Theriogenology* 1984; 21:591-600.

Driancourt MA, Fry RC, Clarke IJ, Cahill LP. Follicular-growth and regression during the 8 days after hypophysectomy in sheep. *Journal Of Reproduction & Fertility* 1987; 79:635-641.

Driancourt MA, Thatcher WW , Terqui M, Andrieu D. Dynamics of ovarian follicular development in cattle during the estrous-cycle, early-pregnancy and in response to pmsg. *Domestic Animal Endocrinology* 1991a; 8:209-221.

Driancourt MA. Follicular dynamics in sheep and cattle. *Theriogenology* 1991b; 35:55-79.

Dufour J, Cahill LP, Mauleon P. Short- and long-term effects of hypophysectomy and unilateral ovariectomy on ovarian follicular populations in sheep. *J Reprod Fert* 1979; 57:301-309.

Duvall E, Wyllie AH. Death and the cell. *Immunology Today* 1986; 7:115-118.

Elvin JA, Clark AT, Wang P, Wolfman NM, Matzuk MM. Paracrine actions of growth differentiation factor-9 in the mammalian ovary. *Mol. Endocrinol.* 1999; 13: 1035-1048.

Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* 1998; 391:43-50.

Eppig JJ. FSH stimulates hyaluronic acid synthesis by oocyte-cumulus cell complexes from mouse preovulatory follicles. *Nature* 1979; 281:483-484.

Eppig JJ, Freter RR, Ward-Bailey PF, Schultz RM. Inhibition of oocyte maturation in the mouse: participation of cAMP, steroid hormones, and a putative maturation-inhibitory factor. *Dev.Biol.* 1983; 100:39-49.

Eppig JJ, Chesnel F, Hirao Y, O'Brien MJ, Pendola FL, Watanabe S, Wigglesworth K. Oocyte control of granulosa cell development: how and why. *Hum.Reprod.* 1997; 12:127-132.

Equine Embryo Transfer IV. Proceedings of the 4th International Symposium on Equine Embryo Transfer. Reims, France, 16-18 January 1997. *Equine.Vet.J.Suppl.* 1997; 6-112.

Evans AC, Duffy P, Hynes N, Boland MP. Waves of follicle development during the estrous cycle in sheep. *Theriogenology* 2000; 53:699-715.

Evans JW. Fsh-secretion patterns in cycling mares - ultradian rhythms. *Journal of Equine Veterinary Science* 1990; 10:203-207.

Evans MJ, Irvine CH. Serum concentrations of FSH, LH and progesterone during the oestrous cycle and early pregnancy in the mare. *J.Reprod.Fertil.Suppl.23* 1975; 193-200.

Evans MJ, Loy RG, Taylor TB, Barrows SP. Effects of exogenous steroids on serum FSH and LH, and on follicular development in cyclic mares. *J. Reprod. Fertil. Suppl.* 1982; 32: 205-212.

Fay JE, Douglas RH. Changes in thecal and granulosa cell LH and FSH receptor content associated with follicular fluid and peripheral plasma gonadotrophin and steroid hormone concentrations in preovulatory follicles of mares. *J.Reprod.Fertil.Suppl.* 1987; 35:169-181.

Felsenfeld G. Chromatin as an essential part of the transcriptional mechanism. *Nature* 1992; 355:219-224.

Fissore RA, He CL, Vande Woude GF. Potential role of mitogen-activated protein kinase during meiosis resumption in bovine oocytes. *Biol.Reprod.* 1996; 55:1261-1270.

Fitzgerald BP, I'Anson H, Loy RG, Legan SJ. Changes in pulsatile LH secretion and the positive and negative feedback actions of oestradiol after administration of a GnRH agonist in the ovariectomized ewe. *J.Reprod.Fertil.* 1985; 73:433-440.

Fitzgerald BP, Micka AF, Meyer SL. Suppression of circulating concentrations of luteinizing-hormone (lh) in ovariectomized (ovx) mares by constant administration of an agonist analog (wy-40972) of gonadotropin-releasing-hormone (gnrh). *J Reprod Fert* 1991; 743-743.

Fitzgerald BP, Peterson KD , Silvia PJ. Effect of constant administration of a gonadotropin-releasing hormone agonist on reproductive activity in mares: preliminary evidence on suppression of ovulation during the breeding season. *Am.J.Vet.Res.* 1993a; 54:1746-1751.

Fitzgerald BP, Meyer SL, Affleck KJ, Silvia PJ. Effect of constant administration of a gonadotropin-releasing hormone agonist on reproductive activity in mares: induction of ovulation during seasonal anestrus. *Am.J.Vet.Res.* 1993b; 54:1735-1745.

Flaws JA, Kugu K, Trbovich AM, Desanti A, Tilly KI, Hirshfield AN, Tilly JL. Interleukin-1-beta-converting enzyme-related proteases (irps) and mammalian-cell death - dissociation of irp-induced oligonucleosomal endonuclease activity from morphological apoptosis in granulosa-cells of the ovarian follicle. *Endocrinology* 1995; 136:5042-5053.

Fortune JE. Ovarian follicular growth and development in mammals. *Biol Reprod* 1994; 50:225-232.

Fowler J, Cohen L, Jarvis P. *Practical statistics for field biology*. 2nd ed. Chichester: John Wiley & Sons, 1998: 1-227.

Fraser HM, Laird NC, Blakeley DM. Decreased pituitary responsiveness and inhibition of the luteinizing hormone surge and ovulation in the stumptailed monkey (Macaca arctoides) by chronic treatment with an agonist of luteinizing hormone-releasing hormone. *Endocrinology* 1980; 106:452-457.

Fraser HM, Baird DT, McRae GI, Nestor JJ, Vickery BH. Suppression of luteal progesterone secretion in the stumptailed macaque by an antagonist analogue of luteinizing hormone releasing hormone. *J.Endocrinol.* 1985; 104:R1-4.

Fraser HM, Lunn SF, Morris KD, Deghenghi R. Initiation of high dose gonadotrophin-releasing hormone antagonist treatment during the late follicular phase in the macaque abolishes luteal function irrespective of effects upon the luteinizing hormone surge. *Human Reproduction* 1997; 12:430-435.

Fry RC, Driancourt MA. Relationships between follicle-stimulating hormone, follicle growth and ovulation rate in sheep. *Reprod.Fertil.Dev.* 1996; 8:279-286.

Fulka J, Jr., Okolski A. Culture of horse oocytes in vitro. *J.Reprod.Fertil.* 1981; 61:213-215.

Fulka J, Jr., Jung T, Moor RM. The fall of biological maturation promoting factor (MPF) and histone H1 kinase activity during anaphase and telophase in mouse oocytes. *Mol.Reprod.Dev.* 1992; 32:378-382.

Garcia MC and Ginther OJ. Regulation of plasma LH by estradiol and progesterone in ovariectomized mares. *Biol. Reprod.* 1978; 19: 447-453.

Garrett WM, Guthrie HD. Expression of androgen receptors and steroidogenic enzymes in relation to follicular-growth and atresia following ovulation in pigs. *Biol Reprod* 1996; 55:949-955.

Garrett WM, Guthrie HD. Steroidogenic enzyme expression during preovulatory follicle maturation in pigs. *Biol Reprod* 1997; 56:1424-1431.

Garza F, Jr., Thompson DL, Jr., St George RL, Reville-Moroz SI. Effects of dihydrotestosterone benzoate administration on gonadotropin secretion in ovariectomized pony mares. *J.Anim.Sci.* 1985; 61:240-244.

Garza F, Jr., St.George RL , Mitchell PS, Thompson DL, Jr. Effect of active immunization against estrogen on gonadotropin response to testosterone propionate treatment in ovariectomized pony mares. *J.Anim.Sci.* 1989; 67:226-231.

Garza F, Jr., Thompson DL, Jr., French DD, Wiest JJ, St.George RL, Ashley KB, Jones LS, Mitchell PS, McNeill DR. Active immunization of intact mares against gonadotropin- releasing hormone: differential effects on secretion of luteinizing hormone and follicle-stimulating hormone. *Biol.Reprod.* 1986a; 35:347-352.

Garza F, Jr., Thompson DL, Jr., St George RL, French DD. Androgen and estradiol effects on gonadotropin secretion and response to GnRH in ovariectomized pony mares. *J.Anim.Sci.* 1986b; 62:1654-1659.

Gastal EL, Gastal MO, Bergfelt DR, Ginther OJ. Role of diameter differences among follicles in selection of a future dominant follicle in mares. *Biol Reprod* 1997a; 57:1320-1327.

Gastal EL, Gastal MO, Bergfelt DR, Ginther OJ. Role of diameter differences among follicles in selection of a future dominant follicle in mares. *Biol.Reprod.* 1997b; 57:1320-1327.

Gastal EL, Gastal MO, Wiltbank MC, Ginther OJ. Relationship of follicle deviation with intrafollicular and systemic estradiol concentrations in mares. *Theriogenology* 1999a; 51:300-300.

Gastal EL, Gastal MO, Wiltbank MC, Ginther OJ. Follicle Deviation and Intrafollicular and Systemic Estradiol Concentrations in Mares. *Biol.Reprod.* 1999b; 61:31-39.

Gastal EL, Gastal MO, Ginther OJ. Experimental assumption of dominance by a smaller follicle and associated hormonal changes in mares. *Biol.Reprod.* 1999c; 61:724-730.

Gastal EL, Bergfelt DR, Nogueira GP, Gastal MO, Ginther OJ. Role of Luteinizing Hormone in Follicle Deviation Based on Manipulating Progesterone Concentrations in Mares. *Biol.Reprod.* 1999d; 61:1492-1498.

Gastal EL, Donadeu FX, Gastal MO, Ginther OJ. Echotextural changes in the follicular wall during follicle deviation in mares. *Theriogenology* 1999e; 52:803-814.

Gastal EL, Bergfelt DR, Nogueira GP, Gastal MO, Ginther OJ. Role of luteinizing hormone in follicle deviation based on manipulating progesterone concentrations in mares. *Biol Reprod* 1999f; 61:1492-1498.

Gastal EL, Gastal MO, Nogueira GP, Bergfelt DR, Ginther OJ. Temporal interrelationships among luteolysis, FSH and LH concentrations and follicle deviation in mares. *Theriogenology* 2000; 53:925-940.

Ginther OJ. Occurrence of anestrus, estrus, diestrus, and ovulation over a 12-month period in mares. *Am.J.Vet.Res.* 1974; 35:1173-1179.

Ginther OJ. Folliculogenesis during the transitional period and early ovulatory season in mares. *J.Reprod.Fertil.* 1990a; 90:311-320.

Ginther OJ, Bergfelt DR. Effect of GnRH treatment during the anovulatory season on multiple ovulation rate and on follicular development during the ensuing pregnancy in mares. *J.Reprod.Fertil.* 1990b; 88:119-126.

Ginther OJ, Bergfelt DR. Associations between FSH concentrations and major and minor follicular waves in pregnant mares. *Theriogenology* 1992a; 38:807-821.

Ginther OJ. Ginther OJ, ed. *Reproductive Biology of the mare*. 1992b:

Ginther OJ, Bergfelt DR. Ultrasonic characterization of follicular waves in mares without maintaining identity of individual follicles. *Journal of Equine Veterinary Science* 1992c; 12:349-354.

Ginther OJ. Major and minor follicular waves during the equine estrous-cycle. *Journal of Equine Veterinary Science* 1993a; 13:18-25.

Ginther OJ, Bergfelt DR. Growth of small follicles and concentrations of fsh during the equine estrous-cycle. *J Reprod Fert* 1993b; 99:105-111.

Ginther OJ, Bergfelt DR. Growth of small follicles and concentrations of FSH during the equine oestrous cycle. *J.Reprod.Fertil.* 1993c; 99:105-111.

Ginther OJ, Wiltbank MC, Fricke PM, Gibbons JR, Kot K. Selection of the dominant follicle in cattle. *Biol.Reprod.* 1996; 55:1187-1194.

Ginther OJ, Kot K, Kulick LJ, Wiltbank MC. Emergence and deviation of follicles during the development of follicular waves in cattle. *Theriogenology* 1997a; 48:75-87.

Ginther OJ, Kot K, Kulick LJ, Wiltbank MC. Sampling follicular fluid without altering follicular status in cattle: oestradiol concentrations early in a follicular wave. *J.Reprod.Fertil.* 1997b; 109:181-186.

Ginther OJ, Bergfelt DR, Kulick LJ, Kot K. Pulsatility of systemic FSH and LH concentrations during follicular-wave development in cattle. *Theriogenology* 1998; 50:507-519.

Gold R, Schmied M, Giegerich G, Breitschopf H, Hartung HP, Toyka KV, Lassmann H. Differentiation between cellular apoptosis and necrosis by the combined use of in situ tailing and nick translation techniques. *Lab.Invest.* 1994; 71:219-225.

Gong JG, Bramley TA, Gutierrez CG, Peters AR, Webb R. Effects of chronic treatment with a gonadotropin-releasing- hormone agonist on peripheral concentrations of fsh and lh, and ovarian- function in heifers. *J Reprod Fert* 1995; 105:263-270.

Gong JG, Campbell BK, Bramley TA, Gutierrez CG, Peters AR, Webb R. Suppression in the secretion of follicle-stimulating hormone and luteinizing hormone, and ovarian follicle development in heifers continuously infused with a gonadotropin-releasing hormone agonist. *Biol.Reprod.* 1996; 55:68-74.

Gore-Langton RE, Armstrong DT. Follicular steroidogenesis and its control. In: Knobil E, Neill JD, eds. *The Physiology of Reproduction*. New York, Raven Press, 1994:571-627.

Gosden R, Krapez J, Briggs D. Growth and development of the mammalian oocyte. *BioEssays* 1997a; 19:875-882.

Gosden R, Spears N. Programmed cell death in the reproductive system. *Br.Med.Bull.* 1997b; 53:644-661.

Goud PT, Goud AP, Qian C, Laverge H, Van der Elst J, De Sutter P, Dhont M. In-vitro maturation of human germinal vesicle stage oocytes: role of cumulus cells and epidermal growth factor in the culture medium. *Hum.Reprod.* 1998; 13:1638-1644.

Goudet G, Bezard J, Duchamp G, Gerard N, Palmer E. Equine oocyte competence for nuclear and cytoplasmic in vitro maturation: effect of follicle size and hormonal environment. *Biol.Reprod.* 1997; 57:232-245.

Goudet G, Bezard J, Belin F, Duchamp G, Palmer E, Gerard N. Oocyte competence for In vitro maturation is associated with histone H1 kinase activity and is influenced by estrous cycle stage in the mare. *Biol.Reprod.* 1998a; 59:456-462.

Goudet, G. Resumption and completion of meiosis in equine oocytes. *Serono Symposia, Conference Proceeding*; 1998b: 177-194.

Goudet G, Belin F, Bezard J, Gerard N. Intrafollicular content of luteinizing hormone receptor, alpha- inhibin, and aromatase in relation to follicular growth, estrous cycle stage, and oocyte competence for in vitro maturation in the mare. *Biol.Reprod.* 1999; 60:1120-1127.

Grant SA, Hunter MG, Foxcroft GR. Morphological and biochemical characteristics during ovarian follicular development in the pig. *J.Reprod.Fertil.* 1989; 86:171-183.

Greenwald GS, Roy SK. Follicular development and its control. In: Knobil E, Neil JD, eds. *The physiology of reproduction*. New York, Raven Press, 1994:

Grimes RW, Matton P, Ireland JJ. A comparison of histological and non-histological indices of atresia and follicular function. *Biol.Reprod.* 1987; 37:82-88.

Grondahl C, Hyttel P, Grondahl ML, Eriksen T, Gotfredsen P, Greve T. Structural and endocrine aspects of equine oocyte maturation in- vivo. *Molecular Reproduction And Development* 1995a; 42:94-105.

Grondahl C, Hyttel P, Grondahl ML, Eriksen T, Gotfredsen P, Greve T. Structural and endocrine aspects of equine oocyte maturation in vivo. *Mol.Reprod.Dev.* 1995b; 42:94-105.

Grondahl C, Hansen TH, Hossaini A, Heinze I, Greve T, Hyttel P. Intracytoplasmic sperm injection of in vitro-matured equine oocytes. *Biol.Reprod.* 1997; 57:1495-1501.

Grupen CG, Nagashima H, Nottle MB. Role of epidermal growth factor and insulin-like growth factor-I on porcine oocyte maturation and embryonic development in vitro. *Reprod.Fertil.Dev.* 1997; 9:571-575.

Guignot F, Ottogalli M, Yvon JM, Magistrini M. Preliminary observations in in vitro development of equine embryo after icsi. *Reprod Nutr Dev* 1998; 38:653-663.

Guignot F, Bezard J, Palmer E. Effect of time during transport of excised mare ovaries on oocyte recovery rate and quality after in vitro maturation. *Theriogenology* 1999; 52:757-766.

Guthrie HD, Bolt DJ. Changes in plasma follicle-stimulating hormone, luteinizing hormone, estrogen and progesterone during growth of ovulatory follicles in the pig. *Domest.Anim.Endocrinol.* 1990; 7:83-91.

Guthrie HD, Welch GR, Cooper BS, Zakaria AD, Johnson LA. Flow cytometric determination of degraded deoxyribonucleic-acid in granulosa-cells to identify atretic follicles during preovulatory maturation in the pig. *Biol Reprod* 1994; 50:1303-1311.

Guthrie HD, Cooper BS, Welch GR, Zakaria AD, Johnson LA. Atresia in follicles grown after ovulation in the pig - measurement of increased apoptosis in granulosa-cells and reduced follicular- fluid estradiol-17-beta. *Biol Reprod* 1995a; 52:920-927.

Guthrie HD, Grimes RW, Cooper BS, Hammond JM. Follicular atresia in pigs - measurement and physiology. *Journal of Animal Science* 1995b; 73:2834-2844.

Guthrie HD, Cooper BS. Follicular atresia, follicular-fluid hormones, and circulating hormones during the midluteal phase of the estrous-cycle in pigs. *Biol Reprod* 1996; 55:543-547.

Hale AJ, Smith CA, Sutherland LC, Stoneman VE, Longthorne VL, Culhane AC, Williams GT. Apoptosis: molecular regulation of cell death. *Eur.J.Biochem.* 1996; 236:1-26.

Harris TG, Robinson JE, Evans NP, Skinner DC, Herbison AE. Gonadotropin-releasing hormone messenger ribonucleic acid expression changes before the onset of the estradiol-induced luteinizing hormone surge in the ewe. *Endocrinology* 1998; 139:57-64.

Herrmann M, Lorenz HM, Voll R, Grunke M, Woith W, Kalden JR. A rapid and simple method for the isolation of apoptotic DNA fragments. *Nucleic. Acids. Res.* 1994; 22: 5506-5507.

Hess KA, Chen L, Larsen WJ. Inter-alpha-inhibitor binding to hyaluronan in the cumulus extracellular matrix is required for optimal ovulation and development of mouse oocytes. *Biol.Reprod.* 1999; 61:436-443.

Hillier SG. Hormonal control of folliculogenesis and luteinization. In: Findlay JK, ed. *Molecular biology of the female reproductive system*. London, Academic Press, 1994:1-37.

Hinrichs K. The relationship of follicle atresia to follicle size, oocyte recovery rate on aspiration, and oocyte morphology in the mare. *Theriogenology* 1991a; 36:157-168.

Hinrichs K. The relationship of follicle atresia to follicle size, oocyte recovery rate on aspiration, and oocyte morphology in the mare. *Theriogenology* 1991b; 36:157-168.

Hinrichs K, Schmidt AL, Selgrath JP. Atlas of chromatin configurations of germinal vesicle-stage and maturing horse oocytes. *Equine Veterinary Journal Supplement* 1993a; 15:60-63.

Hinrichs K, Schmidt AL, Friedman PP, Selgrath JP, Martin MG. In vitro maturation of horse oocytes: characterization of chromatin configuration using fluorescence microscopy. *Biol.Reprod.* 1993b; 48:363-370.

Hinrichs K, Martin MG, Schmidt AL, Friedman PP. Effect of follicular components on meiotic arrest and resumption in horse oocytes. *J.Reprod.Fertil.* 1995; 104:149-156.

Hinrichs K, Williams KA. Relationships among oocyte-cumulus morphology, follicular atresia, initial chromatin configuration, and oocyte meiotic competence in the horse. *Biol.Reprod.* 1997; 57:377-384.

Hinrichs K, Schmidt AL. Meiotic competence in horse oocytes: interactions among chromatin configuration, follicle size, cumulus morphology and season. *Biol Reprod* 2000; 62:1402-1408.

Hirshfield AN. Comparison of granulosa cell proliferation in small follicles of hypophysectomized, prepubertal, and mature rats. *Biol.Reprod.* 1985; 32: 979-987.

Hirshfield AN. Development of follicles in the mammalian ovary. *Int.Rev.Cytol.* 1991; 124:43-101.

Hodges JK, Green DI, Cottingham PG, Sauer MJ, Edwards C, Lightman SL. Induction of luteal regression in the marmoset monkey (Callithrix jacchus) by a gonadotrophin-releasing hormone antagonist and the effects on subsequent follicular development. *J.Reprod.Fertil.* 1988; 82:743-752.

Hsu SY, Lai RJ, Finegold M, Hsueh AJ. Targeted overexpression of Bcl-2 in ovaries of transgenic mice leads to decreased follicle apoptosis, enhanced folliculogenesis, and increased germ cell tumorigenesis. *Endocrinology* 1996; 137:4837-4843.

Hsueh AJ, Erickson GF. Extrapituitary action of gonadotropin-releasing hormone: direct inhibition ovarian steroidogenesis. *Science* 1979; 204:854-855.

Hsueh AJ, Billig H, Tsafriri A. Ovarian follicle atresia: a hormonally controlled apoptotic process. *Endocr.Rev.* 1994; 15:707-724.

Hughes FM, Jr., Gorospe WC. Biochemical identification of apoptosis (programmed cell death) in granulosa cells: evidence for a potential mechanism underlying follicular atresia. *Endocrinology* 1991; 129:2415-2422.

Huhtaniemi IT, Catt KJ, Clayton RN. Newborn and immature rat testes contain gonadotropin-releasing hormone (GnRH) receptors, and their testosterone production is stimulated by a GnRH agonist in vitro. *Mol.Cell Endocrinol.* 1985; 40:41-44.

Hyland JH, Wright PJ, Clarke IJ, Carson RS, Langsford DA, Jeffcott LB. Infusion of gonadotrophin-releasing hormone (GnRH) induces ovulation and fertile oestrus in mares during seasonal anoestrus. *J.Reprod.Fertil.Suppl.* 1987; 35:211-220.

Hynes AC, Sreenan JM, Kane MT. Modulation of the effects of fsh, androstenedione, epidermal growth factor (egf) and insulin-like growth factor i (igf-i) on bovine granulosa

cells by gcif, a growth-inhibitory factor of low molecular mass from bovine follicular fluid. *J Reprod Fert* 1996; 108:193-197.

Hynes AC, Kane MT, Sreenan JM. Immunization of rats and sheep against granulosa cell-inhibitory factor from bovine follicular fluid increases the number of large follicles in rats and the ovulation rate in sheep. *J.Reprod.Fertil.* 1999; 116:35-41.

Imig JL, Juengel JL, Salfen BE, Youngquist RS, Hamilton SA, Smith MF, Garverick HA. Evidence for a role of oligonucleosome formation (apoptosis) in follicular atresia in cattle. *Biol Reprod* 1993; 48:-abstract 245

Ireland JJ, Roche JF. Development of antral follicles in cattle after prostaglandin- induced luteolysis - changes in serum hormones, steroids in follicular-fluid, and gonadotropin receptors. *Endocrinology* 1982; 111:2077-2086.

Ireland JJ, Roche JF. Growth and differentiation of large antral follicles after spontaneous luteolysis in heifers: changes in concentration of hormones in follicular fluid and specific binding of gonadotropins to follicles. *J.Anim.Sci.* 1983; 57:157-167.

Irvine CH, Alexander SL. A novel technique for measuring hypothalamic and pituitary hormone secretion rates from collection of pituitary venous effluent in the normal horse. *J.Endocrinol.* 1987; 113:183-192.

Irvine CH, Alexander SL. Secretory patterns and rates of gonadotropin-releasing hormone, follicle-stimulating hormone, and luteinizing hormone revealed by intensive sampling of pituitary venous blood in the luteal phase mare. *Endocrinology* 1993; 132:212-218.

Irvine CH, Alexander SL. The dynamics of gonadotrophin-releasing hormone, LH and FSH secretion during the spontaneous ovulatory surge of the mare as revealed by intensive sampling of pituitary venous blood. *J.Endocrinol.* 1994; 140:283-295.

Irvine CHG. The non-pregnant mare: a review of some current research and of the last 25 years of endocrinology. *Biology of Reproduction Monograph Series 1: Equine Reproduction VI* 1995; 343-360.

Irvine CH, Alexander SL. Patterns of secretion of GnRH, LH and FSH during the postovulatory period in mares: mechanisms prolonging the LH surge. *J.Reprod.Fertil.* 1997; 109:263-271.

Irvine CH, Turner JE, Alexander SL, Shand N, van Noordt S. Gonadotrophin profiles and dioestrous pulsatile release patterns in mares as determined by collection of jugular blood at 4 h intervals throughout an oestrous cycle. *J.Reprod.Fertil.* 1998; 113:315-322.

Jewgenow K, Heerdegen B, Muller K. In vitro development of individually matured bovine oocytes in relation to follicular wall atresia. *Theriogenology* 1999; 51:745-756.

Johnson AL, Bridgham JT, Witty JP, Tilly JL. Susceptibility of avian ovarian granulosa-cells to apoptosis is dependent upon stage of follicle development and is related to endogenous levels of bcl-xlong gene-expression. *Endocrinology* 1996; 137:2059-2066.

Jolly PD, Tisdall DJ, Heath D, Lun S, McNatty KP. Apoptosis of bovine granulosa-cells occurs in healthy follicles or is an early event in follicle atresia. *Biol Reprod* 1994a; 50:184-184.

Jolly PD, Tisdall DJ, Heath DA, Lun S, McNatty KP. Apoptosis in bovine granulosa-cells in relation to steroid- synthesis, cyclic adenosine-3',5'-monophosphate response to follicle-stimulating-hormone and luteinizing-hormone, and follicular atresia. *Biol Reprod* 1994b; 51:934-944.

Jolly PD, Smith PR, Heath DA, Hudson NL, Lun S, Still LA, Watts CH, McNatty KP. Morphological evidence of apoptosis and the prevalence of apoptotic versus mitotic cells in the membrana granulosa of ovarian follicles during spontaneous and induced atresia in ewes. *Biol.Reprod.* 1997a; 56:837-846.

Jolly PD, Tisdall DJ, De'ath G, Heath DA, Lun S, Hudson NL, McNatty KP. Granulosa cell apoptosis, aromatase activity, cyclic adenosine 3',5'- monophosphate response to gonadotropins, and follicular fluid steroid levels during spontaneous and induced follicular atresia in ewes. *Biol.Reprod.* 1997b; 56:830-836.

Kaipia A, Chun SY, Eisenhauer K, Hsueh AJ. Tumor necrosis factor-alpha and its second messenger, ceramide, stimulate apoptosis in cultured ovarian follicles. *Endocrinology* 1996; 137:4864-4870.

Kaipia A, Hsueh AJ. Regulation of ovarian follicle atresia. *Annu.Rev.Physiol.* 1997; 59:349-63:349-363.

Kenney RM, Condon W, Ganjam VK, Channing C. Morphological and biochemical correlates of equine ovarian follicles as a function of their state of viability or atresia. *J.Reprod.Fertil.Suppl.* 1979; 163-171.

Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br.J.Cancer* 1972; 26:239-257.

Kerr JFR, Winterford CM, Harmon BV. Morphological criteria for identifying apoptosis. In: Anonymous*Cell biology: a laboratory handbook*. Academic Press, Inc, 1994:319-329.

Kim JM, Boone DL, Auyeung A, Tsang BK. Granulosa cell apoptosis induced at the penultimate stage of follicular development is associated with increased levels of Fas and Fas ligand in the rat ovary. *Biol.Reprod.* 1998; 58:1170-1176.

King WA, Bezard J, Bousquet D, Palmer E, Betteridge KJ. The meiotic stage of preovulatory oocytes in mares. *Genome* 1987; 29:679-682.

Kubiak JZ. Mouse oocytes gradually develop the capacity for activation during the metaphase II arrest. *Dev.Biol.* 1989; 136:537-545.

Law AS, Baxter G, Logue DN, O'Shea T, Webb R. Evidence for the action of bovine follicular fluid factor(s) other than inhibin in suppressing follicular development and delaying oestrus in heifers. *J.Reprod.Fertil.* 1992; 96:603-616.

Lawler DF, Armstrong DG, Baxter G, Hogg C, Watson ED. Localisation of mRNA encoding luteinising hormone receptor in the equine follicle and corpus luteum. *7th international symposium on equine reproduction* 1998; 57-58.

LeFevre. *Reproduction Nutrition Developpement* 1989; 29:523-532.

Lincoln GA, Fraser HM. Compensatory response of the luteinizing-hormone (LH)-releasing hormone (LHRH)/LH pulse generator after administration of a potent LHRH antagonist in the ram. *Endocrinology* 1987; 120:2245-2250.

Loeken MR, Channing CP, D'Eletto R, Weiss G. Stimulatory effect of luteinizing hormone upon relaxin secretion by cultured porcine preovulatory granulosa cells. *Endocrinology* 1983; 112:769-771.

Lorenzo PL, Illera MJ, Illera JC, Illera M. Role of egf, igf-i, sera and cumulus cells on maturation in- vitro of bovine oocytes. *Theriogenology* 1995; 44:109-118.

Mais V, Kazer RR, Cetel NS, Rivier J, Vale W, Yen SS. The dependency of folliculogenesis and corpus luteum function on pulsatile gonadotropin secretion in cycling women using a gonadotropin- releasing hormone antagonist as a probe. *J.Clin.Endocrinol.Metab.* 1986; 62:1250-1255.

Mattson BA, Albertini DF. Oogenesis: chromatin and microtubule dynamics during meiotic prophase. *Mol.Reprod.Dev.* 1990; 25:374-383.

McConnell JM, Campbell L, Vincent C. Capacity of mouse oocytes to become activated depends on completion of cytoplasmic but not nuclear meiotic maturation. *Zygote.* 1995; 3:45-55.

McGrath SA, Esquela AF, Lee SJ. Oocyte-specific expression of growth/differentiation factor-9. *Mol. Endocrinol.* 1995; 9: 131-136.

McKinnon AO, Voss JL. *Equine reproduction.* London: Lea & Febiger, 1993: 1-1137.

McNatty KP, Hillier SG, van den Boogaard AM, Trimbos-Kemper TC, Reichert LE, Jr., van Hall EV. Follicular development during the luteal phase of the human menstrual cycle. *J.Clin.Endocrinol.Metab.* 1983; 56:1022-1031.

McNatty KP, Lun S, Heath DA, Kieboom LE, Henderson KM. Influence of follicular atresia on LH-induced cAMP and steroid synthesis by bovine thecae interna. *Mol.Cell Endocrinol.* 1985; 39:209-215.

McNeilly AS, Picton HM, Campbell BK, Baird DT. Gonadotropic control of follicle growth in the ewe. *J Reprod Fert* 1991; 177-186.

Meinecke B, Gips H, Meinecketillmann S. Progestagen, androgen and estrogen-levels in plasma and ovarian follicular-fluid during the estrous-cycle of the mare. *Animal Reproduction Science* 1987; 12:255-265.

Mihm M, Good TE, Ireland JL, Ireland JJ, Knight PG, Roche JF. Decline in serum follicle-stimulating hormone concentrations alters key intrafollicular growth factors involved in selection of the dominant follicle in heifers. *Biol.Reprod.* 1997; 57:1328-1337.

Miller AT, Picton HM, Craigon J, Hunter MG. Follicle dynamics and aromatase activity in high-ovulating Meishan sows and in Large-White hybrid contemporaries. *Biol.Reprod.* 1998; 58:1372-1378.

Miller AT, Picton HM, Hunter MG. Suppression of ovarian activity in the gilt and reversal by exogenous gonadotrophin administration. *Anim.Reprod.Sci.* 1999; 54:179-193.

Mizunuma H, Liu X, Andoh K, Abe Y, Kobayashi J, Yamada K, Yokota H, Ibuki Y, Hasegawa Y. Activin from secondary follicles causes small preantral follicles to remain dormant at the resting stage. *Endocrinology* 1999; 140:37-42.

Montovan SM, Daels PF, Rivier J, Hughes JP, Stabenfeldt GH, Lasley BL. The effect of a potent gnrh agonist on gonadal and sexual- activity in the horse. *Theriogenology* 1990; 33:1305-1321.

Moodie GR, Graham EF. The effect of incubating sheep ovaries for various times and temperatures on oocyte maturation rates in vitro. *Biol Reprod* 1989; 40:-abstract 53

Moor RM, Trounson AO. Hormonal and follicular factors affecting maturation of sheep oocytes in vitro and their subsequent developmental capacity. *J.Reprod.Fertil.* 1977; 49:101-109.

Moor RM, Hay MF, Dott HM, Cran DG. Macroscopic identification and steroidogenic function of atretic follicles in sheep. *J.Endocrinol.* 1978; 77:309-318.

Moor RM, Osborn JC, Cran DG, Walters DE. Selective effect of gonadotrophins on cell coupling, nuclear maturation and protein synthesis in mammalian oocytes. *J.Embryol.Exp.Morphol.* 1981; 61:347-65:347-365.

Moor RM, Crosby IM. Temperature-induced abnormalities in sheep oocytes during maturation. *J.Reprod.Fertil.* 1985; 75:467-473.

Motlik J, Crozet N, Fulka J. Meiotic competence in vitro of pig oocytes isolated from early antral follicles. *J.Reprod.Fertil.* 1984; 72:323-328.

Motlik J, Fulka J. Factors affecting meiotic competence in pig oocytes. *Theriogenology* 1986; 25:87-96.

Murdoch WJ. Programmed cell death in preovulatory ovine follicles. *Biol.Reprod.* 1995; 53:8-12.

Murphy MG, Boland MP, Roche JF. Pattern of follicular growth and resumption of ovarian activity in post-partum beef suckler cows. *J.Reprod.Fertil.* 1990; 90:523-533.

Nagamine N, Nambo Y, Nagata S, Nagaoka K, Tsunoda N, Taniyama H, Tanaka Y, Tohei A, Watanabe G, Taya K. Inhibin secretion in the mare: localization of inhibin alpha, betaA, and betaB subunits in the ovary. *Biol.Reprod.* 1998; 59:1392-1398.

Nagyova E, Prochazka R, Vanderhyden BC. Oocytectomy does not influence synthesis of hyaluronic acid by pig cumulus cells: retention of hyaluronic acid after insulin- like growth factor-I treatment in serum-free medium. *Biol.Reprod.* 1999; 61:569-574.

Nahum R, Beyth Y, Chun SY, Hsueh AJ, Tsafriri A. Early onset of deoxyribonucleic acid fragmentation during atresia of preovulatory ovarian follicles in rats. *Biol.Reprod.* 1996; 55:1075-1080.

Naito K, Hawkins C, Yamashita M, Nagahama Y, Aoki F, Kohmoto K, Toyoda Y, Moor RM. Association of p34cdc2 and cyclin B1 during meiotic maturation in porcine oocytes. *Dev.Biol.* 1995; 168:627-634.

Nambo Y, Kaneko H, Nagata S, Oikawa M, Yoshihara T, Nagamine N, Watanabe G, Taya K. Effect of passive immunization against inhibin on FSH secretion, folliculogenesis and ovulation rate during the follicular phase of the estrous cycle in mares. *Theriogenology* 1998; 50:545-557.

Naor Z, Atlas D, Clayton RN, Forman DS, Amsterdam A, Catt KJ. Interaction of fluorescent gonadotropin-releasing hormone with receptors in cultured pituitary cells. *J.Biol.Chem.* 1981; 256:3049-3052.

Nestor JJ, Ho TL, Tahilramani R, McRae GI, Vickery BH. Long acting LHRH agonists and antagonists. In: Labrie F, Belanger A, Dupont A, eds. *LHRH and its analogues. Basic and clinical aspects*. New York, Exerpta medica, 1984:24-35.

Nuttinck F, Collette L, Massip A, Dessy F. Histologic and autoradiographic study of the in-vitro effects of fgf- 2 and fsh on isolated bovine preantral follicles - preliminary investigation. *Theriogenology* 1996; 45:1235-1245.

Oberhammer F, Fritsch G, Schmied M, Pavelka M, Printz D, Purchio T, Lassmann H, Schulte-Hermann R . Condensation of the chromatin at the membrane of an apoptotic nucleus is not associated with activation of an endonuclease. *J.Cell Sci.* 1993; 104:317-326.

Oberhammer FA, Hochegger K , Froschl G, Tiefenbacher R, Pavelka M. Chromatin condensation during apoptosis is accompanied by degradation of lamin A+B, without enhanced activation of cdc2 kinase. *J.Cell Biol.* 1994; 126:827-837.

Okolski A, Bezard J, Magistrini M, Palmer E. Maturation of oocytes from normal and atretic equine ovarian follicles as affected by steroid concentrations. *J.Reprod.Fertil.Suppl.* 1991; 44:385-92:385-392.

Palmer E, Driancourt MA. Use of ultrasound echography in equine gynecology. *Theriogenology* 1980; 13:203-216.

Palmer E, Quellier P. Uses of LHRH and analogues in the mare. *Proc 11th Int Congr Anim Reprod & AI, Dublin* 1988; 5:339-346.

Palmer E, Bezard J, Magistrini M, Duchamp G. In vitro fertilization in the horse. A retrospective study. *J.Reprod.Fertil.Suppl.* 1991; 44:375-84:375-384.

Pantke P, Hyland J, Galloway DB, MacLean AA, Hoppen HO. Changes in luteinizing hormone bioactivity associated with gonadotrophin pulses in the cycling mare. *J.Reprod.Fertil.Suppl.* 1991; 44:13-18.

Perez GI, Tao XJ, Tilly JL. Fragmentation and death (a.k.a. apoptosis) of ovulated oocytes. *Mol.Hum.Reprod.* 1999; 5:414-420.

Pesce M, Defelici M. Apoptosis in mouse primordial germ-cells - a study by transmission and scanning electron-microscope. *Anatomy And Embryology* 1994; 189:435-440.

Peters KE, Bergfeld EG, Cupp AS, Kojima FN, Mariscal V, Sanchez T, Wehrman ME, Grotjan HE, Hamernik DL, Kittok RJ. Luteinizing hormone has a role in development of fully functional corpora lutea (CL) but is not required to maintain CL function in heifers . *Biol.Reprod.* 1994; 51:1248-1254.

Pickering SJ, Braude PR, Johnson MH, Cant A, Currie J. Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. *Fertil.Steril.* 1990; 54:102-108.

Picton HM, Tsonis CG, McNeilly AS. FSH causes a time-dependent stimulation of preovulatory follicle growth in the absence of pulsatile LH secretion in ewes chronically treated with gonadotrophin-releasing hormone agonist. *J Endocr* 1990a; 126:297-307.

Picton HM, Tsonis CG, McNeilly AS. The antagonistic effect of exogenous LH pulses on FSH-stimulated preovulatory follicle growth in ewes chronically treated with a gonadotrophin-releasing hormone agonist. *J.Endocrinol.* 1990b; 127:273-283.

Pierson RA, Ginther OJ. Follicular population dynamics during the estrous cycle of the mare. *Animal Reproduction Science* 1987; 14:219-231.

Pincus G, Enzmann EV. The comparative behavior of mammalian eggs in vivo and in vitro. *Journal of Experimental Medicine* 1935; 62:665-675.

Pineda MH, Ginther OJ. Inhibition of estrus and ovulation in mares treated with an antiserum against an equine pituitary fraction. *Am.J.Vet.Res.* 1972a; 33:1775-1780.

Pineda MH, Ginther OJ, McShan WH. Regression of corpus luteum in mares treated with an antiserum against an equine pituitary fraction. *Am.J.Vet.Res.* 1972b; 33:1767-1773.

Pineda MH, Garcia MC, Ginther OJ. Effect of antiserum against an equine pituitary fraction on corpus luteum and follicles in mares during diestrus. *Am.J.Vet.Res.* 1973; 34:181-183.

Plata-Madrid H, Loch WE, Youngquist RS, Thompson Jr DL, Bennett-Wimbush KG, Wilkerson C, Bouchard G, Smith MF, Braun WF, Aveiro JJ. Control of FSH, follicular development and estrus synchronization in the mare with steroid-free follicular fluid. *Theriogenology* 1992; 37:817-838.

Porter MB, Cleaver BD, Peltier M, Robinson G, Sharp DC. The effect of pulsatile gonadotropin-releasing hormone and estradiol administration on luteinizing hormone and follicle- stimulating hormone concentrations in pituitary stalk-sectioned ovariectomized pony mares. *Domest.Anim.Endocrinol.* 1997; 14:275-285.

Quirk SM, Cowan RG, Joshi SG, Henrikson KP. Fas antigen-mediated apoptosis in human granulosa/luteal cells. *Biol.Reprod.* 1995; 52:279-287.

Ralph JH, Telfer EE, Wilmut I. Bovine cumulus cell expansion does not depend on the presence of an oocyte secreted factor. *Mol. Reprod. Dev.* 1995; 42: 248-253.

Ratts VS, Flaws JA, Kolp R, Sorenson CM, Tilly JL. Ablation of bcl-2 gene-expression decreases the numbers of oocytes and primordial follicles established in the postnatal female mouse gonad. *Endocrinology* 1995; 136:3665-3668.

Richard FJ, Sirard MA. Effects of harvest methods of bovine oocytes cocultured with follicular hemisections in-vitro on nuclear maturation. *Theriogenology* 1996; 46:1243-1250.

Rieger D, Luciano AM, Modina S, Pocar P, Lauria A, Gandolfi F. The effects of epidermal growth factor and insulin-like growth factor I on the metabolic activity, nuclear maturation and subsequent development of cattle oocytes in vitro. *J.Reprod.Fertil.* 1998; 112:123-130.

Rippel RH, Johnson ES. Inhibition of HCG-induced ovarian and uterine weight augmentation in the immature rat by analogs of GnRH. *Proc.Soc.Exp.Biol.Med.* 1976; 152:432-436.

Robertson DM, Foulds LM, Fry RC, Cummins JT, Clarke I. Circulating half-lives of follicle-stimulating hormone and luteinizing hormone in pituitary extracts and isoform fractions of ovariectomized and intact ewes. *Endocrinology* 1991; 129:1805-1813.

Roser JF, Hughes JP. Prolonged pulsatile administration of gonadotrophin-releasing hormone (GnRH) to fertile stallions. *J.Reprod.Fertil.Suppl.* 1991; 44:155-168.

Roy SK, Greenwald GS. Follicular development through preantral stages - signaling via growth-factors. *J Reprod Fert* 1996; 83-94.

Salfen BE, Cresswell JR, Xu ZZ, Bao B, Garverick HA. Effects of the presence of a dominant follicle and exogenous oestradiol on the duration of the luteal phase of the bovine oestrous cycle. *J.Reprod.Fertil.* 1999; 115:15-21.

Salustri A, Yanagishita M, Hascall VC. Synthesis and accumulation of hyaluronic acid and proteoglycans in the mouse cumulus cell-oocyte complex during follicle- stimulating hormone- induced mucification. *J.Biol.Chem.* 1989; 264:13840-13847.

Salustri A, Yanagishita M, Hascall VC. Mouse oocytes regulate hyaluronic acid synthesis and mucification by FSH-stimulated cumulus cells . *Dev.Biol.* 1990; 138:26-32.

Savio JD, Keenan L, Boland MP, Roche JF. Pattern of growth of dominant follicles during the oestrous cycle of heifers. *J.Reprod.Fertil.* 1988; 83:663-671.

Schroeder AC, Johnston D, Eppig JJ. Reversal of postmortem degeneration of mouse oocytes during meiotic maturation in vitro. *J.Exp.Zool.* 1991; 258:240-245.

Schwartz LM, Osborne BA. Programmed cell death, apoptosis and killer genes. *Immunol.Today* 1993; 14:582-590.

Schwartzman RA, Cidlowski JA . Apoptosis: the biochemistry and molecular biology of programmed cell death. *Endocr.Rev.* 1993; 14:133-151.

Shabpareh V, Squires EL, Seidel GE, Jasko DJ. Methods for collecting and maturing equine oocytes in-vitro . *Theriogenology* 1993; 40:1161-1175.

Sharp DC, Grubaugh WR, Weithenauer J, Davis SD, Wilcox CJ. Effects of steroid administration on pituitary luteinizing hormone and follicle-stimulating hormone in ovariectomized pony mares in the early spring: pituitary responsiveness to gonadotropin-releasing hormone and pituitary gonadotropin content. *Biol.Reprod.* 1991; 44:983-990.

Shaw JM, Trounson AO. Parthenogenetic activation of unfertilized mouse oocytes by exposure to 1,2-propanediol is influenced by temperature, oocyte age, and cumulus removal. *Gamete Res.* 1989; 24:269-279.

Siegel S. *Nonparametric statistics for the behavioural sciences*. London: McGraw-Hill International book company, 1956: 1-312.

Singh J, Adams GP. Histomorphometry of dominant and subordinate bovine ovarian follicles. *Anat.Rec.2000.Jan.1;258(1):58-70.* 2000; 258:58-70.

Sirois J, Ball BA, Fortune JE. Patterns of growth and regression of ovarian follicles during the oestrous cycle and after hemiovariectomy in mares. *Equine Veterinary Journal* 1989; Supplement 8:43-48.

Sirois J, Kimmich TL, Fortune JE. Steroidogenesis by equine preovulatory follicles: Relative roles of theca interna and granulosa cells . *Endocrinology* 1991; 128:1159-1166.

Song L, Porter DG, Coomber BL. Production of gelatinases and tissue inhibitors of matrix metalloproteinases by equine ovarian stromal cells In vitro. *Biol.Reprod.* 1999; 60:1-7.

Sorensen RA, Wassarman PM. Relationship between growth and meiotic maturation of the mouse oocyte. *Dev.Biol.* 1976; 50:531-536.

Squires EL, Garcia RH, Ginther OJ, Voss JL, Seidel GE. Comparison of equine pituitary extract and follicle-stimulating hormone for superovulating mares. *Theriogenology* 1986; 26:661-670.

Squires EL. Diagnostic-techniques and assisted reproductive technology. *Veterinary Clinics Of North America-Equine Practice* 1996; 12:

Sun DY, Jiang S, Zheng LM, Ojcius DM, Young JD. Separate metabolic pathways leading to DNA fragmentation and apoptotic chromatin condensation. *J.Exp.Med.* 1994; 179:559-568.

Sunderland SJ, Crowe MA, Boland MP, Roche JF, Ireland JJ. Selection, dominance and atresia of follicles during the estrous- cycle of heifers. *J Reprod Fert* 1994; 101:547-555.

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 1999; 397:441-446.

Suzuki DT, Griffiths AJF, Lewontin RC. Chromosome theory of inheritance. In: *An introduction to genetic analysis*. 1989:39-46. Publisher WH Freeman, New York.

Telfer EE, Christy C, Binnie JP. Oocytes secrete a factor(s) that suppresses apoptosis in granulosa cells in murine preantral follicles. *Journal of Reproduction and Fertility Abstract Series* 1999; 9-10.

Thanki KH, Channing CP. Influence of serum, estrogen, and gonadotropins upon growth and progesterone secretion by cultures of granulosa cells from small porcine follicles. *Endocr.Res.Commun.* 1976; 3:319-333.

Thompson DL, Jr, Godke RA, Squires EL. Testosterone effects on mares during synchronization with altrenogest: FSH, LH, estrous duration and pregnancy rate. *J. Anim. Sci.* 1983; 56: 678-686.

Thompson DL, Jr., McNeill DR, Wiest JJ, St.George RL, Jones LS, Garza F, Jr. Secretion of luteinizing hormone and follicle stimulating hormone in intact and ovariectomized mares in summer and winter . *J.Anim.Sci.* 1987; 64:247-253.

Thompson DL, Jr., Garza F, Jr., St George RL, Rabb MH, Barry BE, French DD. Relationships among LH, FSH and prolactin secretion, storage and response to secretagogue and hypothalamic GnRH content in ovariectomized pony mares administered testosterone, dihydrotestosterone, estradiol, progesterone, dexamethasone or follicular fluid. *Domest.Anim.Endocrinol.* 1991; 8:189-199.

Tilly JL, Kowalski KI, Johnson AL, Hsueh AJ. Involvement of apoptosis in ovarian follicular atresia and postovulatory regression. *Endocrinology* 1991; 129:2799-2801.

Tilly JL, Billig H, Kowalski KI, Hsueh AJ. Epidermal growth factor and basic fibroblast growth factor suppress the spontaneous onset of apoptosis in cultured rat ovarian granulosa cells and follicles by a tyrosine kinase- dependent mechanism. *Mol.Endocrinol.* 1992; 6:1942-1950.

Tilly JL, Hsueh AJ. Microscale autoradiographic method for the qualitative and quantitative analysis of apoptotic DNA fragmentation. *J.Cell Physiol.* 1993; 154:519-526.

Tilly JL. Apoptosis and ovarian function. *Rev.Reprod.* 1996; 1:162-172.

Tirone E, D'Alessandris C, Hascall VC, Siracusa G, Salustri A. Hyaluronan synthesis by mouse cumulus cells is regulated by interactions between follicle-stimulating hormone (or epidermal growth factor) and a soluble oocyte factor (or transforming growth factor beta1). *J.Biol.Chem.* 1997; 272:4787-4794.

Torner, A. and Alm, H. Meiotic configuration of horse oocytes in relation to the morphology of the cumulus-oocyte complex. Sharp, D. C. and Bazer, F. W. Monograph series 1(VI), 253-259. 1995. USA, Edwards Brothers.

Tortonese DJ, Lewis PE, Papkoff H, Inskeep EK. Roles of the dominant follicle and the pattern of oestradiol in induction of preovulatory surges of LH and FSH in prepubertal heifers by pulsatile low doses of LH. *J.Reprod.Fertil.* 1990; 90:127-135.

Townson DH, Pierson RA, Ginther OJ. Characterization of plasma progesterone concentrations for 2 distinct luteal morphologies in mares. *Theriogenology* 1989; 32:197-204.

Tsafriri A, Pomerantz SH. Regulation of the development of meiotic competence and of the resumption of oocyte maturation in the rat. *Symp.Soc.Exp.Biol.* 1984; 38:25-43:25-43.

Tsafriri A, Dekel N. Molecular mechanisms in ovulation. In: Findlay JK, ed. *Molecular biology of the female reproductive system*. London, Academic press, 1994:207-258.

Tucker KE, Henderson KA, Duby RT. In vitro steroidogenesis by granulosa-cells from equine preovulatory follicles. *J Reprod Fert* 1991; suppl 44:45-55.

Turzillo AM, Fortune JE. Suppression of the secondary fsh surge with bovine follicular-fluid is associated with delayed ovarian follicular development in heifers. *Journal Of Reproduction & Fertility* 1990; 89:643-653.

Van Blerkom J, Davis PW. DNA strand breaks and phosphatidylserine redistribution in newly ovulated and cultured mouse and human oocytes: occurrence and relationship to apoptosis. *Hum.Reprod.* 1998; 13:1317-1324.

Vanderhyden BC, Telfer EE, Eppig JJ. Mouse oocytes promote proliferation of granulosa-cells from preantral and antral follicles invitro. *Biol Reprod* 1992; 46:1196-1204.

Vanderhyden BC, Macdonald EA . Mouse oocytes regulate granulosa cell steroidogenesis throughout follicular development. *Biol.Reprod.* 1998; 59:1296-1301.

Wang XN and Greenwald GS. Hypophysectomy of the cyclic mouse. II. Effects of follicle-stimulating hormone (FSH) and luteinizing hormone on folliculogenesis, FSH and human chorionic gonadotropin receptors, and steroidogenesis. *Biol Reprod* 1993; 48: 595-605.

Wassarman PM, Letourneau GE. Meiotic maturation of mouse oocytes in vitro: association of newly synthesized proteins with condensing chromosomes. *J.Cell Sci.* 1976a; 20:549-568.

Wassarman PM, Josefowicz WJ, Letourneau GE. Meiotic maturation of mouse oocytes in vitro: inhibition of maturation at specific stages of nuclear progression. *J.Cell Sci.* 1976b; 22:531-545.

Wassarman PM, Albertini DF. The mammalian ovum. In: Knobil E , Neill JD, eds. *The physiology of reproduction*. New York, Raven press, 1994:79-122.

Watson ED, Colston M, Broadley C. LH and progesterone concentrations during diestrus in the mare and the effect of hCG. *Theriogenology* 1995; 43:1325-1337.

Whitmore HL, Wentworth BC, Ginther OJ. Circulating concentrations of luteinizing hormone during estrous cycle of mares as determined by radioimmunoassay. *Am.J.Vet.Res.* 1973; 34:631-636.

Wickramasinghe D, Ebert KM , Albertini DF. Meiotic competence acquisition is associated with the appearance of M-phase characteristics in growing mouse oocytes. *Dev.Biol.* 1991; 143:162-172.

Wu B, Ignotz G, Currie WB, Yang X. Dynamics of maturation-promoting factor and its constituent proteins during in vitro maturation of bovine oocytes. *Biol.Reprod.* 1997; 56:253-259.

Wyllie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* 1980a; 284:555-556.

Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. *Int.Rev.Cytol.* 1980b; 68:251-306:251-306.

Xu Z, Garverick HA, Smith GW, Smith MF, Hamilton SA, Youngquist RS. Expression of follicle-stimulating hormone and luteinizing hormone receptor messenger ribonucleic acids in bovine follicles during the first follicular wave. *Biol.Reprod.* 1995; 53:951-957.

Zamzami N, Hirsch T, Dallaporta B, Petit PX, Kroemer G. Mitochondrial implication in accidental and programmed cell death: apoptosis and necrosis. *J.Bioenerg.Biomembr.* 1997; 29:185-193.

Zeron Y, Pearl M, Borochov A, Arav A. Kinetic and temporal factors influence chilling injury to germinal vesicle and mature bovine oocytes. *Cryobiology.* 1999; 38:35-42.

Zhang JJ, Boyle MS, Allen WR, Galli C. Recent studies on in vivo fertilisation of in vitro matured horse oocytes. *J Reprod Fert* 1989; Supplement 8:101-104.

Zuccotti M, Rossi PG, Martinez A, Garagna S, Forabosco A, Redi CA. Meiotic and developmental competence of mouse antral oocytes. *Biol.Reprod.* 1998; 58:700-704.