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Follicular Development Patterns of Pregnant Mares During the First Half of Gestation.

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Louisiana State University and Agricultural & Mechanical College

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FOLLICULAR DEVELOPMENT PATTERNS OF PREGNANT MARES DURING THE FIRST HALF OF GESTATION

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Interdepartmental Program in Veterinary Medical Sciences through the Department of Veterinary Physiology, Pharmacology and Toxicology

by Marius Meintjes
BVSc., University of Pretoria, 1988
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ABSTRACT

Information gained by studying follicular wave and endocrinological patterns of pregnant mares, may be useful in understanding reproductive wastage, infertility, control of the estrous cycle and physiological function of equine chorionic gonadotropin (eCG). Furthermore, supplementary follicular development, characteristic of the early equine pregnancy, may be used to harvest oocytes for gamete intrafallopian tube transfer and in vitro fertilization (IVF) procedures. The objectives of this study were to: characterize follicular wave patterns during the first half of the equine pregnancy; relate follicular development patterns to serum levels of estradiol-17β, progesterone, FSH and eCG; evaluate the effect of fetal genotype on follicular dynamics; evaluate the repeatability and safety of transvaginal oocyte recovery procedures on pregnant mares; evaluate the viability of recovered oocytes by a novel IVF procedure; and test the applicability of this aspiration and IVF technology in the Burchell’s zebra. Three follicular aspiration treatments were applied to mares pregnant with horse and mule fetuses. The most striking difference in follicular development patterns was observed between mule and horse pregnancies. Mares pregnant with mules did not have an equivalent follicular activity peak corresponding to peak levels of mule eCG as did mares pregnant with horse fetuses. Mares carrying horse pregnancies had markedly reduced follicular activity during the post-eCG period when compared with that of mule pregnancies. Results presented suggest that the phenomenon of reduced follicular activity in mares carrying horse fetuses involves desensitization of the ovary by exposure to high concentrations of horse eCG and a direct inhibitory effect of horse eCG on FSH. Although repeated aspiration of follicles influenced follicular wave parameters and hormone profiles, the ongoing pregnancy was not endangered. Zona-drilled, IVF oocytes from pregnant mares were cultured to morula and blastocyst
stages, indicating that these oocytes were viable. To our knowledge, this is the first report of successful IVF of equine oocytes collected from pregnant donors. The oocyte aspiration procedure was equally effective, safe and repeatable in zebra mares. The oocytes of zebra mares similarly produced blastocyst-stage embryos after exposure to the in vitro maturation, IVF and in vitro culture procedures developed for horse oocytes.
INTRODUCTION

Pregnancy loss in the mare is a major economic concern for the horse breeding industry (Ginther, 1985; Forde et al., 1987; Voller et al., 1991). When normal fertile mares are mated, only 64% of ovulated oocytes result in a term pregnancy; whereas, in subfertile mares the term pregnancy rate can be as low as 12% (Ginther, 1992). It is well documented that reproductive efficiency declines with increasing age in the mare as indicated by decreased pregnancy rates and increased embryo-loss rates in older mares (Woods, et al., 1987; Chevalier-Clement, 1989). This decline in fertility may be attributed to age related changes in the uterus (Carnevale et al., 1994), changes in hormonal and follicular wave characteristics (Carnevale et al., 1993a, 1994; Ginther et al., 1993) or defective oocytes (Carnevale & Ginther, 1994; Brinsko et al., 1995). Also, prized mares of a breed often do not produce embryos with standard embryo flushing techniques. Thus, a new method is proposed for harvesting the genetic potential of these valuable mares by the collection of oocytes prior to ovulation, followed by in vitro fertilization in a laboratory setting and transferring of these in vitro-fertilized embryos to cyclic recipient mares.

There are several additional advantages that could be gained from in vitro fertilization (IVF) in the horse. This approach may: 1) provide a supply of embryos at different stages of development for embryo or genetic micromanipulation or could be used as a model for studying early events of fertilization; 2) provide an useful in vitro test for stallion fertility; and 3) expand the reproductive potential of valuable mares and endangered equine species.

In 1978, the first human baby was born in England as a result of IVF (Steptoe & Edwards, 1978). Subsequently, researchers in Pennsylvania produced the first live calf from an IVF procedure in 1981 (Brackett et al., 1982b). Since then, IVF has
become a standard method for treating infertility in human couples (Trounson et al., 1994). In the last few years, IVF in cattle has become an invaluable tool for advancing embryo research (Hasler, 1992). Recently, there has been a keen interest in using this technology to treat valuable, clinically infertile cows (Gordon & Lu, 1990; Looney et al., 1994). There is only limited evidence of confirmed IVF of equine oocytes, with only one report in the scientific literature of a live foal born in 1990 in France, after surgical oviductal transfer of an early stage embryo (Palmer et al., 1991). For IVF to become commercially viable in the equine industry, IVF-derived embryos need to be obtained routinely and cultured to later morula or blastocyst stages to allow nonsurgical transfer to recipient mares. Research on equine IVF procedures has been behind that of other species, partially because of the difficulty to obtain viable oocytes. Ovaries obtained from horse slaughter plants originate from less than adequate animals, and often the oocytes deteriorate before they arrive at the IVF laboratory (Fayrer-Hosken et al., 1993). For the development of a commercially-viable IVF procedure for the horse, a successful and repeatable in vivo oocyte recovery technique must be developed for in-field use.

In recent years, efforts have been made to develop in situ oocyte recovery methods in the mare. Various approaches that have been used to recover oocytes include recovery through flank surgery (Betteridge et al., 1982; McKinnon et al., 1988) and paralumbar needle puncture of follicles with manipulation of the ovary per rectum (Hinrichs & Kenney, 1987; Palmer et al., 1987). These methods are considered to be invasive, often cumbersome and to have low repeatability due to adhesion and scar tissue formation in donor mares.

Recently, as in humans (Dellenbach et al., 1985) and cattle (Pieterse et al., 1988, 1991; Kruip et al., 1991), repeated, nonsurgical ultrasound-guided follicular
aspiration procedures have been attempted to recover oocytes from cycling mares (Brück et al., 1992; Cook et al., 1992, 1993b). Pregnancies resulted from transvaginally aspirated in vivo matured oocytes after gamete intrafallopian tube transfer (GIFT) (Carnevale & Ginther, 1994; Ray et al., 1994). However, for this approach to be successful, one to six oocytes need to be transferred to a single recipient. Only the preovulatory dominant follicle can be aspirated, since the subordinate follicles are atretic and contain degenerative oocytes (Lacker et al., 1987; Okolski et al., 1991; Barrisco et al., 1992; Bergfelt & Ginther, 1993; Findlay, 1994). Furthermore, transvaginal aspiration of preovulatory follicles probably would never yield a 100% recovery rate for cyclic mares, since recovery rates reported to date have varied between 25% (Brück et al., 1992) and 84% (Ray et al., 1994).

Superovulation seems to be one approach to produce multiple oocytes from one donor mare. However, the mare is not easily superovulated with exogenous gonadotropins. The use of follicle stimulating hormone (FSH) and other gonadotropic agents have only produced limited ovarian superstimulation in mares (Woods & Ginther, 1983; McCue et al., 1993; Hinrichs, 1994), and the amount of some of these hormones required for a superovulatory response is nearly 70 times that used to induce supplemental follicular growth in cattle (Ginther, 1992).

Many researchers agree that multiple follicular development occur in the ovaries of the mare during the first 4 months of pregnancy (Allen, 1974; Squires, 1979; Urwin & Allen, 1982b). With the high efficiency of current in vitro maturation (IVM) procedures of equine oocytes (Hinrichs et al., 1993; Okolski et al., 1993; Alm & Torner, 1994), the collection of multiple uniform immature oocytes from a pregnant donor for IVF or GIFT may be feasible as long as the ongoing pregnancy is not jeopardized.
Although several hypotheses exist about the physiological role of equine chorionic gonadotropin (eCG) and several biological effects of eCG have been identified, the precise role of this well-described hormone of pregnancy has not yet been elucidated (Allen, 1984; Manning et al., 1987; Anderson, 1988; McFarlane et al., 1991a). The high level of ovarian activity in the pregnant mare is commonly attributed to the presence of eCG, however, considerable follicular development is present (before day 20 of pregnancy) long before the first detection of eCG in the circulating plasma (days 35 to 40 of pregnancy) (Cole & Hart, 1930). Several authors have suggested that rhythmic waves of follicular activity occur during early pregnancy in the horse (Evans & Irvine, 1975; Allen, 1982), and that these waves are probably associated with similar rhythmic surges of follicle stimulating hormone (FSH) in the peripheral plasma. Using transrectal ultrasonography, the relationship between circulating levels of FSH and waves of follicular activity have recently been investigated up to day 50 of pregnancy (Bergfelt & Ginther, 1992; Ginther & Bergfelt, 1992a). Bearing in mind that the endocrine patterns in the pregnant mare change dynamically during the course of pregnancy (Ginther, 1992), a definite need exists to characterize this intricate relationship between the hormones of pregnancy and ovarian activity through later stages of pregnancy.

By studying the temporal relationships between follicular wave and endocrinological patterns of pregnancy in the horse, new information may be found that can be useful in the understanding of reproductive wastage, subfertility, artificial control of the estrous cycle and pregnancy, the physiological function of eCG and basic physiology of the equine pregnancy. The use of interspecies and extraspecies pregnancies in equids are valuable tools when the role of eCG is studied, since the
FSH:LH ratio (Stewart et al., 1977; Aggarwal et al., 1980) and quantities of eCG (Allen, 1982, 1984; Allen et al., 1993) differ dramatically between these pregnancies.

If the oocytes from the follicles of pregnant mares are viable and could indeed be used for GIFT and IVF procedures, accurate characterization of follicular waves of pregnancy will be essential to ensure optimal timing and frequency of repeated oocyte collections. The timing will be critical to achieve maximum oocyte recovery and to ensure that oocytes are collected from nonatretic follicles.

This study was therefore designed to: 1) accurately characterize follicular wave patterns during the first half of pregnancy (days 21 to 150) in the mare; 2) temporally relate the established patterns of follicular development to corresponding serum levels of estradiol-17β, progesterone, FSH and eCG; 3) evaluate the effect of genotype of the fetus on the follicular dynamics of pregnancy; 4) evaluate the efficacy, repeatability and safety of transvaginal oocyte recovery procedures on cycling and pregnant horse mares; 5) evaluate the viability of recovered oocytes by a novel in vitro fertilization procedure; and 6) to test the applicability of this aspiration and IVF technology in free ranging and semi-captive Burchell's zebra.
CHAPTER I
LITERATURE REVIEW

Follicular Activity in the Mare

During earlier studies on in situ follicle development in the horse, information was obtained by rectal palpation (Allen, 1975a, 1977; Squires et al., 1983), surgical or laparoscopic visualization of the ovaries (Witherspoon, 1975), since ultrasound scanners were not yet available to the research community. The anatomy of the horse ovary is species specific in that the follicle-rich ovarian cortex is entirely surrounded by the medullary tissue, except for a small area called the ovulatory fossa (Stabenfeldt et al., 1975; Witherspoon, 1975). For this reason, corpora lutea and smaller follicles are situated under the surface of the ovary and can not be readily palpated. The frequency at which ovarian activity can be monitored by surgery or laparoscopy is limited by the invasiveness of these procedures. Pertinent information can be gained by the physical (Hinrichs, 1991; Okolski et al., 1991) and histological (Van Niekerk et al., 1975; Barrisco et al., 1992) examination of surgically removed ovaries (Pineda et al., 1973) or from ovaries obtained at slaughter (Wesson & Ginther, 1981). A weakness in the studies on horse ovaries obtained from slaughterhouses is that the stage of the cycle can not be accurately determined, often only estimated by the presence and size of luteal and follicular structures.

With the availability of ultrasound technology in the 1980s and its application to the reproductive biology of the mare, many previously unanswered questions could now be addressed and results from earlier studies confirmed (Ginther, 1992). Frequent noninvasive visualization of the ovaries makes it possible to identify individual follicles (Bergfelt & Ginther, 1993), obtain accurate follicular sizes and to track the dynamic follicular populations over a period of time (Ginther, 1993). Furthermore, accurate
assessments can be made of small (2 to 20 mm) follicles (Ginther & Bergfelt, 1993), the ovulatory process can be closely monitored (Pierson & Ginther, 1985b) and the development, maintenance and regression of the corpus luteum can be evaluated (Pierson & Ginther, 1985a; Daels et al., 1992). In recent years, ultrasonography contributed markedly to the current knowledge of follicular dynamics in the mare.

A Model for Folliculogenesis

In the human and other vertebrate species, the gametogenic potential of the ovary is established early in fetal development, although it only starts to function as an endocrine organ at puberty. Primordial germ cells undergo rapid proliferation and give rise to millions of oocytes in the cortex of the ovary, however, by the time of puberty each human ovary may only be populated by ~250,000 oocytes. These oocytes are surrounded by a single layer of rectangular granulosa cells to form a primordial follicle (Hadley, 1988). Similarly, 18,000 and 60,000 primordial follicles were identified per ovary in mares and cows, respectively (Ginther, 1992).

This pool of primordial follicles is essentially quiescent and subject to minimal atresia. Multiple primordial follicles are recruited to commit to growth and form primary follicles. The number of primordial follicles that will commit to growth is not under the control of gonadotropins, but may be influenced by other nonendocrine factors such as the species, local ovarian bloodflow and dietary energy (Swinker et al., 1993; Findlay, 1994). Once committed to growth, the granulosa cells become cuboidal, and these primary follicles can not revert back to form primordial follicles (Hadley, 1988; Findlay, 1994).

It is speculated that these primary follicles continuously emerge and regress to provide a reservoir for larger follicles, and that this basal activity occurs (not gonadotropin dependent) during all reproductive states including pregnancy,
pseudopregnancy, diestrus and anestrus. However, it is not clear if the level of this underlying activity is constant for the different reproductive states or at different seasons of the year. Furthermore, it is unclear if primordial follicle recruitment occurs in a synchronized manner where groups of follicles enter the growth phase, or whether the follicles are recruited independently (Ginther, 1992).

A small species-characteristic number of developing follicles emerges from the committed reservoir of primary follicles during a hormone sensitive time window from which only one or several will eventually ovulate. The remaining follicles of the group will undergo atresia. The number and quality of secondary and tertiary follicles in the developing follicular wave is thus dependent on the availability of primary follicles in the recruitment pool. It is assumed that there is a definite hierarchy among committed follicles according to their developmental status. Interactions between the developmental status and other endocrine, paracrine and autocrine factors probably determine the ovulatory fate of the follicle (Lacker et al., 1987).

Follicles that enter the follicular wave go through several chronological steps of differentiation. Committed follicles form a follicular antrum (secondary follicle) and the granulosa cells become gonadotropin responsive (first to FSH and then to LH) but these have not yet become gonadotropin dependent. Thereafter, these follicles will become gonadotropin dependent (e.g., become atretic if the concentration of FSH drops below 1 ng per ml in sheep) and a high rate of follicular atresia will be evident (Findlay, 1994). Finally, the granulosa cells of the ovulatory (dominant) follicle(s) will express LH receptors (can survive a FSH concentration below 1 ng per ml) and ovulate in the presence of an LH surge (Findlay, 1994). In the absence of an LH surge, the ovulatory follicle(s) will also undergo atresia (Badinga et al., 1994; Findlay, 1994). It takes 4 to 5 months for an individual primordial follicle to develop to an ovulatory
Folicicle according to studies in sheep (Betteridge et al., 1989). It can be assumed to be similar for the mare (Ginther, 1992).

**Follicular Waves**

**Follicular waves of nonequine species**

The existence of follicular wave growth patterns has first been characterized in cattle by using sequential rectal ultrasound imaging (Savio et al., 1988; Sirois & Fortune, 1988). Follicular development in cattle was characterized by the synchronous recruitment, emergence and development of two (Ginther et al., 1989c; Knopf et al., 1989) or three (Savio et al., 1988; Sirois & Fortune, 1988) successive cohorts of follicles during the estrous cycle. This synchronous development of a group of follicles is termed a follicular wave.

In the cow, a single follicle is selected from each cohort to develop to a large dominant follicle within the wave, while the development of the remaining follicles (secondary follicles) are suppressed by local and systemic mechanisms and become atretic (Turzillo & Fortune, 1993; Findlay, 1994). Usually, the dominant follicle of the waves during diestrus will be anovulatory and only the one associated with estrus (exposed to the preovulatory LH surge) will ovulate. It appears that different populations of heifers differ in the proportion of two-wave and three-wave interovulatory intervals. Some studies (Savio et al., 1988; Sirois & Fortune, 1988) have consistently demonstrated a predominance (80%) of three-wave interovulatory intervals; whereas, others experienced a high frequency (81%) of two-wave interovulatory intervals (Ginther et al., 1989b, c).

The mean length of two-wave interovulatory intervals of cattle are shorter (20.4 days) than those of three-wave interovulatory intervals (22.8 days)(Ginther et al., 1989c. Also, the presence of a third wave is associated with a longer luteal phase than
for animals with only two waves. As a general rule, the viable dominant follicle present at the time of luteolysis (third wave for three-wave animals and second wave for two-wave animals) will become the ovulatory follicle so that for all interovulatory intervals, luteal regression occurs after emergence of the ovulatory wave. Furthermore, the first wave of the next cycle in cattle do not emerge until near or at the day of ovulation (Ginther et al., 1989c). The factors responsible and the clinical implications of the occurrence of two-wave interovulatory intervals in some animals and three-wave follicular intervals in others, have not yet been elucidated.

According to subsequent studies (Ginther et al., 1989b; Bergfelt et al., 1991), the presence of a longer luteal phase in three-wave cycling animals and the ovulation of the viable dominant follicle present at the time of luteolysis, was a strong indication that waves of anovulatory follicular activity will occur at regular intervals until the corpus luteum regresses. The periodic emergence of anovulatory follicular waves should therefore, continue during periods of progesterone dominance such as pseudopregnancy, pregnancy or nonpregnant progesterone-treated animals.

There is evidence of follicular activity during early pregnancy in cows (Guilbault et al., 1986; Pierson & Ginther, 1986). Indeed, periodic waves of anovulatory follicular activity were identified in nonpregnant progesterone-treated heifers (Bergfelt et al., 1991) as well as in pregnant Holstein heifers between days 0 and 70 of pregnancy with a constant interwave interval (8.5 to 9.8 days) (Ginther et al., 1989b). A dominant follicle of any follicular wave during pregnancy did not begin to regress before the emergence of a new wave, indicating that a developing dominant follicle is not only instrumental in the suppression of its own subordinate follicles, but also to the dominant follicle of the previous wave. These effects are apparently exerted through systemic and not local intraovarian pathways (Ginther et al., 1989a). The significance
of continued follicular activity during early pregnancy is not clear, but may serve to keep the animal in a constant state of reproductive readiness in the case of luteolysis and pregnancy loss.

Besides the cow, evidence for the follicular wave phenomenon has also been documented in other large domesticated animals. Results from transrectal ultrasound studies in the ewe, implies that two waves of follicular activity occur during the 16- to 18-day estrous cycle (Ravindra et al., 1993; Shrick et al., 1993). Follicular development was also studied by transrectal ultrasonography in four consecutive cycles in Saanen goats. Four consecutive follicular waves were identified in 75% of the interovulatory intervals with ovulation occurring from the dominant follicle of wave number 4 (Ginther & Kot, 1994). In the other 25% of interovulatory intervals, some waves merged together and could not be identified as individual follicular waves. Apparent follicular dominance was only expressed in some waves, with dominance identified more frequently in the first and fourth waves (Ginther & Kot, 1994).

Waves of follicular activity were similarly identified in pregnant and nonpregnant llamas, a domesticated member of the new world camelids. Llamas are considered to be induced ovulators and therefore, unmated animals should not ovulate. Adams and co-workers (1990), have reported that the interwave interval for unmated (nonpregnant, anovulatory), vasectomy-mated (nonpregnant, ovulatory) and pregnant (anovulatory) llamas was 19.9, 19.7 and 14.8 days, respectively. Lactation was associated with a 2.5 day shorter interwave interval across all treatment groups in this study. Furthermore, lactation and pregnancy caused reduced prominence of dominant follicles. However, it was clear that pertinent follicular waves occur in llamas for all three of these reproductive states (Adams et al., 1990).
It has been reported in the pig that successive populations of primordial follicles are recruited into a pool of intermediate size follicles during diestrus, and that ovulatory follicles are obtained from this pool after luteolysis (Greenwald & Moor, 1989). Evidence was also presented in this study that follicular populations were greater in pregnant than in nonpregnant sows. However, in contrast to other species so far presented, a recent transrectal ultrasound study by Ryan et al., (1994), found that the proportion of different size category follicles in sows remained constant up to day 20 of pregnancy and day 15 of the estrous cycle. It was concluded that synchronous follicular recruitment occurs only around day 15 of the estrous cycle and that follicular waves could not be identified during earlier stages of the estrous cycle or during early pregnancy.

**Follicular waves in the mare**

The ovaries and therefore, the preovulatory follicles in other species are relatively small if compared with those of the horse. A typical preovulatory follicular diameter for the cow will be 14 mm (Ginther et al., 1989c), for the goat 9 mm (Ginther & Kot, 1994) and for the pig 6 mm (Ryan et al., 1994). The preovulatory follicle of the horse can be expected to ovulate when it reaches a diameter of 34 to 47 mm (Bergfelt & Ginther, 1993). These large follicles can be evaluated per rectum, thus researchers were able to gather in situ information on follicular dynamics in the mare long before the availability of ultrasound (Allen, 1975a). Even after the arrival of ultrasound technology, the large follicles and distinct expression of dominance made the horse an excellent model to the study of folliculogenesis. Detailed study of follicular development patterns in cycling (Ginther, 1993) and pregnant (Ginther & Bergfelt, 1992a) mares with the use of ultrasound, created a need for more specific definitions for different types of follicular waves.
Follicular wave definitions

A major follicular wave refers to a cohort of follicles that initially grow in synchrony at a similar growth rate but dissociate somewhere during the growth process into a dominant follicle and subordinate follicles that will most often become atretic. An inherent selection mechanism is responsible for the dissociation and should, therefore, be part of this definition (Ginther, 1992, 1993; Findlay, 1994). A major follicular wave in the mare is thought to be equivalent to the follicular wave as it is described for the cow. A major follicular wave that emerges during late estrus or early diestrus to give rise to a dominant diestrus anovulatory follicle or diestrus ovulation (secondary ovulation) is called a secondary major follicular wave (Bergfelt & Ginther, 1993). Alternatively, the major follicular wave can emerge during mid-diestrus and give rise to the primary or estrus-associated ovulation. In this case, it will be called the primary major follicular wave (Ginther, 1992, 1993). A minor follicular wave is characterized by the failure of the largest follicle of the wave to attain a typical preovulatory size and the apparent partial or total absence of dissociation and subsequent dominance (Bergfelt & Ginther, 1993; Ginther, 1993).

Primary major follicular wave

Techniques of study that provided information that led to the confirmation of the existence of primary major follicular waves in the mare include transrectal palpation, evaluation of excised ovaries, forcing the emergence of a new wave, sequential ultrasonic categorizing of follicles by size and, ultrasonic identification and tracking of individual follicles (Ginther, 1992, 1993).

Monitoring the follicular development in cycling pony mares by transrectal palpation indicated that the diameter of the largest follicle and number of follicles >20 mm in diameter were low before day 10 of the estrous cycle after which the diameter
of the largest follicle and number of follicles >20 mm in diameter progressively increased to reach a maximum close to ovulation (Ginther, 1979). Results of rectal palpation in the horse also has identified increased follicular activity at diestrus that will eventually lead to ovulation (King & Evans, 1988). Based on another palpation study with mares, Evans & Irvine (1975) distinctly characterized two waves of follicular growth with the second period of activity originating during mid-diestrus.

It has been possible to characterize the phenomenon of follicular dominance by rectal palpation over the years. An increase in the number of large follicles occurred during the middle of the estrous cycle after which only the largest follicle continued to increase in diameter (Ginther, 1992). The other large follicles started to decrease in diameter starting 6 days prior to ovulation. Similarly, it was found that the number of large follicles decreased approximately 6 days before ovulation in jennies (Vandeplassche et al., 1981). Since only larger follicles are generally palpable, the exact time of emergence of the primary wave and the interrelationship between individual follicles could not be identified in these studies.

Studies on excised ovaries allows the histological evaluation of different sizes of follicles (Hinrichs, 1991; Okolski et al., 1991). Using this approach, no pony mares had a nonatretic follicle that was larger than 10 mm in diameter at day 6 of the estrous cycle; whereas, all mares in this study had a nonatretic follicle larger than 10 mm in diameter at day 14 of the estrous cycle. These observations indicate that only small viable follicles were present at the beginning of the estrous cycle but larger viable follicles were present on the ovaries after day 14. In the same study on preovulatory mares, the largest follicle continued to increase in size as ovulation approached; whereas, the atretic follicles were smaller than the viable follicles at day 14 indicating a dissociation between the dominant follicle and subordinate follicles (Driancourt et al.,
1982). When ovaries are obtained from a slaughterhouse rather than from ovariectomies for study, estimation of the stage of the estrous cycle is likely not to be accurate for data collection.

One can study the development of a fresh follicular wave by forcing the emergence of a new wave in several ways. These include removing the ovary with the dominant follicle and then observing the reaction of the contralateral ovary; suppressing all follicular activity with exogenous steroids followed by withdrawal of the steroid treatment (Ginther, 1992, 1993); inducing new waves of follicular activity with prostaglandin treatment (Silvia et al., 1987a); or selective dominant follicle aspiration (Hinrichs et al., 1991). With complete removal of the preovulatory follicle in pony mares by hemiovariectomy, a new wave of follicular development gave rise to another follicle that ovulated 15 days later (Driancourt & Palmer, 1984). When the same approach was followed in horse mares, the interval to subsequent ovulation was 13.7 days (Sirois et al., 1989). When follicular activity was suppressed with daily injections of progesterone, the time from the last injection to ovulation was 8 days (Palmer, 1978). In another study, however, the interovulatory interval was reported to be 13.3 days when prostaglandins were administered daily for 8 days starting on the day of ovulation (Silvia et al., 1987a). A French researcher reported a very similar short follicular phase (treatment to ovulation) of 8 days after inducing luteolysis with prostaglandins during diestrus (Palmer, 1978). Generally, it appears that most ovulation synchronization protocols in the horse that are based on the principle of follicular suppression result in a treatment-to-ovulation interval of 10 to 14 days (Ginther, 1992).

Part of discrepancies in the interval from treatment to ovulation can likely be explained by the timing (diameter of the preovulatory follicle) of the treatment. If
prostaglandin is given during diestrus when a large viable follicle is present on either ovary, then the follicle will become the preovulatory follicle rather than small follicles of a following wave, resulting in a short treatment-to-ovulation interval. In contrast, if prostaglandins are given when the larger follicles are already atretic or when only small follicles are present, one can expect the interval from treatment to ovulation to be significantly longer with a typical treatment-to-ovulation interval of 10 to 14 days (Ginther, 1992).

This same principal is well illustrated in a study where dominant follicles were aspirated through the paralumbar fossa when they were either 30 to 34 mm, 35 to 39 mm, or 40 to 44 mm in diameter (Hinrichs et al., 1991). The incidence of a secondary follicle (post-aspiration) ovulating in 10 days from aspirating the primary preovulatory follicle was 55, 33 and 14% for the three treatments, respectively. This implies that most ovulations in the 30 to 34 mm follicle treatment group (aspirated earlier in the estrous cycle) originated from follicles of the original primary wave; whereas, most ovulations in the remaining two treatment groups originated from a subsequent follicular wave.

The first approach of ultrasound studies on the follicular populations of mares was to track the number of follicles in various categorized size groups during the estrous cycle. This approach confirmed the results of previous palpation studies (Ginther, 1992). In a typical study, the number of large follicles and the diameters of the two largest follicles began to increase after day 10 of the estrous cycle (Pierson & Ginther, 1987). Furthermore, the number of large follicles and the diameter of the second largest follicle began to decrease 6 days prior to ovulation, substantiating the existence of follicular dominance as evidenced by divergence between the dominant follicle and the subordinate follicles, with resultant atresia of these subordinate follicles.
at least 6 days before ovulation. The same data were used and the follicles sorted from largest to smallest for both ovaries for each day of the estrous cycle (excluding the dominant follicle)(Ginther & Bergfelt, 1993). They were then divided into three to five tiers of six follicles each. A significant increase in the mean diameters followed by a significant decrease was used to identify waves of follicular activity within each of the follicle tiers for each mare. Ginther & Bergfelt (1993) have concluded that all mares had a primary major follicular wave and that the primary wave emerged 6 days after ovulation (Ginther & Bergfelt, 1993). The time of emergence of the primary major follicular wave as well as the exact time of morphological divergence between the dominant follicle and its subordinates could now be determined. However, it should be noted that individual follicular waves can be obscured by using this approach because follicular waves tend to overlap so that large follicles from one wave often survive well into the next wave (Betteridge et al., 1982).

Monitoring individual follicles makes it possible to identify specific waves of follicular activity, even when waves overlap (i.e., growing, static and regressing phases can be present on the same ovary at the same time). A close estimation can be made of the time of emergence of the follicles from the cohort that comprise the follicular wave, growth rate of the dominant follicle, divergence between the dominant and subordinate follicles and the time of ovulation or regression of the wave. In a detailed study on the follicular development patterns during the Spring transitional phase, it was found that erratic follicular activity was followed by one to three anovulatory major follicular waves (Ginther, 1990). The next wave did not emerge before the dominant follicle of the previous wave became static. Only after emergence of the new wave was regression of the previous dominant follicle evident. A fairly constant interwave interval of 10.8 days was observed and the size of the dominant follicle gradually
increased with subsequent waves until ovulation. The mean interval from the cessation of growth of the subordinate follicles (divergence) to ovulation was 6.8 days. The emergence of the major ovulatory wave (first ovulation of the season) was clearly detected at mid-cycle (Ginther, 1990). These results indicate that the primary major follicular wave in the horse may be very similar to those of heifers where it is proposed that the growing dominant follicle will not only suppress its own subordinate follicles, but will also cause regression of the static dominant follicle of the previous wave and prevent the emergence of a new follicular wave. These effects may be exerted through systemic as well as local pathways (Ginther et al., 1989a).

In a subsequent study during the breeding season, the primary wave emerged as a cohort of follicles with an average diameter of 12 mm on day 7.4 post-ovulation (Ginther, 1993). The mean interval from emergence of the wave to divergence between the dominant and subordinate follicles was 7.4 days and the interval from divergence to ovulation was 7.2 days. In contrast, the same group reported the emergence of the primary follicular wave in the mare to be day 12 of the estrous cycle (Bergfelt & Ginther, 1993). Both of the studies were performed at the same time of the year on the same breed of horses, but slightly different criteria to identify emergence of a wave and methods for analyzing the data were used.

In the first study (Ginther, 1993), individual follicles could be identified when they were 12 mm in diameter and in the second study (Bergfelt & Ginther, 1993) individual follicles were only considered when they were 15 mm in diameter. By applying the growth rates of follicles after they have been identified by ultrasound, it was estimated that the follicles that emerged as part of the primary wave (detected by ultrasound at 12 to 15 mm in diameter) actually began as 2 to 3 mm follicles a few days before ovulation at the end of the previous estrous cycle (Ginther & Bergfelt, 1993).
Although maintaining identity of individual follicles allows detailed evaluation of follicular dynamics, it is labor intensive, subject to human error and may even become impossible when excessive follicular activity is present. If follicular wave information is to be used in a practical setting (e.g., timing of oocyte collection procedures for assisted reproduction, timing of estrous synchronization treatments), a more practical approach will be needed. Recently a mathematical approach has been described to characterize ultrasonic-derived follicular data without maintaining follicular identity (Ginther & Bergfelt, 1992b). The diameters of the three largest follicles on each ovary were used to determine the days of emergence of each follicular wave on the basis of significant increases of follicular diameters after removing values for the largest (dominant) follicles. This mathematical method of characterizing follicular waves may be inadequate when multiple ovulations are involved because of the complexity of the diameter profiles of multiple dominant follicles (Ginther, 1993).

Secondary major follicular wave

After a study on the endocrinological patterns of the estrous cycle and rectal palpation of associated follicular development, it was concluded that two waves of follicular growth occur during the estrous cycle (Evans & Irvine, 1975). Similarly, follicular activity during the first half of the estrous cycle and subsequent diestrus ovulations have been indicated as possible causes of multiple ovulations in the mare (Stabenfeldt et al., 1975). Unlike other species, horses are able to ovulate while under progesterone dominance, such as diestrus and pregnancy (Stabenfeldt et al., 1975; Ginther, 1993). It has been noted that the dose of progesterone needed for the suppression of estrus is much lower than that needed for the suppression of ovulation in the mare (Palmer, 1978). Ultrasonic evaluation of the ovaries of heifers clearly demonstrated that two or three waves of follicular activity occur during an estrous cycle
and that the dominant follicle of the second or third wave will often become the ovulatory follicle (Sirois & Fortune, 1988; Ginther et al., 1989c) These and other studies (Stabenfeldt et al., 1972; Vandeplassche et al., 1979) indicated that there may be additional follicular activity besides the primary estrus-associated ovulatory wave in the mare.

The presence of secondary major waves during the equine estrous cycle have been well characterized in a series of recent ultrasound studies. Two major follicular waves (secondary and primary) were detected in 11 of 25 interovulatory intervals of Appaloosa and Quarter Horse mares (Ginther, 1992). The other 14 interovulatory intervals had only the one primary ovulatory follicular wave. Similarly, secondary waves occurred in eight of 34 estrous cycles of Spring cycling mares (Ginther, 1993), in six of nine first estrous cycles of the year (Ginther, 1990) and in three of 15 Quarter Horse mares, also during the first half of the breeding season (Ginther & Bergfelt, 1993).

As previously discussed, the primary wave emerges during mid-diestrus and the dominant follicle from this wave ovulates during estrus. If present, the secondary major wave emerges during late estrus or early diestrus. In one study, the mean day of emergence of secondary waves was day -1.4 before the previous ovulation (Ginther, 1993). The dominant follicle of the secondary wave may result in a diestrus ovulation (Stabenfeldt et al., 1975), become anovulatory and regress (Ginther, 1992), or continue to grow and ovulate in synchrony with the dominant follicle of the primary wave at estrus (Ginther, 1990; Bergfelt & Ginther, 1993). It has been reported that the length of estrous cycles was shorter (18.2 days) for Thoroughbred mares with one wave than for mares with two waves (21.6 days)(Sirois et al., 1989). A similar study in Appaloosa and Quarter Horse mares failed to find this relationship between the number
of follicular waves per estrous cycle and estrous cycle length (Ginther, 1990). When primary and secondary major waves were compared, it seemed that dissociation between the dominant and subordinate follicles occurred, on average, earlier for secondary waves. Correspondingly, the dominant follicle of the secondary wave reached its maximal diameter earlier and did not attain as large of a diameter as the dominant follicle of the primary wave (Ginther, 1993).

Twinning in the mare and therefore, the incidence of double ovulations occurs more frequently in certain family lines and breeds of horses. Double ovulations and twin pregnancies has long been known to be highly repeatable within mares. In a rectal palpation study, multiple ovulations occurred in 19% of Thoroughbreds, 9% of Quarter Horses and 8% of Appaloosas (Ginther, 1982). Also, distinction was made between synchronous (1 day apart) and asynchronous (2 to 10 days apart) double ovulations with twins originating more frequently from asynchronous double ovulations (nine of 57) than from synchronous double ovulations (zero of 39). Approximately 50% of multiple ovulations in these females was found to be synchronous and 50% to be asynchronous (Squires et al., 1987).

The incidence, origin and nature of double ovulations can now be explained, at least in part, by the existence of primary and secondary follicular waves. After monitoring individual follicles by ultrasound, Ginther (1992) reported that ponies and Quarter Horses usually have only one major follicular wave; whereas, in a combined study with Thoroughbreds, Appaloosas and Quarter Horses, 11 of 25 mares had two follicular waves. In addition, the incidence of diestrous ovulations was greater in Thoroughbreds than in Quarter Horses and pony mares. It appears that the higher incidence of diestrus ovulations in Thoroughbreds can be explained by the more frequent occurrence of two follicular waves in this breed (Ginther, 1992). The origin
of multiple synchronous ovulations in a recent follicular wave study (Ginther, 1993) were as follows: 1) two ovulations from the same primary wave; 2) two ovulations from the primary wave and one from the secondary wave, which was delayed to ovulate in synchrony with the two from the primary wave at estrus; and 3) one ovulation from the primary wave and one from the primary wave of the previous anovulatory estrous cycle.

Asynchronous ovulations can originate from the same follicular wave, or more commonly from diestrous ovulations (Ginther, 1992; Bergfelt & Ginther, 1993). Diestrous ovulations are potentially fertile and planned breeding at this time can give rise to a pregnancy (Hughes & Stabenfeldt, 1977). Heritability of double ovulations and twinning may, therefore, be attributed, in part, to the heritability of the occurrence of secondary follicular waves and diestrus ovulations. In a study on cyclic Thoroughbred follicular development patterns (Sirois et al., 1989), three of five mares with a secondary wave had a diestrous ovulation; whereas, in a similar study with Quarter Horses, none of the secondary waves (six mares) ovulated (Ginther, 1990). These observations indicate that although certain breeds have a relatively high incidence of secondary follicular waves, the capability of the secondary waves to ovulate may be a separate factor.

Minor follicular wave

Differences between major and minor follicular waves were summarized by Bergfelt & Ginther (1993) and Ginther & Bergfelt (1993) as follows: 1) the largest follicle of a major wave will reach its maximum diameter (mean of 9 days) later after wave emergence than the largest follicle of a minor wave (mean of 4 days); 2) the maximum diameter (mean of 42 mm) of the largest follicle of a major wave will be larger than the maximum diameter (mean of 21 mm) of the largest follicle of a minor
wave; 3) follicular dominance would be evident in major waves as indicated by a wide
difference in maximum diameter between the largest and second largest follicles (mean
of 18 mm); whereas, the two largest follicles of a minor wave would differ by a mean
of only 3 mm (Bergfelt & Ginther, 1993); and 4) major primary waves originate during
mid-diestrus, major secondary waves originate during early diestrus or late estrus and
minor waves can originate at any time during the estrous cycle (Ginther & Bergfelt,
1993). It appears that the presence of one major follicular wave leading to the estrus-
associated ovulation is the predominant follicular growth pattern in the mare, although
in some mares and in some breeds modifications may occur including the development
of secondary and minor follicular waves (Bergfelt & Ginther, 1993; Ginther, 1993).

There are indications that mares exhibit greater follicular activity during the
Spring than during later estrous cycles of the breeding season (Pierson & Ginther,
1987; Ginther, 1990, 1993). Pony mares have bimodal FSH-profiles during the estrous
cycles of the first half of the breeding season but have only unimodal profiles (low FSH
values during the first half of the estrous cycle) during the second half of the breeding
season (Ginther, 1979). Also, the mean diameter of the largest follicle during the first
half of diestrus has been reported to be larger for mares during the first half of the
breeding season than for mares during the second half of the breeding season (Pierson
& Ginther, 1987). The relationship of increased follicular activity during the first half
of the breeding season to follicular waves has not yet been adequately characterized.

In the special case during the first ovulation of the year, it was noted that the
interval from divergence of the dominant follicle (dominant follicle from the
subordinate follicles) to ovulation was longer than for subsequent ovulations (Ginther,
1990). Excluding the first ovulation of the season, the length of the interovulatory
intervals and all examined characteristics of the primary follicular waves were not
different between Fall and Spring estrous cycles in mares. It was concluded that the increased follicular activity detected in the first half of the breeding season was mainly due to the more frequent occurrence of secondary and minor follicular waves (Ginther, 1993). In general, the frequency of minor follicular waves was low and occurred in four of 12 (Bergfelt & Ginther, 1993) and eight of 34 estrous cycles (Ginther, 1993).

**Follicular waves of pregnancy**

The presence of follicular activity has been well documented in early pregnant mares (Allen, 1975a; Squires & Ginther, 1975; Urwin & Allen, 1982b; Allen, 1984). The follicular and luteal activity of the pregnant mare are the greatest during days 50 to 150 of pregnancy, with peak follicular activity corresponding with the time of peak eCG concentrations in the maternal circulation. After disappearance of eCG from the circulation, the follicular activity will drastically decrease and remain at very low values during the last third of pregnancy (Wesson & Ginther, 1980). Traditionally, these follicular growth patterns have been attributed to the presence of eCG in the circulation (Stewart et al., 1977; Urwin, 1983). However, considerable growth and development of follicles occur during days 17 and 30 of pregnancy, well before days 35 to 40 when eCG is first detected in the circulation of pregnant mares (Bain, 1967; Van Rensburgh & Van Niekerk, 1968).

It was observed that regular intervals separated peak periods of follicular activity of mares on days 2, 12, 22, 32 and 42 of pregnancy (Van Rensburgh & Van Niekerk, 1968). Similarly, peaks of ovulation were apparent at 40 to 42, 54 to 56 and 63 to 66 days after conception (Allen, 1974). Also, periodic emergence of follicular waves every 8.5 to 9.8 days has been described for early pregnant heifers (Ginther et al., 1989b; Bergfelt et al., 1991). Also, major primary, major secondary and minor follicular waves have been well described for cycling mares (Ginther, 1990, 1993;
Individual follicles were identified and monitored by transrectal ultrasonography between days 0 and 50 of pregnancy in nine riding-type horses (Bergfelt & Ginther, 1992). Two to three sequential, distinct follicular waves with the dominant follicle reaching 35 to 52 mm in diameter were identified in three of these mares. The mean interwave interval was 13.4 days. Also, three other mares had only one wave with a dominant follicle of a large size (35 mm in diameter). Other apparent waves in these three mares and in the remaining three mares did not reach 35 mm in diameter and follicular dominance was not evident. The mean days of wave emergence during early pregnancy were days 5.5, 19.2 and 29, respectively. Divergence between the dominant and subordinate follicles occurred 2 days after wave emergence. In this study the wave characteristics ranged from the rhythmical occurrence of major follicular waves with a large follicular diameter to the sporadic occurrence of less prominent, minor waves where dominance was not evident. Although minor waves were not characterized in this study, it seems that approximately 50% of the follicular waves were minor waves.

In a subsequent study, a mathematical procedure was developed to characterize minor and major follicular waves in nine Quarter Horses between days 0 and 40 of pregnancy, without the necessity to maintain identity of individual follicles (Ginther & Bergfelt, 1992a). From 17 waves detected, 11 (65%) were identified as major waves with a mean diameter of 42 mm and six (35%) were considered to be minor waves with a mean diameter of 27 mm. Using the mathematical method to identify the waves of follicular activity, the day of emergence of a wave could be identified 2 days earlier than for the individual identity method. Therefore, divergence between the dominant follicle and the second largest follicle was significant only between the 4th to 6th day.
after wave emergence, 2 days later than for the previous study (Ginther & Bergfelt, 1992a).

In an effort to further characterize the nature of major and minor follicular waves during early pregnancy in the mare, 21 riding-type mares between 11 and 40 days of pregnancy were monitored by ultrasound and the data analyzed by using the previous validated mathematical method, where individual follicular identity was not required (Ginther & Bergfelt, 1992a). Eighteen major waves (51%) and 17 minor waves (49%) were identified during the experimental period. The interval from wave emergence to divergence of the dominant follicle and the subordinate follicles was several days longer for solitary waves (no adjacent waves) than for waves following consecutively. When major and minor waves of pregnancy were compared, minor waves had larger follicles on the day of emergence and significantly more variation in the day of attainment of maximal diameter of the largest follicle. Also, minor waves differed from major waves in the diameter of the largest follicle (means of 27 and 42 mm, respectively) and the difference between the diameter of the largest and second largest follicle was much less (means of 4 and 20 mm for minor and major waves, respectively). Similar to the minor wave of cyclic mares, the minor wave of pregnancy attained maximal diameter several days earlier than the dominant follicle of a major wave. The interwave interval for minor waves (7.8 days) was shorter than the interwave interval for major waves (11.7 days)(Ginther & Bergfelt, 1992a).

It seems that follicular activity is highly variable among mares during the first 50 days of pregnancy, ranging from periodically to sporadically occurring major follicular waves in some mares to only minor waves in others (Ginther & Bergfelt, 1992a). The variable follicular wave activity and interwave intervals found during early pregnancy in mares are in contrast to the fairly constant interwave intervals of \( \approx 9 \) days.
that were observed in early pregnant heifers (Ginther et al., 1989b). The major waves of pregnancy in mares contain a dominant follicle that may ovulate, become hemorrhagic or regress (Bergfelt & Ginther, 1992).

It has been well established that the mare is a seasonal breeder with the onset of the ovulatory season in the Spring at a time of increasing photoperiod. Season has a pronounced effect on the follicular population as well as on the accompanying endocrinological patterns (Freedman et al., 1979; Turner et al., 1979b; Silvia et al., 1986, 1987b; King et al., 1993). It has been reported that follicular activity was greater between days 20 and 40 of pregnancy for mares mated in the Summer than for mares mated in the Fall (Bergfelt & Ginther, 1986).

Indications are that the ovarian activity during pregnancy can be influenced by the season of the year too. The number of secondary corpora lutea during pregnancy appeared to be lower for late-mated than for Spring-mated pony mares (Wesson & Ginther, 1980). Similarly, a marked seasonal effect has been observed with increased ovarian size, follicular content and ovulation rates for mares conceiving between the end of April and the beginning of July if compared with mares that conceived between the middle of July and the end of February. However, no such seasonal effect has been detected for the circulating levels of eCG, although the occurrence of ovulations during pregnancy was restricted to May to October (Allen, 1974, 1975a). The effects of season on the follicular development patterns of pregnancy provide indirect support that pituitary gonadotropins (markedly affected by season) rather than eCG is responsible for these development patterns (Ginther, 1992).
The relationship between circulating concentrations of FSH and follicular waves

FSH and follicular waves in cycling mares

The mean concentrations of circulating FSH during the estrous cycle of the mare have been characterized by low levels during estrus that will increase during diestrus and decrease again ≈8 days before ovulation (Miller et al., 1980; Urwin & Allen, 1983; Alexander & Irvine, 1987; Thompson et al., 1987a). However, it was demonstrated that surges of FSH occur at 10- to 12-day intervals during the estrous cycle (Evans & Irvine, 1975; Foster et al., 1979; Urwin & Allen, 1982a). A surge of FSH rather than a spike was identified in mares during late estrus/early diestrus and again during mid-diestrus (Evans & Irvine, 1975; Foster et al., 1979). These surges of FSH were correlated with two waves of follicular growth that occurred at the same time when serum FSH concentrations were elevated in mares (Van Rensburgh & Van Niekerk, 1968; Evans & Irvine, 1975). Despite individual mare variability, a surge of FSH preceded ovulation in mares by 10 to 13 days, almost without exception (Evans & Irvine, 1975). The increased follicular development associated with the mid-diestrus surge of FSH could be blocked in females by the administration of antiserum against an equine pituitary fraction (Pineda et al., 1973).

It seems that estrous cycle FSH profiles can exhibit unimodal, bimodal or trimodal patterns depending on individual mare variation, age, time of the year, breed (also ponies and horses), sampling frequency and microheterogeneity (Ginther, 1992). In a study on circulating FSH concentrations in pony mares throughout the breeding season, it was noted that on average two FSH surges occurred early in the breeding season but that only one surge occurred later in the breeding season (Turner et al., 1979a). It is evident that the surges observed from daily sample collection are only portions of much more frequent pulses of FSH (Alexander & Irvine, 1987). It is
proposed that blood samples need to be obtained at intervals of 3 minutes or less to accurately describe ultradian FSH rhythms (Evans, 1990).

Equine FSH exists in the pituitary as a family of molecular forms that can be separated by charge (microheterogeneity). The microheterogeneity of FSH stems largely from varying degrees of sialylation of carbohydrate side chains that affects receptor binding and therefore, biological activity, circulatory half-life and consequently also assay binding. Although the significance of this phenomenon is not clear, the possibility of a physiological role for FSH microheterogeneity can not be ignored (Matteri & Papkoff, 1988; Shand et al., 1991). Unlike progesterone and estradiol, FSH does not follow a consistent modality (Garza et al., 1986). One must therefore be careful to make deductions from mean profiles as individual hormone patterns can be masked (Ginther, 1992).

Similar FSH profiles are expected during the estrous cycle of the mare and the jenny (Urwin, 1983; Henry et al., 1987). However, when the patterns of FSH during the estrous cycle of horse mares and jennies were compared, the peak heights of the FSH surges were greater in the jennies than in the horse mares, and there was less uniformity in the amplitude and regularity of these surges (Urwin, 1983).

Correspondingly, close associations have been reported between FSH and follicular waves in cycling heifers (Adams et al., 1992). The importance of the role of FSH in follicular wave development in the mare has been clearly demonstrated by the suppression of circulating levels of FSH and concurrent diminished follicular development following treatment with a proteinaceous fraction of equine follicular fluid (Bergfelt & Ginther, 1985). Recently, individual FSH surges were compared with follicular waves within mares and FSH and follicular changes were evaluated over mares (Bergfelt & Ginther, 1993). There was a significant increase in mean daily
plasma FSH concentrations 6 days before the emergence of a primary wave and 4 days before the emergence of a minor wave. This is in agreement with a later report on increased numbers and diameters of 2 to 3 mm follicles during the estrous cycle of the mare in temporal association with increased concentrations of circulating FSH (Ginther & Bergfelt, 1993). The concentrations of FSH decreased significantly 2 days after the emergence of a primary follicular wave (Bergfelt & Ginther, 1993). In this particular study, the duration of individual FSH surges had a mean of 4.5 days and the time period between individual surges of FSH was 3 to 7 days (Bergfelt & Ginther, 1993). It was concluded by these authors that the increase in mean daily FSH concentrations 4 to 6 days before the emergence of a wave was attributable to a significant increase in the frequency and magnitude of individual surges. Selection of the dominant follicle of the primary wave occurred in association with decreasing mean FSH concentrations. It is unknown whether the decline of FSH was functionally related to the dominant follicle selection process or if it drops as a result of suppression by inhibin or related substances from the newly selected dominant follicle (Bergfelt & Ginther, 1993; Roser et al., 1994).

Fertility in mature mares has been shown to decrease with advancing age and the noted subfertility was related, in part, to the time period of embryonic development before the embryo actually entered the uterus (Carnevale et al., 1993b; Brinsko et al., 1995). Although a shortage of circulating FSH was suspected as a possible cause of age-related subfertility in mares, no differences in mean concentrations of FSH were found between old and younger mares (Carnevale et al., 1994). Similar to the findings in menopausal women, the defect in old mares appears to be related to the ovary itself (oocyte quality, ovarian responsiveness to FSH) rather than to the availability of circulating FSH (Ginther et al., 1993; Carnevale et al., 1994).
FSH and follicular waves in pregnant mares

The coincidental effects of season on circulating FSH concentrations in ovariectomized mares (Freedman et al., 1979) and on the follicular development patterns of pregnancy (Allen, 1974) with the lack of a similar seasonal effect on eCG production, provide indirect support that pituitary gonadotropins rather than eCG is responsible for follicular development during early pregnancy (Ginther, 1992). This concept is further supported by receptor binding site studies that demonstrated that the ovarian tissue of the mare possesses very low binding affinities for eCG with almost no binding occurring at the FSH receptor site (Stewart & Allen, 1979; Manning et al., 1987).

When circulating levels of FSH during early pregnancy was suppressed by the administration of charcoal-extracted equine follicular fluid, the number of small, medium and large follicles as well as the diameter of the largest two follicles have been reported to be markedly reduced (Bergfelt & Ginther, 1986). Also, there is a marked synchronized decline in both the number of large follicles and in the circulating levels of FSH during the second half of pregnancy (Allen, 1974, 1982; Urwin & Allen, 1982b).

The observation that regular intervals separated peak periods of follicular activity on days 2, 12, 22, 32 and 42 of pregnancy, lead to the assumption that if pituitary gonadotropins rather than eCG was responsible for the rhythmic follicular growth patterns, the concentrations of the gonadotropins should also peak at 10-day intervals (Van Rensburgh & Van Niekerk, 1968; Miller et al., 1980). Indeed, Evans & Irvine (1975) found that five fold surges of FSH occurred at regular 10- to 11-day intervals during early pregnancy in the mare. With surges of FSH occurring during days 10 and 20 of pregnancy, it seems that the rhythmic release of FSH is not inhibited
by the high levels of progesterone frequently detected during this time period (Evans & Irvine, 1975). Similar FSH surges have been identified at regular intervals throughout the first 100 days of pregnancy in jenny donkeys as well as in mares (Urwin, 1983).

A study on the follicular dynamics between days 0 and 50 of the equine pregnancy demonstrated a close association between the increase in FSH concentrations and the emergence of follicular waves (Bergfelt & Ginther, 1992). A significant increase in the mean FSH concentrations occurred between 4 and 5 days before emergence of a major wave, reached maximal concentrations 3 days before emergence and maintained these maximal concentrations for 5 days. Dissociation between the dominant follicle and subordinate follicles of major waves of pregnancy was associated with a marked decline in circulating FSH concentrations 2 days after wave emergence. As with both cycling mares (Bergfelt & Ginther, 1993) and cycling heifers (Ginther et al., 1989c; Adams et al., 1992), it is not clear if the decrease in FSH concentrations associated with the divergence is functionally related to the follicle selection process or if it is due to inhibin-mediated suppression of the already selected dominant follicle.

In a related study of follicular development patterns in mares during the first six weeks of pregnancy, a significant decrease in FSH levels similarly occurred after the third day of major wave emergence but a significant decrease did not occur subsequent to the day of minor wave emergence (Ginther & Bergfelt, 1992a). Also in this study, a mean increase of FSH was temporally associated with the emergence of both minor and major follicular waves. In mares with predominantly minor waves, the mean circulating FSH concentrations were higher over the experimental period than for mares with major follicular waves. It is suggested that perhaps the overall circulating FSH
concentrations were lower in mares with large follicles (major follicular waves), due to the suppression of FSH mediated by the dominant follicle.

**Follicle and Follicular Fluid Studies**

During the growth period of a follicular wave, the follicles are exposed to various endocrine (e.g., FSH and LH), paracrine (between follicles) and autocrine (within follicle) stimuli that lead to differential production of steroids, proteins and growth factors with the eventual selection of the dominant ovulatory follicle (Sharp *et al.*, 1991; Squires *et al.*, 1992; Weedman *et al.*, 1993; Findlay, 1994). A series of events follow that lead to the ultimate rupture of the follicle with the release of a fertilizable oocyte. Accurate identification of the preovulatory follicle will permit further detailed research on the time course and mechanism of the selection process (Fay & Douglas, 1987). A better understanding of the endocrine and paracrine events surrounding follicular wave development may aid in the manipulation of the estrous cycle (estrous synchronization, superstimulation)(Fay & Douglas, 1987; Strois *et al.*, 1990) and in the collection of developmentally competent gametes during assisted reproduction (Watson & Sertich, 1990; Hinrichs, 1991).

The effect of follicular fluid contents on the course of endocrinological and follicular events of the estrous cycle is well demonstrated in studies where different fractions of follicular fluid were administered to alter the follicular development patterns and circulating FSH profiles at various stages of the estrous cycle (Miller *et al.*, 1981; Driancourt *et al.*, 1991). Treatment of mares with steroid-free follicular fluid appeared to induce atresia in larger follicles and initiated the emergence of a new follicular wave. When mares were treated with a combination of progesterone and estradiol, the development of a new follicular wave was delayed. The large follicles present at the time of treatment continued to develop after steroid treatment withdrawal.
The inhibition and delay of follicular growth after these treatments was in part due to the suppression of circulating FSH concentrations. In the mare, progesterone treatment by itself had no effect on circulating FSH concentrations or follicular wave development (Plata-Madrid et al., 1992). Substances in follicular fluid that may be responsible for some of the demonstrated effects include estradiol, androgens, progesterone, growth factors, inhibin and various activins (Roser et al., 1994).

Studying the follicular fluid and follicular walls of follicles during three stages of the estrous cycle, it was found that the presumptive preovulatory follicle was the largest in size, contained the largest amount of protein in the granulosa cell component, was the most vascular, possessed the highest granulosa cell LH/hCG receptor content and contained the greatest follicular fluid estradiol concentration. The FSH and LH concentrations in follicular fluid reflected plasma concentrations with follicular fluid LH being highest in the presumptive preovulatory follicle. As follicles were sampled closer to ovulation, androstenedione and testosterone concentrations increased markedly in the follicular fluid, reflecting endogenous stimulation of androgen biosynthesis by LH (LH surge occurs several days before ovulation and continue until after ovulation). Progesterone concentrations tended to be higher and estradiol was 30- to 50-fold higher in the follicular fluid of the preovulatory follicle, when compared with the non-ovulatory follicles (Fay & Douglas, 1987). The pattern of steroid production changes as follicular maturation proceeds and granulosa cells from estrous follicles do contain an active aromatase system that will convert androgens to estradiol (Watson & Hinrichs, 1988; Tucker et al., 1991; Amri et al., 1993). The clear increase in the number of LH receptors in the presumptive ovulatory follicle 14 days after ovulation may indicate an increased responsiveness of this follicle to basal levels of LH (increased androgen biosynthesis) and further, that the dominant follicle has already been selected.
14 days after ovulation (Fay & Douglas, 1987). It appears that these physiological markers are useful and accurate in the identification of the ovulatory follicle.

Insulin-like growth factor (IGF-1) is known to have dramatic stimulatory effects on the steroidogenic and mitogenic potential of granulosa and theca cells in vitro. It was recently reported that the concentrations of IGF-1 increase in the follicular fluid of large follicles closer to ovulation and that concentrations of IGF-1 are positively correlated with concentrations of estradiol and androstenedione. It was suggested that these follicles change their permeability to plasma IGF-1, which may enhance follicular differentiation (Spicer et al., 1991). Follicles from mares in vernal transition lack the ability to produce normal amounts of steroids, and are usually anovulatory. This phenomenon is explained by the LH dependency of steroidogenesis and the lack of LH in the circulation at this time (Davis & Sharp, 1990). It may be that LH is also responsible for the increased permeability of selected dominant follicles to IGF-1. The zona pellucida as well as the fertilized embryo are potentially antigenic to the maternal immune system. Furthermore, it is believed that ovulation is a sterile inflammatory process that should be curbed before it proceeds to a full scale immune response (Espey, 1980; Watson & Hinrichs, 1988; Watson & Zanecosky, 1990). Indications are that the preovulatory follicular fluid in the mare is increasingly suppressive to lymphocytes as the time of ovulation approaches and that this immunosuppression is associated with an altered response to lymphokine stimulation (Watson & Zanecosky, 1990).

**Equine Chorionic Gonadotropin**

Between days 36 to 38 of the equine pregnancy, the chorionic girdle (specialized area of the chorion) attaches to the uterine epithelium after which trophoblastic cells from the girdle invade the endometrium to form the endometrial cups (Allen, 1982,
Endometrial cups comprise a series of small, white, ulcer-like, endometrial outgrowths in a circle around the conceptus in close association with the placental chorionic girdle. The invasion process occurs rapidly within a 24- to 48-hour time period (Allen, 1982). Immediately after the initial invasion of chorionic girdle cells, a maternal immune response is launched and leucocytes begin to accumulate in the endometrial stroma around each cup. However, it seems that the leucocytes are unable to penetrate into the cups before days 80 to 90 of pregnancy when generalized regression of the cups are evident. At approximately days 120 to 150 of pregnancy, most of the endometrial cup tissue will be necrotic with sloughing of the cups into the uterine lumen (Allen, 1982, 1984).

It has long been established that eCG is produced by the endometrial cups. The profile of circulating levels of eCG during pregnancy is temporally related to and can be explained by the maternal-endometrial cup interaction. Low levels of eCG are first detected in the maternal circulation between days 36 and 40 of pregnancy followed by a rapid rise to a well-defined peak between days 55 and 70. Circulating concentrations of eCG then decline gradually until it disappears from the circulation between days 120 and 150 of pregnancy (Nett et al., 1975; Allen, 1984). The rise of eCG concentrations in the circulation is much more rapid than the decline (Ginther, 1992).

**Equine chorionic gonadotropin structure and bioactivity**

The three equine gonadotropins LH, FSH and eCG share a common α-subunit polypeptide chain. Unlike the gonadotropins of other species, equine LH (eLH) and eCG also has the same β-subunit polypeptide chain (Combarnous et al., 1991; Sherman et al., 1991; McDowell et al., 1993). In contrast to human chorionic gonadotropin and LH, the expression of eCG and eLH are mediated by the same gene and by a common
transcriptional initiation site in the equine placenta and pituitary, respectively (Sherman et al., 1991).

Although eLH and eCG are chemically so similar, they do have different biological effects (Aggarwal et al., 1980; Combarnous et al., 1991). Both eLH and eCG will exhibit FSH-like activity in heterologous species but only LH-like activity in equine systems. However, eCG accomplishes only 4% of the binding activity of eLH to horse LH receptors and causes a 20-fold less stimulation of testosterone production in rat Leidig cells. Since both eLH and eCG are glycoprotein hormones, it is believed that the differences in the binding and biological activities are the result of differences in their carbohydrate moieties (Combarnous et al., 1991). The horse eCG molecule has the highest carbohydrate and sialic acid content if compared with the horse pituitary gonadotropins. This characteristic may account for some of its specific biological activities and for its exceptionally long biological half-life in the horse of 6 days (Allen, 1982; Manning et al., 1987).

The biological potency of eCG varied during different stages of pregnancy as well as among mares (González-Menció et al., 1978). The natural existence in pregnant mare serum of isoforms of eCG were demonstrated with differences in chemical composition as well as in biological and immunological activity. It has been proposed that the most active isoform resembles eCG previously isolated from pregnant mare serum and that the other isoform with lower activity was similar to eCG isolated from trophoblastic tissue (Aggarwal et al., 1980). Several studies using bioassays and radioimmunoassays have demonstrated that eCG exhibits both FSH-like and LH-like activities (Farmer & Papkoff, 1979; Aggarwal et al., 1980). The ratio of the two activities did not vary significantly during stages of pregnancies or among mares (Ginther, 1992). However, Canadian researchers demonstrated that the FSH activity
of eCG was greater at days 71 and 104 of gestation than at day 39 (Manning et al., 1987).

Furthermore, considerable difference can be detected in the FSH to LH ratio of eCG produced by different genotypes of equid fetuses. This difference in ratios is used to explain the different biological responses of the maternal ovaries to eCG produced by the conceptuses of intraspecies (e.g., normal horse pregnancy), interspecies (e.g., mule or hinny pregnancy) or extraspecies (e.g., horse embryo transferred to a jenny) pregnancies (Allen, 1982). Chorionic gonadotropin of the horse, donkey, mule and hinny has a FSH:LH ratio of 1.2, 0.2, 0.7 and 0.5, respectively (Stewart et al., 1977).

Factors that may influence circulating levels of eCG

Fetal genotype

Fetal genotype has been shown to have a profound influence on the total amount of eCG secreted during pregnancy as well as on the rate of disappearance of eCG from the maternal circulation (Allen, 1975b; Manning et al., 1987). In mares carrying mule conceptuses (sired by a donkey), peak serum concentrations of eCG will be five- to 10-fold lower than for mares carrying horse conceptuses. Also, eCG will disappear from the circulation as early as day 70 to 80 of pregnancy compared with disappearance at days 120 to 150 of pregnancy for horse conceptuses. Conversely, the peak serum concentrations of eCG in the circulation of jennies carrying hinny pregnancies (sired by a horse stallion) will be five to eight times higher than for jennies carrying normal intraspecies donkey conceptuses.

The reason for these marked differences can be found in the morphological differences in the endometrial cups (Allen, 1975b, 1982, 1984). In mares carrying mule fetuses, the endometrial cups are individually much smaller than what would be expected in a normal horse pregnancy. The smaller cups and smaller total endometrial
cup mass result from the much narrower chorionic girdle found on the mule fetus. Furthermore, the maternal leucocyte reaction is greatly increased in mares carrying a mule fetus, resulting in premature destruction and sloughing of the endometrial cups. In donkeys carrying hinny pregnancies, the chorionic girdle on the hinny fetus is much broader, therefore, the individual cups and total endometrial mass are much larger than would be expected in a normal donkey pregnancy. As observed in the mare carrying a mule fetus, the immune reaction against the cups of a hinny fetus is also significantly increased, however, the ability of the leukocytes to penetrate the hinny cups and kill the endometrial cup cells are greatly reduced.

Although there are very large differences in eCG production between mares carrying horse and mule conceptuses, the amount of secondary luteal development, as estimated by peripheral plasma progesterone concentrations between 40 and 150 days of gestation was not found to be different (Allen, 1984). In a donkey carrying a hinny fetus, the increased circulating levels of eCG cause dramatic overstimulation of the maternal ovaries, resulting in many secondary corpora lutea with circulating progesterone concentrations typically as high as 300 to 800 ng per ml between days 50 and 100 of pregnancy. As mentioned, horse chorionic gonadotropin has a FSH:LH ratio of 1.2. The FSH:LH ratio of mule and hinny chorionic gonadotropin has been reported to be 0.7 and 0.5, respectively, and that of donkey chorionic gonadotropin is 0.2 (Allen, 1984; Roser et al., 1984; McFarlane et al., 1991b).

It is proposed that it is the quality (higher LH-like activity) and not the quantity of the eCG that permits donkeys and mares pregnant with mules to induce enough secondary corpora lutea so that the circulating levels of progesterone are equivalent to those of normal horse pregnancies with much higher levels of eCG (Allen, 1984). Receptor binding studies indicated that horse eCG has very low (1 to 3%) affinity for
its own gonadal gonadotropin receptors, unlike for gonadal gonadotropin receptors of other species (Stewart & Allen, 1979). It is further suggested that this low affinity intraspecies gonadal receptor binding characteristic of horse eCG functions to protect the mare against the hyperstimulatory effect of her own chorionic gonadotropin. In the case of a hinny pregnancy, when the ovaries of the jenny mother are confronted with larger quantities of a chorionic gonadotropin with a different FSH:LH ratio than that of normal donkey chorionic gonadotropin, the protection mechanism is overcome and massive ovarian hyperstimulation results (Allen, 1984).

**Individual mare variability and maternal uterine environment**

It is well documented that the time of first detection of eCG in the maternal circulation is fairly constant among mares, however, peak concentrations of this placental hormone may differ as much as 10 times between individual animals (Ginther, 1992). These big differences are probably related to the total amount of endometrial cup tissue that develops in the endometrium of the mare. This is dependent on the proportion of the total surface area of endometrium that can make close contact with the chorionic girdle tissue at the time of chorionic girdle cell invasion. The degree of folding of the endometrium to form primary and secondary rugae can vary enormously between individual animals. It is proposed that mares with a highly folded endometrium and deep crypts will expose a smaller effective surface area of the endometrium to the chorionic girdle. Fewer girdle cells will invade the exposed endometrium resulting in fewer and smaller endometrial cups (Allen, 1982).

Pregnancies were established in a horse mare and jenny donkey, each carrying one half of the same interspecies mule embryo that was bisected as a morula prior to transfer (Allen et al., 1993). The circulating eCG concentrations in the jenny carrying the mule conceptus was much higher than those of the horse carrying the other identical
twin conceptus. The same pattern is seen when unrelated mule embryos are transferred to horse mares and jenny donkeys. When a hinny pregnancy is established (donkey mother), endometrial cup development is enhanced. When a donkey embryo is transferred to a horse mare, endometrial cups fail to develop and the pregnancy usually fails before day 100 of gestation. The horse uterus, therefore, appears to be inhibitory to any conceptus that contains a donkey component; whereas, the donkey uterus appears to be stimulatory to any conceptus with a horse component. It is suggested that the donkey uterus is more permissive than the horse uterus to chorionic girdle cell invasion and endometrial cup formation originating from foreign inter- and extraspecies pregnancies.

**Paternal genotype**

When eCG was measured in the sera of 227 Belgian mare pregnancies, two populations of sires could be identified. Some sires consistently produced pregnancies with high titers of eCG and others produced pregnancies with low titers of eCG. Furthermore, it was possible to select sires and mares to increase eCG production (Manning et al., 1987). It was demonstrated that the width and overall development of the chorionic girdle are closely governed by the paternal genotype. Horse and hinny conceptuses (horse father) display a broad, thick chorionic girdle that will give rise to large active endometrial cups and subsequent high levels of circulating eCG. Conversely, a much narrower chorionic girdle will develop on mule and donkey conceptuses that result in smaller endometrial cups after endometrial invasion and, therefore, much lower levels of circulating eCG (Allen et al., 1993).

**Ratio of mare size to endometrial cup mass**

It seems to be a constant finding that peak eCG levels are higher in small horse and pony breeds when compared with those of larger mares (Allen, 1982). The size
of the conceptuses of horse and pony mares has been reported to be similar at the time of chorionic girdle cell invasion, and it can therefore be expected that the mass of the endometrial cups will be similar (Ginther, 1992). However, smaller pony mares have a smaller blood volume and eCG has a long half-life (~6 days)(Allen, 1982). Therefore, eCG levels can be expected to be higher in mares with a smaller body size as the ratio of mare blood volume to endometrial cup mass will be smaller.

**Parity status**

It was noted that the peak eCG concentrations tended to decline over a 4-year period in a group of pony mares that were mated to the same stallion and carried a pregnancy in each of the 4 years (Allen, 1982). This may be related to the observation that mares with a second consecutive mule pregnancy exhibit lower serum eCG levels for a shorter time period. The reason given was the invasiveness of the endometrial cup formation that initiated an anamnestic cellular response. This immune reaction was more intense in the second pregnancy (Antczak & Allen, 1984; Anderson, 1988).

**Twins and postpartum estrus**

Mares with twin fetuses will have two sets of endometrial cups and will, therefore, generally produce more eCG. However, mares with unilateral twins will not produce much more eCG than mares with singletons. This may be, in part, due to the spatial limitations presented by the single uterine horn. The low levels of eCG in unilateral twins probably also reflects the large area of ineffective apposition between the two conceptuses (trophoblastic-trophoblastic instead of uterine-trophoblastic contact)(Ginther, 1992). The circulating concentrations of eCG were higher in mares that conceived at foal estrous than mares that conceived at a later postpartum estrus (Bell & Bristol, 1991).
Functional roles of eCG

Rhythmic surges of FSH rather than eCG are believed to be responsible for the follicular development patterns during early pregnancy in the mare based on the following: 1) considerable ovarian follicular activity is evident in pregnant mares long before days 35 to 40 when eCG is first detected in the circulation (Allen, 1974; Evans & Irvine, 1975; Squires & Ginther, 1975); 2) there is a definite difference in follicular activity between mares mated early in the breeding season and mares mated during the second half of the breeding season but no obvious seasonal difference in eCG production (Allen, 1974; Ginther, 1992); 3) receptor binding studies have shown that the affinity for eCG is as low as 1 to 3% for horse gonadal gonadotropin receptors but higher in other species (Stewart & Allen, 1979); 4) eCG has an almost exclusive LH-like activity in the horse although it will exert significant FSH-like effects in other species (Aggarwal et al., 1980; Roser et al., 1984; McFarlane et al., 1991b); and 5) the rhythmic occurrence of FSH surges during early pregnancy are temporally related to follicular waves (Bergfelt & Ginther, 1992; Ginther & Bergfelt, 1992a).

However, ovarian size and the total follicular diameters start to increase as eCG appears in the peripheral circulation, peak in close temporal relationship to detected peak circulating eCG concentrations and decline rapidly with the coincidental disappearance of eCG from the circulation. Furthermore, the majority of ovulations during pregnancy in mares occur between days 40 and 70 post-fertilization, and coincide with peak circulating concentrations of eCG. It is therefore believed that pituitary FSH stimulates follicular wave growth during early pregnancy and that eCG by virtue of its LH activity induces a final growth spurt with resultant luteinization or ovulation of FSH-differentiated follicles to form the secondary and accessory corpora lutea in the pregnant mare (Allen, 1974, 1984). It is concluded that FSH is the primary
stimulus of follicular development during pregnancy, with eCG assuming a synergistic role in the process.

The amount of progesterone produced by the primary corpus luteum of pregnancy steadily declines from days 15 to 40 of pregnancy after which a resurge in the progesterone secretory ability of the primary corpus luteum occurs, coincidently with the appearance of eCG in the circulation (Squires & Ginther, 1975). Furthermore, the primary and secondary corpora lutea will remain functional while eCG secretion continues but regress soon after it disappears from the circulation. Systemic circulating concentrations of progesterone and eCG have been shown to be significantly correlated.

When eCG was added to in vitro cultures of primary, secondary and accessory corpora lutea obtained from pregnant mares, progesterone production was increased (Squires, 1979). In the event of an extraspecies transfer of a donkey embryo into the uterus of a horse, no eCG is produced and no surge of systemic progesterone concentrations is detected after day 40 of pregnancy, in contrast as to what would be expected in a normal horse pregnancy (Allen, 1982; Allen et al., 1993). From these findings, it seems that eCG is at least one of the major luteotropins of pregnancy in the horse. It has been demonstrated that eCG also stimulates luteal estrogen synthesis in the corpora lutea of early pregnancy in the mare, which may have crucial functions in the homeostasis of early pregnancy (Daels et al., 1990, 1991).

The question arises if the presence of eCG is absolutely essential to successfully maintain a pregnancy. Although, the most donkey-in-horse pregnancies were aborted before day 100 of pregnancy, even with exogenous progesterone and eCG supplementation, a small percentage of these pregnancies were carried to term (Allen, 1982, 1984). Part of this pregnancy failure syndrome was attributed to an exaggerated local immune response and a total lack of conceptus implantation. The eCG molecule
has the highest carbohydrate and sialic acid content when compared with hCG or any of the pituitary gonadotropins, having a half-life of 6 days. It is proposed that this highly sialated glycoprotein may provide immunoprotection by either forming a physical barrier that covers the surface of the trophoblast or repels the maternal leucocytes. This may explain the failure of most donkey-in-horse pregnancies where chorionic girdle cell penetration and endometrial cup formation was not possible. However, the short time relative to the whole pregnancy that eCG is present in the circulation does not support this hypothesis (Allen, 1982).

Traditionally it has been proposed that the formation of secondary corpora lutea and the increased progesterone circulating due to eCG production by the endometrial cups were essential to bridge a potential gap between progesterone production by the primary corpus luteum and progestogen production by the fetal-placental unit. However, it is now accepted that the primary corpus luteum of pregnancy may remain functional up to day 160 of gestation (Squires & Ginther, 1975), with fetal-placental pregnanes starting to appear in the peripheral circulation as early as day 60 of pregnancy (Ginther, 1992). The solution to the question if the primary and secondary luteotrophic function of eCG is essential to maintain pregnancy may be partly answered by the finding that when ovaries are removed from mares at 50 to 70 days of pregnancy (transition between ovarian and fetal-placental steroid production), only some mares will abort. Also, circulating levels of progesterone in early pregnancy are highly variable among mares. Therefore, the eCG-steroid production system may be essential only for those mares with marginal circulating progesterone levels (Ginther, 1992).

It is believed that eCG does not have any negative effect on the pituitary gonadotropins, such as FSH, since surges of FSH continue to occur even at times when eCG reach peak concentrations in the circulation (Urwin & Allen, 1982b; Urwin, 1983;
As in normal horse pregnancies, similar fluctuating FSH patterns occur in transferred donkey-in-horse pregnancies where no eCG is produced. In contrast, there appears to be a definite decline in circulating concentrations of FSH coincidently with the steep increase in eCG production around day 40 of pregnancy in donkeys carrying extraspecies horse pregnancies (Urwin & Allen, 1982b). It was suggested that this suppressive effect on FSH was an indirect effect of the massive production of progesterone (up to 800 ng per ml) by the hyperstimulated ovaries rather than a direct effect of eCG on FSH. However, the decline in FSH levels in donkeys carrying extraspecies horse conceptuses was temporally closer associated with the rapid rise of eCG than with the rise in circulating levels of progesterone.

Urwin & Allen (1982b) concluded that in this type of pregnancy only, can eCG exert a negative effect on circulating levels of FSH (Urwin & Allen, 1982b). It is a common clinical finding that mares that lose their pregnancy after endometrial cup formation will not start to cycle and ovulate until complete regression of the endometrial cups has occurred, resulting in the disappearance of eCG from the peripheral circulation (Allen, 1982, 1984). This suggests that FSH is not allowed to induce the emergence of a new follicular wave until eCG is no longer present.
CHAPTER II
FOLLICULAR DYNAMICS OF THE PREGNANT HORSE

Introduction

When fertile cycling mares are mated, only 64% of ovulated oocytes may result in a term pregnancy (Ginther, 1992). In subfertile and old mares the percentage of ovulated oocytes that result in a term pregnancy may drop to 12% (Ginther, 1992). It is common practice to breed mares at their first postpartum estrus (foal heat) to attain a pregnancy early in the year. Furthermore, many mares revert to ovarian inactivity after foal heat, if they have foaled during the anestrus or transitional seasons (Loy, 1980). Owners of valuable mares are, therefore, reluctant to offer their animals for conventional embryo transfer because of the possibilities of procedure-related complications or not getting the mare pregnant in that breeding season. One alternative for obtaining supplementary gametes to maximize the reproductive potential of a prized mare without jeopardizing an ongoing pregnancy is to collect quality oocytes from pregnant mares (Meintjes et al., 1994), followed by in vitro fertilization and transfer of the in vitro fertilized embryos to cyclic recipient mares.

It has been well documented that considerable follicular activity occurs in the ovaries of the pregnant mare, especially during days 50 to 150 of gestation (Van Rensburgh & Van Niekerk, 1968; Allen, 1975a; Evans & Irvine, 1975; Squires & Ginther, 1975). Similarly to pregnant heifers (Ginther et al., 1989b) and cycling mares (Bergfelt & Ginther, 1993; Ginther, 1993), rhythmic follicular waves were identified in early pregnant horses up to day 50 of gestation, with interwave intervals of 11.7 to 13.4 days (Bergfelt & Ginther, 1992; Ginther & Bergfelt, 1992a). These follicular waves of pregnancy were closely associated with surges of FSH, with a significant
increase in FSH occurring 4 to 5 days before emergence of a follicular wave in the mare (Bergfelt & Ginther, 1992).

Based on histological findings, between 51 and 73% of follicles on ovaries collected by surgery (Barrisco et al., 1992) or at slaughter (Okolski et al., 1991) were atretic at any time during the estrous cycle. The high percentage of atretic follicles can be explained by the simultaneous presence of growing, static and regressing follicular waves on the ovary. In addition, follicular dominance has been identified in 51% (Ginther & Bergfelt, 1992a) and 65% (Bergfelt & Ginther, 1992) of follicular waves of early pregnancy and, therefore, most follicles of the growing wave become atretic after the expression of dominance. It seems that the harvesting of oocytes from pregnant mares for assisted reproduction technologies should be performed on growing follicular waves, before the expression of follicle dominance in the wave.

It is now accepted that FSH is the primary stimulus for the rhythmic occurrence of follicular waves during early pregnancy, with eCG only serving a synergistic function (Evans & Irvine, 1975; Urwin & Allen, 1982b; Ginther & Bergfelt, 1992a). Only the last LH-dependent growth spurt of FSH-differentiated follicles and subsequent luteinization or ovulation of these follicles during pregnancy are attributed to eCG (Allen, 1974, 1984). However, peak ovarian and follicular size have been reported to coincide with peak levels of circulating concentrations of eCG (Allen, 1974) and after disappearance of eCG from the circulation, follicular activity will drastically decrease and obtain very low values during the last half of pregnancy (Wesson & Ginther, 1980). Although not the primary stimulus, eCG may modulate the follicular population during early pregnancy and should, therefore, be included in studying ovarian follicular dynamics of pregnancy. Interspecies (e.g., mule and hinny pregnancies) and extraspecies (e.g., zebra or horse embryo transferred to a jenny donkey recipient)
pregnancies are valuable research tools when the role of eCG in pregnancy is considered, because the biological activities of eCG (Stewart et al., 1977; Aggarwal et al., 1980) and quantities of eCG (Allen et al., 1993) that are produced differ markedly between these pregnancies.

The objectives of this study were as follows: 1) to characterize the follicular development patterns up to day 150 of pregnancy by applying different follicular aspiration treatments; 2) to temporally relate the established patterns of follicular activity to corresponding circulating serum levels of estradiol-17\(\beta\), progesterone, FSH and eCG; and 3) to evaluate the effect of mule and horse fetal genotypes on the follicular patterns of early pregnancy in the mare.

**Materials and Methods**

**Animal management**

Eighteen mares of mixed breeds and good body condition (372 to 569 kg) were used in this experiment. Before breeding, all mares had normal-length estrous cycles and were maintained on Bermudagrass and rye grass pastures in the summer and winter months, respectively. During the winter, each animal was fed an additional ration of 2 kg ground corn and oats twice daily to help maintain body condition.

**Experimental design**

Half of the mares (n=9) were inseminated with the semen of one Arab stallion and the other half (n=9) were inseminated with donkey jack semen. Pregnancy status was confirmed by the presence of an embryonic vesicle at 14 days post-insemination and again at day 21 of gestation when the animals were allotted to the experiment. When animals were confirmed pregnant between April 1993 through March 1994, they were randomly assigned to three follicular aspiration treatments.
The three aspiration treatments were: 1) a one time sham aspiration (n=4) in the second month of gestation (days 33 to 68) corresponding with a follicular wave peak where the aspiration needle was positioned in the ovary without puncturing any follicles (Treatment I); 2) aspiration of all follicles with a diameter ≥20 mm (n=7) every time when a follicular wave reached a peak with the first aspiration performed on day 21 of pregnancy regardless of the follicular status (Treatment II); and 3) aspiration of all follicles ≥4 mm (n=7) when a follicular wave reached a peak with the first aspiration similarly performed on day 21 regardless of follicular status (Treatment III). During a single aspiration procedure, aspiration was attempted on all follicles on both ovaries that met the criteria for the specific treatment group.

It was hypothesized that when only follicles ≥20 mm in diameter are aspirated (Treatment II), the negative effect of follicular dominance will be reduced with more follicular waves occurring closer to each other, resulting in an overall increase in the total follicular activity. Furthermore, it was hypothesized that when all follicles are aspirated (Treatment III), the follicle reserves in the ovaries will eventually be depleted with follicular waves occurring progressively further apart, resulting in an overall decrease in the total follicular activity.

Follicular development and fetal heart beat were monitored with a 5 MHZ rectal ultrasound transducer by a single operator three times per week in all mares during days 21 to 150 of pregnancy. Consecutive sagittal ultrasonic sections of each ovary were printed using a thermal printer (Sony®, model no. UP850, Tokyo). Care was taken between ultrasonic examination sessions to consistently scan an ovary from dorsal to ventral and from lateral to medial to ease follicular identification. Using these prints, the identity of all follicles ≥5 mm were maintained and the maximum diameter
determined for each follicle at the time of each examination on both ovaries of each animal for the duration of the experiment.

After each examination session, the individual maximal diameters of follicles for each ovary were plotted over time so that an updated follicular wave growth profile could be maintained. Because day to day decisions had to be made to apply follicular aspiration treatments at peaks of developing follicular waves, follicular waves could not be identified by statistical methods. Therefore, a follicular wave was considered to include all follicular development that occurred from the time the largest follicle was retrospectively identifiable by ultrasound until this follicle attained maximum diameter.

Follicular aspiration treatments were implemented whenever a follicular wave on either ovary reached a peak so that the number of aspiration treatments performed was equal to the number of follicular waves of pregnancy that met the criteria for the aspiration treatment in each horse (excluding the control treatment). By inspecting the individual follicular wave growth profiles on the diameter-time graph, a distinction could be made between major follicular waves (divergence between the largest and second largest follicle, resulting in a large difference in their diameters) and minor follicular waves (no divergence evident with small differences in the diameters of the largest and second largest follicle)(Ginther & Bergfelt, 1992a; Ginther, 1993).

A major follicular wave was considered to peak when the dominant follicle maintained the same diameter or decreased in diameter between two consecutive ultrasound examinations. Similarly, a minor follicular wave was considered to peak when ≥80% of the follicles in the follicular wave maintained the same diameter or decreased in diameter between two subsequent transrectal ultrasonic examinations.
Animal preparation for follicular aspiration

All mares were premedicated with 0.01 mg/kg of detomidine (Dormosedan®, Smith Kline Beecham, West Chester, PA), 0.05 mg/kg of atropine (Anpro Pharmaceutical, Arcadia, CA), 0.1 mg/kg of butorphanol (Torbugesic®, Fort Dodge Laboratories, Fort Dodge, IA) and 1 mg/kg of flunixin meglumine (Banamine®, Schering Corporation, Kenilworth, NJ) prior to the aspiration procedure. Once sedated, the mare was restrained in a modified holding chute (padded cattle squeeze chute) to prevent excess movement. The rectum of the mare was then manually evacuated, a tail bandage applied and the perineal area aseptically prepared.

Follicle aspiration procedure

Follicular aspiration was performed transvaginally with a 12-gauge stainless steel needle under ultrasound guidance using an Aloka® 500-V ultrasound unit (Corometrics Medical Systems, Wallingford, CT). The transducer (with the aspiration needle) was inserted into the vagina and the ovary rectally manipulated against the distal end of the transducer, so that the puncture line (displayed on the monitor screen) transected the hypoechoic follicle through its longest axis. The aspiration needle (500 mm in length) was advanced through the anterior vaginal wall into the positioned ovary and follicular antrum to aspirate all follicles on each ovary that complied with the parameters set for the specific aspiration treatment. Because oocytes were simultaneously recovered for related studies (Chapters III and IV), the follicles were not only punctured but also flushed to improve oocyte recovery rates. After an follicular aspiration procedure, all mares were closely monitored for 24 to 48 hours for signs of colic and also examined by transrectal ultrasonography up to 100 days after the last aspiration to verify pregnancy status. The details of the follicular aspiration procedure are described in Chapter III.
Follicular data analyses

Follicular waves were identified on each ovary and from the individual profiles on each ovary, an overall follicular wave profile was compiled for each individual mare (see Appendix A). When two follicular wave peaks on the same ovary or on opposite ovaries were more than 3 days apart, they were considered to be separate waves and both were included in the overall follicular wave profile for that mare. When two peaks on the same ovary occurred 3 or less days apart, it was assumed that they originated from the same wave and only the follicle that covered the greatest number of days from ultrasonic identification until reaching maximum diameter was included in the overall follicular wave profile. This follicle had the largest diameter in all cases.

When two peaks from opposite ovaries occurred 3 or less days apart they were assumed to originate from the same hormonal stimulus and similarly, only the wave that covered the greatest number of days from ultrasonic identification until reaching maximum diameter was included in the overall follicular wave profile. The longest wave generally also attained the largest diameter. Preference for inclusion in the overall follicular wave pattern was still given to the longest wave for the few waves where the longest wave did not also attain the largest diameter, as the difference between the largest follicle maximum diameters of the smaller longer wave and the larger shorter wave was not marked. Distinction was made for data analysis between follicular waves where the largest follicle had a maximum diameter of ≥17 mm and waves where the largest follicle did not reach a diameter of 17 mm.

Using the overall follicular wave profile for each horse, four follicular wave parameters were identified and compared between the three aspiration treatments, pregnancy types and the left and right ovaries. The four parameters were: 1) the maximum follicular diameter of the largest follicle on a follicular wave; 2) wave length
(number of days from the retrospective identification of the largest follicle of a wave to the time when this follicle attained maximum diameter); 3) interwave interval (number of days between two consecutive wave peaks); and 4) the interval in days from wave peak to the emergence of a next wave. Furthermore, three diameter (mm) or period (days) ranges were assigned to each parameter and the number of follicular waves that fit in each range was further grouped according to the wave number as they occurred from day 21 to 150 of pregnancy. By classifying the follicular waves according to chronological wave number of pregnancy in the respective diameter or period ranges within each parameter, frequency distribution patterns were obtained (Appendix B).

Besides chronological follicular wave number, the time effect of the ongoing pregnancy on follicular dynamics was also considered by dividing days 21 to 150 of pregnancy in two (days 21 to 85 and days 86 to 150) or four (days 21 to 60, 61 to 90, 91 to 120 and 121 to 150) time periods. The number of follicular waves ≥17 mm that occurred between days 21 to 85 and days 86 to 150 of pregnancy was compared between pregnancy types and follicular aspiration treatments. The wave lengths, interwave intervals, maximum follicular diameter and the interval from wave peak to the emergence of a following wave was evaluated over the four time periods. Also, the follicular aspiration treatments and pregnancy types were compared within each time period.

A total follicular diameter value was calculated for each individual animal for each day of ultrasound evaluation (three times per week) by adding the individual follicular diameters of all follicles (≥5 mm in diameter) on both ovaries. The means of these values were compared between horse and mule pregnancies during days 21 to 150 of pregnancy.
Other parameters that were evaluated include the total number of days during days 21 to 150 of pregnancy that was covered by follicular waves (maximum of 130 days), by waves with the largest follicle ≥17 mm in diameter only and the total number of days that were covered by waves from each ovary independently. Also, the mean number of waves and the mean number of waves with the largest follicle ≥17 mm in diameter were compared between the right and left ovary, the three follicular aspiration treatments and pregnancy types.

**Blood sample collection**

Blood samples were collected daily at 0900 hours by jugular venipuncture from each mare between days 21 and 150 of pregnancy. Additionally, hourly samples were collected from each mare for 51 hours around an aspiration procedure in the second month of pregnancy when circulating levels of eCG was expected to rise sharply. For the hourly sample collections, a jugular vein was catheterized with the first sample collected 3 hours before follicular aspiration and the last sample 48 hours after aspiration. Serum samples were collected on ice in glass collection tubes (Monoject Vacutainers®, Sherwood Medical, St Louis, MO, USA) and centrifuged within 30 minutes of collection to separate the serum. All serum samples were stored at -20°C until the time of analysis.

**Hormone analyses**

All the daily and hourly samples were analyzed for FSH. The concentrations of FSH were determined by radioimmunoassay as described previously (Thompson et al., 1983a). The cross-reactivity of eCG in the FSH assay was 0.02%. The circulating concentrations of eCG were determined only between days 30 and 150 of pregnancy. Samples from alternate days were assayed between days 30 and 40 of pregnancy, thereafter, only weekly samples were analyzed until day 150. All horse and mule eCG
samples were analyzed with a radioimmunoassay previously used and validated for horse eCG in this laboratory (Thompson et al., 1982). The cross-reactivity of FSH in the eCG assay was 8.3%.

The amino acid sequence of the horse eCG and LH α- and β-subunits are identical (Combarnous et al., 1991; Sherman et al., 1991; McDowell et al., 1993). Although horse LH and horse eCG have different receptor binding characteristics (Combarnous et al., 1991) and eCG has twice the carbohydrate content of LH (Manning et al., 1987; Ginther, 1992), the cross-reactivity for eCG in equine LH radioimmunoassays is approximately 100% (Thompson et al., 1982) so that horse LH radioimmunoassays are commonly used to determine horse eCG concentrations (Thompson et al., 1982; Urwin, 1983). The only difference between chorionic gonadotropins of equid species is believed to be the carbohydrate structure and content (Combarnous et al., 199; Ginther, 1992) and; therefore, the same horse LH radioimmunoassays have frequently been used to determine the concentrations of horse, donkey and mule chorionic gonadotropin (Allen, 1982; Urwin, 1983; Allen, 1984).

Progesterone concentrations were determined for alternate days between days 21 and 150 of pregnancy. A commercial radioimmunoassay kit for human progesterone (DSL-3400, Diagnostic Systems Laboratories, Webster, Tx) was used for all progesterone determinations in this study. Sample preparation required petroleum ether extraction. This assay has been validated in this laboratory for the analysis of circulating progesterone concentrations in the horse (Thompson, 1995). Estradiol-17β was similarly measured with a commercial radioimmunoassay kit for human estradiol-17β (DSL-4400, Diagnostic Systems Laboratories, Webster, Tx) and samples required diethylether extraction. Circulating concentrations of estradiol-17β were determined for every third day of pregnancy between days 40 and 150 post-ovulation. The
estradiol-17β kit has also been validated in this laboratory for the determination of estradiol-17β in the horse (Thompson, 1995). The average intra- and interassay coefficients of variation for these steroid radioimmunoassays were 5 and 10%, respectively.

Circulating estradiol-17β and eCG concentrations were compared between the pregnancy types. The serum levels of progesterone and FSH were compared between the three follicular aspiration treatments and between the pregnancy types. To evaluate the effect of the follicular aspiration on luteinization and progesterone production, the difference between the progesterone concentration at the morning of the day of aspiration and 6 to 8 days later (only alternate samples analyzed) was determined for the three follicular aspiration treatments and the pregnancy types. To allow for the possible effect of eCG on progesterone production, the change in circulating progesterone concentrations was considered separately for aspiration treatments and pregnancy types between days 21 to 39 (before eCG), 40 to 95 (high concentrations of eCG), and 96 to 150 (after eCG) of pregnancy. Each individual horse FSH profile between days 21 and 150 of pregnancy was evaluated and any visual FSH surges preceding an identified follicular wave peak was recorded. The amplitude (increase above baseline value) of these presumptive FSH surges and the number of days between the peak of the FSH surge and of the follicular wave was compared between the aspiration treatments and pregnancy types.

Also, the relationship between follicular dominance and the size of the largest follicle of a follicular wave to the number of days between the peak of the FSH surge and of the follicular wave was evaluated. Surges of FSH could be correlated to 183 of the 199 identified follicular wave peaks and therefore, FSH surge amplitude and the
number of days between the presumptive FSH peak and the peak of the following follicular wave were evaluated for these 183 waves only.

**Statistical analyses**

The frequency distributions of follicular wave maximum diameter, wave length, interwave interval and the interval in days from wave peak to the emergence of a next wave were analyzed with Fisher's Exact Two-Tailed Test (Metha & Patel, 1983). The number of follicular waves with a largest follicle of at least 17 mm in diameter during different time periods of pregnancy was analyzed by the General Linear Models Procedure (GLM) with pregnancy type and time period as the main effects and pregnancy type by time period the main interaction term (SAS, 1990). The total number of days during the experimental period covered by follicular activity and the number of days covered only by waves with a largest follicle ≥17 mm in diameter were similarly analyzed using the GLM procedure. Follicular aspiration treatments, pregnancy type and aspiration treatment by pregnancy type interaction were included in the model. Separate analyses were performed for the different size follicular waves. Similarly, several GLM procedures were used to analyze the number of days during the experimental period covered by follicular activity and the number of days covered by waves with a largest follicle ≥17 mm in diameter only for the right and left ovary, the number of follicular waves identified on the right and left ovary, the number of waves with the largest follicle ≥17 mm in diameter on the right and left ovary and the number of waves contributed to the overall follicular profile by the right and left ovary.

These analyses included the effect of follicular aspiration treatment and pregnancy type. For each GLM procedure with a significant F-value, individual treatment differences were identified with Scheffe's test or more specific preplanned
comparisons were made between Bonferroni-adjusted Least Squares Means with significance set at $P < 0.05$. (SAS, 1990; Montgomery, 1991).

The hormonal profiles (eCG, estradiol, progesterone and FSH) and total follicular diameter were analyzed by the GLM procedure with follicular aspiration treatment and day of pregnancy, or pregnancy type and day of pregnancy as the main effects. Treatment by day of pregnancy, or pregnancy type by day of pregnancy were used as interaction terms. Specific comparisons between the interaction terms were made by comparison of Bonferroni-adjusted Least Squares Means. The graphs represent raw means, however, areas of significance are indicated on the basis of Least Squares Means. Because of the high individual mare variability in the hormonal profiles and the limited number of animals that could practically be included in the experiment, a level of significance of at least $P \leq 0.10$ was accepted for the hormonal profile graphs, unless otherwise specified. The increase of circulating progesterone concentrations after aspiration for the pregnancy types for different time periods of pregnancy was also analyzed with the GLM procedure. Pregnancy type, pregnancy type nested in horse, period and pregnancy type by period terms were included in this model. Specific differences were detected by comparisons of Bonferroni-adjusted Least Square Means. The increase of circulating progesterone concentrations after aspiration for the three follicular aspiration treatments for different time periods of pregnancy; the effect of dominance, wave size and aspiration treatment on the number of days between the FSH surge and the follicular wave peak; and the effect of aspiration treatments on the FSH-surge amplitude were compared separately by an One Way Analysis of Variance using the GLM procedure. Specific differences for these analyses were also identified by comparing Least Squares Means or by using Tukey's Studentized Range Test (SAS, 1990; Montgomery, 1991).
Results

Frequency distribution of follicular wave size, wave length, interwave interval and interval from wave peak to the emergence of the next follicular wave associated with chronological wave numbers in pregnant mares

Follicular wave size

Follicular waves were grouped in three size ranges according to the maximum diameter of the largest follicle of a follicular wave (Table 2.1). The three ranges were follicular waves with a largest follicle < 17 mm (small), 17 to 34 mm (medium) or >34 mm (large) in diameter. No differences were detected in the frequency distribution of small-, medium- or large-size waves for the three aspiration treatments (also see Appendix B, Figures 1 to 3). However, when the number of waves in each size category was compared within each aspiration treatment, medium-size (17 to 34 mm in diameter) waves were most common for all three treatments. The number of small- and large-size follicular waves was not different in the control aspiration treatment but less (P<0.05) than the medium-size waves. When only follicles ≥20 mm in diameter were aspirated (Treatment II), there were more (P<0.05) medium- than small-size waves and more (P<0.05) small- than large-size waves. There was no difference in the numbers of small- and medium-size waves when all follicles were aspirated (Treatment III) but the number of large-size waves was lower (P<0.05) than the number of medium-size or small-size waves.

There were no differences between the numbers of small and large follicular waves between horse and mule pregnancies (see Appendix B, Figures 4 and 5). However, significantly more (P<0.05) medium-size waves were detected in mule pregnancies. Horse pregnancies were not different in the numbers of small- and medium-size waves, however, mule pregnancies had more (P<0.05) medium- than
Table 2.1. The number of observations (n), percentage of observations (% of obv.) and the mean maximum follicular diameter (mm) of follicular waves of pregnancy for groups < 17 mm, 17 to 34 mm and > 34 mm in diameter for the three follicular aspiration treatments, horse and mule pregnancies and for the right and left ovary.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>&lt;17 mm</th>
<th>17 to 34 mm</th>
<th>&gt;34 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>% of obv.</td>
<td>mm ± SD</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>21*</td>
<td>12.7±2.1</td>
</tr>
<tr>
<td>Asp. ≥20 mm</td>
<td>28</td>
<td>34*</td>
<td>12.0±2.7</td>
</tr>
<tr>
<td>Asp. all</td>
<td>28</td>
<td>38*</td>
<td>11.9±2.5</td>
</tr>
<tr>
<td>Horse preg.</td>
<td>36</td>
<td>38*</td>
<td>12.0±2.4</td>
</tr>
<tr>
<td>Mule preg.</td>
<td>30</td>
<td>27*</td>
<td>12.1±2.7</td>
</tr>
<tr>
<td>Right ovary</td>
<td>55</td>
<td>32*</td>
<td>12.2±2.6</td>
</tr>
<tr>
<td>Left ovary</td>
<td>48</td>
<td>30*</td>
<td>12.8±2.4</td>
</tr>
</tbody>
</table>

Values within rows or columns within each of the three groups of treatments (aspiration, pregnancy type, ovary side) with different superscripts are different (Fisher's Exact Test; P < 0.05).
small-size waves. In both horse and mule pregnancies, the numbers of large waves was significantly less ($P < 0.05$) than the number of waves in both smaller size categories.

A clear pattern of follicular wave size distribution could be identified in horse pregnancies (Appendix B, Figure 4). During the first part of the observation interval (follicular wave numbers 1 and 2) the majority of follicular waves were large. During the later stages of pregnancy (wave numbers 3 to 6), most of the follicular waves were of medium size. At the end of the observation period (wave numbers 7 to 14), almost all the waves were small in size. The same pattern was not identified for mule pregnancies. The distribution of wave sizes stayed the same as pregnancy progressed (Appendix B, Figure 5).

The numbers of follicular waves in the three size groups were not different for the left and right ovary. However, both ovaries seemed to follow the overall pattern where the number of medium-size follicular waves were the highest, small-size waves second highest followed by large waves. The mean diameter of the largest follicle of all small-size follicular waves was 12 mm, for medium-size waves was 25 mm and for large-size follicular waves was 40 mm.

**Follicular wave length**

Similarly to follicular wave diameter, the follicular wave lengths were grouped into three period ranges. All follicular waves that were 4 to 10 (short), 11 to 15 (medium) or >15 (long) days long were grouped together, respectively. When all follicles were aspirated (Treatment III), almost all the follicular waves (97%) were short waves. When only follicles ≥20 mm in diameter were aspirated (Treatment II), 73% of waves was short. Only half (52%) of the waves of the control aspiration treatment (Treatment I) were short waves (Table 2.2).
Table 2.2. The number of observations (n), percentage of observations (% of obv.) and the mean wave length (days) for follicular waves of pregnancy for wave lengths 4 to 10 days, 11 to 15 days and for wave lengths longer than 15 days for the three follicular aspiration treatments, horse and mule pregnancies and for the right and left ovary.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>4 to 10 days</th>
<th>11 to 15 days</th>
<th>&gt; 15 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of obv.</td>
<td>Days ± SD</td>
<td>% of obv.</td>
</tr>
<tr>
<td>Control</td>
<td>23</td>
<td>52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.2±1.5</td>
</tr>
<tr>
<td>Asp. ≥20 mm</td>
<td>59</td>
<td>73&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.1±1.9</td>
</tr>
<tr>
<td>Asp. all</td>
<td>71</td>
<td>97&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.1±1.9</td>
</tr>
<tr>
<td>Horse preg.</td>
<td>64</td>
<td>70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.1±1.9</td>
</tr>
<tr>
<td>Mule preg.</td>
<td>89</td>
<td>83&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.1±1.7</td>
</tr>
<tr>
<td>Right ovary</td>
<td>136</td>
<td>81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.8±1.9</td>
</tr>
<tr>
<td>Left ovary</td>
<td>127</td>
<td>83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.8±1.7</td>
</tr>
</tbody>
</table>

<sup>a,b,c,d</sup> Values within rows or columns within each of the three groups of treatments (aspiration, pregnancy type, ovary side) with different superscripts are different (Fisher's Exact Test; P<0.05).
The number of medium-length waves was not different for Treatments I and II but the number of medium-length waves for both these treatments were higher (P<0.05) than the number of medium-length waves for Treatment III. The pattern for long follicular waves was opposite to that seen for short follicular waves (also see Appendix B, Figures 8 to 10). The number of long waves for Treatment I was higher (P<0.05) than that of Treatments II and III. Furthermore, more (P<0.05) long waves occurred in Treatment II pregnancies than in Treatment III pregnancies. When the distribution of wave lengths was compared within follicular aspiration treatments, the number of short waves was higher (P<0.05) than the number of medium-length and long waves for Treatment I. However, the number of medium and long follicular waves was not different.

Approximately half of the waves from animals in the control aspiration treatment (Treatment I) occurred in the short-wave group, a quarter in the medium wave-length group and another quarter in the long-wave group (Appendix B, Figure 8). The number of short waves was higher (P<0.05) than the number of medium-length waves and the number of long waves when only follicles ≥20 mm were aspirated (Treatment II). Also the number of medium-length waves was greater (P<0.05) than the number of long waves. In general, almost 75% of waves in Treatment II were in the short-wave group with most of the others in the medium-length wave group (Appendix B, Figure 9). The number of short waves was greater (P<0.05) than the number of medium-length or long waves when all the follicles were aspirated from the mares (Treatment III). Follicular waves of mares in Treatment III were predominantly (97%) short waves (Appendix B, Figure 10).

The number of follicular waves in the medium-length wave group was not different between horse and mule pregnancies (Table 2.2). However, horses had more
(P<0.05) long follicular waves and mules had more (P<0.05) short follicular waves (see also Appendix B, Figures 11 and 12). The number of short follicular waves was greater (P<0.05) than the number of medium-length or long waves in horse pregnancies. The numbers of medium and long waves were not different. Similarly, the number of short follicular waves was greater (P<0.05) than the number of medium-length or the number of long waves in mule pregnancies but the number of medium-length waves was also greater (P<0.05) than the number of short waves.

No differences in the distribution frequencies of wave lengths was found for the right and left ovary (see also Appendix B, Figures 13 and 14). Both ovaries had the same distribution pattern of waves where the number of small waves was larger followed by the medium-length and long waves. There were more medium-length waves than long waves. The approximate wave length for all short waves was 7 days. The approximate wave lengths for medium-length and long waves were 12 and 19 days, respectively.

**Interwave interval.**

The three interwave interval ranges designated in this study were as follows: <10 days (short), 10 to 14 days (medium) and >14 days (long). No differences in the interwave interval frequency distribution were detected for the control aspiration treatment (Treatment I) and when only follicles ≥20 mm in diameter were aspirated (Treatment II). However, when all follicles were aspirated (Treatment III), fewer (P<0.05) short interwave intervals and more (P<0.05) medium interwave intervals were detected than for Treatments I and II (Table 2.3). When the interwave intervals were compared within follicular aspiration treatments, both Treatment I and Treatment II had more (P<0.05) short interwave intervals than medium or long interwave intervals. The numbers of medium and long interwave intervals for these two treatments
Table 2.3. The number of observations (n), percentage of observations (% of obv.) and the mean interwave interval (days) of follicular waves of pregnancy for intervals < 10 days long, intervals 10 to 14 days long and for intervals longer than 14 days for the three follicular aspiration treatments, horse and mule pregnancies and for the right and left ovary

<table>
<thead>
<tr>
<th>Treatment</th>
<th>&lt; 10 days</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>% of obv.</td>
<td>Days ± SD</td>
<td>n</td>
<td>% of obv.</td>
<td>Days ± SD</td>
<td>n</td>
<td>% of obv.</td>
<td>Days ± SD</td>
</tr>
<tr>
<td>Control</td>
<td>24</td>
<td>56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.8±1.7</td>
<td>12</td>
<td>28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.2±1.5</td>
<td>7</td>
<td>16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.0±6.5</td>
</tr>
<tr>
<td>Asp. ≥20 mm</td>
<td>50</td>
<td>66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.2±1.6</td>
<td>17</td>
<td>22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.9±1.5</td>
<td>9</td>
<td>12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.8±6.0</td>
</tr>
<tr>
<td>Asp. all</td>
<td>17</td>
<td>25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.4±1.6</td>
<td>40</td>
<td>60&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12.1±1.4</td>
<td>10</td>
<td>15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.4±3.7</td>
</tr>
<tr>
<td>Horse preg.</td>
<td>38</td>
<td>45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.4±1.7</td>
<td>32</td>
<td>38&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>12.2±1.4</td>
<td>15</td>
<td>18&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21.4±6.0</td>
</tr>
<tr>
<td>Mule preg.</td>
<td>53</td>
<td>53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.0±1.6</td>
<td>37</td>
<td>37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.9±1.5</td>
<td>11</td>
<td>11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>18.1±3.8</td>
</tr>
<tr>
<td>Right ovary</td>
<td>63</td>
<td>41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.2±1.6</td>
<td>51</td>
<td>33&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>12.1±1.5</td>
<td>41</td>
<td>26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.5±6.4</td>
</tr>
<tr>
<td>Left ovary</td>
<td>53</td>
<td>38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.1±1.6</td>
<td>48</td>
<td>34&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>11.8±1.4</td>
<td>39</td>
<td>28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.3±13.3</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup> Values within rows or columns within each of the three groups of treatments (aspiration, pregnancy type, ovary side) with different superscripts are different (Fisher's Exact Test; P<0.05).
were not different. In contrast, most (60%) interwave intervals of Treatment III were of medium length and this was more (P<0.05) than the number of short or long interwave intervals. The number of long and medium interwave intervals for Treatment III was not different. In general, ≈60% of the interwave intervals of Treatments I and II were short whereas, 60% of the interwave intervals of Treatment III were of medium length (see also Appendix B, Figures 15 to 17).

The frequency distribution of interwave intervals was not different for horse and mule pregnancies (see also Appendix B, Figures 18 and 19). Within pregnancy type, the numbers of short and medium interwave intervals were not different for horse pregnancies but both the number of short and the number of medium interwave intervals were higher (P<0.05) than the number of long interwave intervals. For mule pregnancies, the number of short interwave intervals was higher (P<0.05) than the number of medium interwave intervals and also, the number of medium interwave intervals was higher (P<0.05) than the number of long interwave intervals.

No differences were detected in the distribution pattern of interwave intervals for the right and for the left ovary (Appendix B, Figures 20 and 21). Both ovaries had a greater number of short and a higher number of medium interwave intervals than the number of long interwave intervals (P<0.05). However, the number of short and the number of medium interwave intervals were not different. The interwave interval for all short interwave intervals was ≈7 days. The interwave interval for medium and long interwave intervals was ≈12 and ≈21 days, respectively (Table 2.3).

**Interval from wave peak to emergence of the next follicular wave**

The interval from a wave peak to the emergence of the next follicular wave was assigned three period ranges (Table 2.4). All intervals <1 day (short), 1 to 5 days (medium) and >5 days (long) were grouped together to form three interval-length
Table 2.4. The number of observations (n), percentage of observations (% of obv.) and the mean interval (days) from wave peak to the start of the next wave for intervals < 1 day long, intervals 1 to 5 days long and for intervals longer than 5 days for the three follicular aspiration treatments and for horse and mule pregnancies

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>% of Days ±SD</th>
<th>n</th>
<th>% of Days ±SD</th>
<th>n</th>
<th>% of Days ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>31</td>
<td>69* -3.1±3.8</td>
<td>11</td>
<td>24b 3.3±1.5</td>
<td>3</td>
<td>7c 9.3±2.5</td>
</tr>
<tr>
<td>Asp. ≥20 mm</td>
<td>46</td>
<td>59* -3.2±3.6</td>
<td>24</td>
<td>31b 3.5±1.2</td>
<td>8</td>
<td>10c 15.0±8.0</td>
</tr>
<tr>
<td>Asp. all</td>
<td>5</td>
<td>7h -2.4±1.8</td>
<td>42</td>
<td>63c 3.3±1.3</td>
<td>20</td>
<td>30a 9.4±4.0</td>
</tr>
<tr>
<td>Horse preg.</td>
<td>36</td>
<td>41* -4.4±4.5</td>
<td>35</td>
<td>40* 3.3±1.2</td>
<td>16</td>
<td>18b 12.4±6.3</td>
</tr>
<tr>
<td>Mule preg.</td>
<td>46</td>
<td>44* -2.1±2.1</td>
<td>42</td>
<td>41* 3.4±1.3</td>
<td>15</td>
<td>15b 9.2±4.5</td>
</tr>
</tbody>
</table>

*be Values within rows or columns within each of the two groups of treatments (aspiration and pregnancy type) with different superscripts are different (Fisher's Exact Test; P<0.05).
groups. When the time interval between the wave peak and the emergence of a following wave was negative, the two waves overlapped so that parts occurred at the same time (see individual wave profiles in Appendix A). No differences were detected in the frequency distribution of this interval for the control treatment (Treatment I) and when only follicles ≥20 mm in diameter were aspirated (Treatment II). However, when all follicles were aspirated (Treatment III), the number of short intervals was lower, the number of medium length intervals was greater and the number of long intervals was larger than for those of Treatments I and II (P<0.05). When the distribution pattern of the interval from a wave peak to the emergence of the next wave was detected within aspiration treatments, both Treatments I and II had more short intervals than medium intervals and more medium-length intervals than long intervals (P<0.05). However, mares in Treatment III had more (P<0.05) medium-length intervals than long intervals and more (P<0.05) long intervals than short intervals (see also Appendix B, Figures 22 to 24).

The number of waves in the different interval length categories was not different for horse and mule pregnancies. The number of intervals in the short interval category and the number of intervals in the medium-length interval category also, were not different for horse and mule pregnancies when the distribution pattern was compared within pregnancy type. However, for both horse and mule pregnancies, the number of intervals between a wave peak and the emergence of a new follicular wave that was long, was less (P<0.05) than the number of intervals in the other two interval-length categories. In general, the interval length across all observations for the short interval group was -3 days so that a wave peak and emergence of a new wave overlapped on average with 3 days. Also, the interval length of the medium-length group seemed to
be approximately 3.5 days and that for the long interval group was longer than 9 days (Table 2.4).

**Distribution of follicular wave size, wave length, interwave interval and interval from wave peak to the emergence of the next follicular wave according to relevant time periods**

The time effect of the ongoing pregnancy on follicular dynamics was not only studied by grouping follicular wave parameters according to chronological wave numbers, but also by approaching the time of pregnancy under study as several relevant longer time sections that contained multiple follicular waves.

**Days 21 to 85 and days 86 to 150 of pregnancy**

When the time of pregnancy under study (days 21 to 150) was divided in two subgroups (days 21 to 85 and days 86 to 150 of pregnancy), the number of complete follicular waves with the largest follicular diameter ≥17 mm in mule pregnancies was less (P < 0.02) between days 21 to 85 (3.8 waves) than between days 86 to 150 (5.1 waves) (Table 2.5). Conversely, more (P < 0.0001) follicular waves with a diameter ≥17 mm occurred during days 21 to 85 (5.2 waves) of horse pregnancies than during days 86 to 150 (1.2 waves). Furthermore, horse pregnancies had more (P < 0.02) follicular waves ≥17 mm in diameter between days 21 and 85 of pregnancy than mule pregnancies and mule pregnancies had more (P < 0.0001) waves between days 86 to 150 of pregnancy than did horse pregnancies.

**Months of pregnancy**

When days 21 to 150 of pregnancy were divided into the second (days 20 to 60), third (days 61 to 90), fourth (days 91 to 120) or fifth (days 121 to 150) month of pregnancy, similar follicular development patterns were noted as when the follicular patterns of early pregnancy were analyzed by grouping follicular wave parameters
Table 2.5. Number of follicular waves with the largest follicle ≥17 mm in diameter during days 21 to 85 and during days 86 to 150 of pregnancy

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Treatment</th>
<th>Pregnancy type</th>
<th>Days 21 to 85</th>
<th>Days 86 to 150</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>control</td>
<td>horse</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>control</td>
<td>horse</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>≥20 mm</td>
<td>horse</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>≥20 mm</td>
<td>horse</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>≥20 mm</td>
<td>horse</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>all</td>
<td>horse</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>13</td>
<td>all</td>
<td>horse</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td>all</td>
<td>horse</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>all</td>
<td>horse</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Mean±SD</td>
<td></td>
<td></td>
<td>5.2±1.1a</td>
<td>1.2±1.3b</td>
</tr>
<tr>
<td>3</td>
<td>control</td>
<td>mule</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>control</td>
<td>mule</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>≥20 mm</td>
<td>mule</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>≥20 mm</td>
<td>mule</td>
<td>3</td>
<td>6</td>
</tr>
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<td>all</td>
<td>mule</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>17</td>
<td>all</td>
<td>mule</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>18</td>
<td>all</td>
<td>mule</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Mean±SD</td>
<td></td>
<td></td>
<td>3.8±1.8c</td>
<td>5.1±2.4a</td>
</tr>
</tbody>
</table>

*a,b,c* Values within rows or columns with different superscripts are different (GLM; P<0.02).
according to chronological wave number. When months of pregnancy were considered, only the pattern of follicular wave parameter changes was noted as pregnancy progressed; however, observed patterns were not statistically analyzed.

The diameter pattern observed for the largest follicle of a follicular wave for the control aspiration treatment (Treatment I) and when all follicles were aspirated (Treatment III) gradually decreased as pregnancy progressed (Figure 2.1). However, when only follicles with a diameter ≥20 mm in diameter were aspirated (Treatment II), the maximum diameter of follicular waves remained constant through months two to four of pregnancy and was only reduced in the fifth month of pregnancy.

Horse pregnancies followed a similar pattern as Treatments I and III where the diameter of the largest follicle decreased gradually from the second month of gestation to the fifth (32 mm to 13 mm in diameter). Mule pregnancies followed a similar pattern to that of Treatment II, where the maximum follicular diameter of a follicular wave stayed fairly constant through months two to four of pregnancy (24 mm, 23 mm and 25 mm, respectively) and then dropped slightly in the fifth month (21 mm) (Figure 2.2). The overall pattern for the mean diameter of the largest follicle of a follicular wave for all the follicular waves in this study was a gradual linear decrease from day 21 to 150 of gestation (decreased from a mean of 29.5 mm at days 21 to 60 to a mean diameter of 18 mm at days 121 to 150)(Figure 2.3).

The distribution pattern of follicular wave lengths for the control aspiration treatment (Treatment I) and when all follicles ≥20 mm in diameter were aspirated (Treatment II), demonstrated a gradual decrease from the second to fifth months of pregnancy (Figure 2.4). The same pattern of decrease was not detected when all follicles were aspirated (Treatment II). However, the shortest wave lengths for
Figure 2.1. Mean maximum diameter (mm) of the largest follicle of a follicular wave for the second to fifth month of pregnancy for the three follicular aspiration treatments.
Figure 2.2. Mean maximum diameter (mm) of the largest follicle of a follicular wave for the second to fifth month of pregnancy for horse and mule pregnancies.
Figure 2.3. Mean maximum diameter (mm) of the largest follicle of a follicular wave for the second to fifth month of pregnancy for all follicular waves.
Figure 2.4. Mean wave length (days) of follicular waves for the second to fifth month of pregnancy for the three follicular aspiration treatments.
Treatments I and II (days 121 to 150 of pregnancy) were the same as the constant but shorter wavelengths of Treatment III (Figure 2.4).

Horse pregnancies generally had longer mean wave lengths in the second and third months of pregnancy than mule pregnancies, however, this difference was no longer evident at the fourth and fifth months (Figure 2.5). The mean wave lengths of mule pregnancies were constant throughout the experimental period (8.5, 8.2, 8.3 and 8.0 days for the second, third, fourth and fifth months of pregnancy, respectively). Although the mean wavelength of the follicular waves across all three aspiration treatments and both pregnancy types in the second month of pregnancy was 9.6 days and in the fifth month was 7.7 days, an overall pattern of gradual decrease from day 20 to 150 of gestation was not striking (Figure 2.6).

Although there were differences in the frequency distribution of interwave intervals between the three follicular aspiration treatments when they were grouped according to chronological wave numbers (Table 2.3 and Appendix B, Figures 15 to 17), the length of the mean interwave intervals for each aspiration treatment did not change much as pregnancy progressed. The longest interwave intervals were observed when all follicles were aspirated (Treatment III) when compared with Treatments I and II (Figure 2.7). Similarly, no obvious difference in the distribution patterns of interwave intervals was evident through the second to fifth months of horse and mule pregnancies (Figure 2.8) or when all interwave intervals of this study were considered in the evaluation (Figure 2.9).

The mean interval from a wave peak to the emergence of the next follicular wave was either negative (overlapping of consecutive waves) or was less than 1 day long from the second to fifth months of pregnancy for the control aspiration treatment (Figure 2.10). This interval was most negative between days 21 and 60 of pregnancy
Figure 2.5. Mean wave length (days) of follicular waves for the second to fifth month of pregnancy for horse and mule pregnancies.
Figure 2.6. Mean wave length (days) of follicular waves for the second to fifth month of pregnancy for all follicular waves.
Figure 2.7. Mean interwave intervals (days) of follicular waves for the second to fifth month of pregnancy for the three follicular aspiration treatments.
Figure 2.8. Mean interwave intervals (days) of follicular waves for the second to fifth month of pregnancy for horse and mule pregnancies.
Figure 2.9. Mean interwave intervals (days) of follicular waves for the second to fifth month of pregnancy for all follicular waves.
Figure 2.10. Mean interval (days) from wave peak to emergence of a new follicular wave for the second to fifth month of pregnancy for the three follicular aspiration treatments.
(-2.4 days), whereafter the interval centered around zero for the remainder of the experimental period (0.4, 0.3 and -0.8 days for months 3, 4 and 5 of pregnancy, respectively). The interval between wave peak and emergence of the next wave appeared to be much longer for all four periods of pregnancy during the experimental period when all follicles (Treatment III) were aspirated (4 to 6 days) than when only follicles with a diameter ≥20 mm were aspirated (-0.3 to 2 days)(Treatment II) or when no follicles were aspirated (-2.4 to 0.3 days)(Treatment I).

In contrast to Treatment I, this interval generally seemed to increase as pregnancy progressed through months 2 to 5 for Treatments II and III (Figure 2.10). Horse and mule pregnancies had opposite distribution patterns of the interval between wave peak and the emergence of a new follicular wave (Figure 2.11). In horse pregnancies the interval appeared to increase from months 2 to 5 (-1.2 days to 4.3 days); whereas, in mule pregnancies the interval decreased (2.9 days to 0.4 days).

The overall tendency for the interval from wave peak to the emergence of the next follicular wave when all intervals in this study were included, followed the same pattern as those of horse pregnancies. The interval increased linearly from the second month (0.8 days) to the fourth month (2.9 days) of pregnancy after which it decreased to attain the same value in the fifth month (2.0 days) as what it had in the third month (2.1 days)(Figure 2.12).

**Number of days covered by follicular wave activity**

Alternatively, follicular activity was quantified by counting the number of days between days 21 and 150 of pregnancy (maximum of 130 days) that was covered by follicular wave (partial or complete waves) activity (only the growing phase was considered). The days covered by all-size follicular waves and the days covered only by waves with a maximal diameter ≥17 mm were considered separately. The mean
Figure 2.11. Mean interval (days) from wave peak to emergence of a new follicular wave for the second to fifth month of pregnancy for horse and mule pregnancies.
Figure 2.12. Mean interval (days) from wave peak to emergence of a new follicular wave for the second to fifth month of pregnancy for all follicular waves.

Days of Pregnancy

Interval From Wave Peak to Start of Next Wave (Days)

Days 21 to 60
Days 61 to 90
Days 91 to 120
Days 121 to 150
number of days covered by all-size follicular waves was the greatest (109.8 days) for Treatment I, lower (P<0.04, compared with Treatment I) for Treatment II (89.9 days) with the least (P<0.002, compared with Treatment I) number of days (74.3 days) covered by any follicular wave activity in Treatment III mares (Table 2.6). When only days covered by follicular waves with the largest follicle ≥17 mm in diameter were considered, the greatest number of days covered was similar to that of Treatment I (92 days), with a tendency (P<0.09, compared with Treatment I) for fewer days covered by waves for Treatment II (63.7 days) and the least (P<0.02, compared with Treatment I) number of days covered by waves for Treatment III (51.3 days). However, the number of days covered by follicular wave activity and the number of days covered only by waves with a largest follicular diameter ≥17 mm were not different between Treatments II and III.

The number of days covered by all-size follicular waves or only by large waves was not different between horse and mule pregnancies (Table 2.6). However, both the number of all-size follicular waves (P<0.10) and the number of waves with a largest follicle ≥17 mm in diameter tended (P<0.07) to be lower for horse pregnancies than for mule pregnancies (Table 2.7).

When the number of days covered by all follicular wave activity or by activity representing only waves with a follicular diameter ≥17 mm in diameter was considered separately for each ovary, no differences were evident between the right and left ovary.

**Number of waves identified on each ovary and the number of waves contributed to the overall individual follicular profiles by right and left ovaries**

Two other parameters were evaluated to determine if there were any differences between the follicular activity of the left and right ovary. These parameters were the number of waves detected on each ovary and the number of waves contributed by each
Table 2.6. Total number of days during the experimental period covered by follicular activity and number of days covered only by waves with the diameter of the largest follicle ≥17 mm

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Treatment</th>
<th>Pregnancy type</th>
<th>Days covered (all follicular waves)</th>
<th>Days covered (waves ≥17 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>control</td>
<td>horse</td>
<td>102</td>
<td>72</td>
</tr>
<tr>
<td>2</td>
<td>control</td>
<td>horse</td>
<td>121</td>
<td>87</td>
</tr>
<tr>
<td>3</td>
<td>control</td>
<td>mule</td>
<td>108</td>
<td>101</td>
</tr>
<tr>
<td>4</td>
<td>control</td>
<td>mule</td>
<td>108</td>
<td>108</td>
</tr>
<tr>
<td>5</td>
<td>≥20 mm</td>
<td>horse</td>
<td>87</td>
<td>73</td>
</tr>
<tr>
<td>6</td>
<td>≥20 mm</td>
<td>horse</td>
<td>57</td>
<td>30</td>
</tr>
<tr>
<td>7</td>
<td>≥20 mm</td>
<td>horse</td>
<td>110</td>
<td>90</td>
</tr>
<tr>
<td>8</td>
<td>≥20 mm</td>
<td>mule</td>
<td>84</td>
<td>77</td>
</tr>
<tr>
<td>9</td>
<td>≥20 mm</td>
<td>mule</td>
<td>96</td>
<td>80</td>
</tr>
<tr>
<td>10</td>
<td>≥20 mm</td>
<td>mule</td>
<td>94</td>
<td>9</td>
</tr>
<tr>
<td>11</td>
<td>≥20 mm</td>
<td>mule</td>
<td>101</td>
<td>87</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td></td>
<td>109.8 ± 8.0*</td>
<td>92 ± 15.9*</td>
</tr>
<tr>
<td>5</td>
<td>≥20 mm</td>
<td>horse</td>
<td>87</td>
<td>73</td>
</tr>
<tr>
<td>6</td>
<td>≥20 mm</td>
<td>horse</td>
<td>57</td>
<td>30</td>
</tr>
<tr>
<td>7</td>
<td>≥20 mm</td>
<td>horse</td>
<td>110</td>
<td>90</td>
</tr>
<tr>
<td>8</td>
<td>≥20 mm</td>
<td>mule</td>
<td>84</td>
<td>77</td>
</tr>
<tr>
<td>9</td>
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<td>mule</td>
<td>96</td>
<td>80</td>
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<td>11</td>
<td>≥20 mm</td>
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<td>101</td>
<td>87</td>
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<tr>
<td>Mean ± SD</td>
<td></td>
<td></td>
<td>89.9 ± 16.9b</td>
<td>63.7 ± 31.3b</td>
</tr>
<tr>
<td>12</td>
<td>all</td>
<td>horse</td>
<td>70</td>
<td>40</td>
</tr>
<tr>
<td>13</td>
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<td>horse</td>
<td>58</td>
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</tr>
<tr>
<td>15</td>
<td>all</td>
<td>horse</td>
<td>90</td>
<td>59</td>
</tr>
<tr>
<td>16</td>
<td>all</td>
<td>mule</td>
<td>73</td>
<td>40</td>
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<td>17</td>
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<td>18</td>
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<td>mule</td>
<td>76</td>
<td>64</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td></td>
<td>74.3 ± 11.0b</td>
<td>51.3 ± 14.6b</td>
</tr>
</tbody>
</table>

Horse pregnancies 84.6 ± 23.0* 59.1 ± 22.0*
Mule pregnancies 91.8 ± 12.9* 71.2 ± 30.6*

* Values within columns with different superscripts are different (GLM; P<0.05).
Table 2.7. Number of follicular waves identified for each horse, for each ovary independently and the number of waves contributed by each ovary to the final individual follicular activity profiles

<table>
<thead>
<tr>
<th>Trt type</th>
<th>Preg. no.</th>
<th>Total no. waves (≥17 mm)</th>
<th>R.O. no. waves (≥17 mm)</th>
<th>L.O. no. waves (≥17 mm)</th>
<th>R.O. contr. overall (≥17 mm)</th>
<th>L.O. contr. overall (≥17 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control horse</td>
<td>9 (5)</td>
<td>8 (5)</td>
<td>8 (5)</td>
<td>7 (4)</td>
<td>2 (1)</td>
<td></td>
</tr>
<tr>
<td>control horse</td>
<td>11 (6)</td>
<td>8 (4)</td>
<td>8 (4)</td>
<td>5 (3)</td>
<td>6 (3)</td>
<td></td>
</tr>
<tr>
<td>control mule</td>
<td>14 (13)</td>
<td>11 (11)</td>
<td>9 (8)</td>
<td>8 (8)</td>
<td>6 (5)</td>
<td></td>
</tr>
<tr>
<td>control mule</td>
<td>10 (10)</td>
<td>10 (10)</td>
<td>9 (8)</td>
<td>7 (7)</td>
<td>6 (3)</td>
<td></td>
</tr>
<tr>
<td>Mean±SD (all) (≥17 mm)</td>
<td>11.0±2.2a</td>
<td>9.3±1.5b</td>
<td>8.5±0.6c</td>
<td>6.8±1.3d</td>
<td>4.3±2.1e</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Trt type</th>
<th>Preg. no.</th>
<th>Total no. waves (≥17 mm)</th>
<th>R.O. no. waves (≥17 mm)</th>
<th>L.O. no. waves (≥17 mm)</th>
<th>R.O. contr. overall (≥17 mm)</th>
<th>L.O. contr. overall (≥17 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥20mm horse</td>
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<td>10 (8)</td>
<td>10 (7)</td>
<td>8 (6)</td>
<td>2 (2)</td>
<td></td>
</tr>
<tr>
<td>≥20mm horse</td>
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<td>6 (3)</td>
<td>6 (3)</td>
<td>4 (2)</td>
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<tr>
<td>≥20mm horse</td>
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<td>13 (9)</td>
<td>13 (10)</td>
<td>6 (4)</td>
<td>8 (7)</td>
<td></td>
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<tr>
<td>≥20mm mule</td>
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<td>8 (7)</td>
<td>8 (7)</td>
<td>3 (3)</td>
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<tr>
<td>≥20mm mule</td>
<td>12 (9)</td>
<td>9 (7)</td>
<td>11 (8)</td>
<td>5 (5)</td>
<td>7 (4)</td>
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</tr>
<tr>
<td>≥20mm mule</td>
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<td>12 (1)</td>
<td>10 (1)</td>
<td>9 (1)</td>
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<td>13 (11)</td>
<td>13 (11)</td>
<td>12 (9)</td>
<td>7 (5)</td>
<td>6 (6)</td>
<td></td>
</tr>
<tr>
<td>Mean±SD (all) (≥17 mm)</td>
<td>11.6±2.6a</td>
<td>10.1±2.7b</td>
<td>10.0±2.4c</td>
<td>6.0±2.2d</td>
<td>5.6±2.0e</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Trt type</th>
<th>Preg. no.</th>
<th>Total no. waves (≥17 mm)</th>
<th>R.O. no. waves (≥17 mm)</th>
<th>L.O. no. waves (≥17 mm)</th>
<th>R.O. contr. overall (≥17 mm)</th>
<th>L.O. contr. overall (≥17 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>all horse</td>
<td>12 (7)</td>
<td>9 (5)</td>
<td>11 (6)</td>
<td>6 (3)</td>
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<td>6 (4)</td>
<td>7 (4)</td>
<td>5 (3)</td>
<td>5 (2)</td>
<td></td>
</tr>
<tr>
<td>all horse</td>
<td>10 (5)</td>
<td>10 (5)</td>
<td>4 (3)</td>
<td>10 (5)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>all horse</td>
<td>8 (5)</td>
<td>6 (2)</td>
<td>4 (4)</td>
<td>4 (1)</td>
<td>4 (4)</td>
<td></td>
</tr>
<tr>
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<td>7 (6)</td>
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<td>11 (9)</td>
<td>11 (9)</td>
<td>10 (9)</td>
<td>4 (3)</td>
<td>7 (6)</td>
<td></td>
</tr>
<tr>
<td>all mule</td>
<td>11 (5)</td>
<td>8 (4)</td>
<td>6 (3)</td>
<td>6 (3)</td>
<td>5 (2)</td>
<td></td>
</tr>
<tr>
<td>Mean±SD (all) (≥17 mm)</td>
<td>10.6±1.4a</td>
<td>8.6±2.0b</td>
<td>7.0±2.7c</td>
<td>6.0±2.1d</td>
<td>4.6±2.2e</td>
<td></td>
</tr>
</tbody>
</table>

(Table cont’d)
<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>All trt's (all)</td>
<td>11.1 ± 2.0</td>
<td>9.3 ± 2.2f</td>
<td>8.5 ± 2.5f</td>
<td>6.2 ± 1.9e</td>
<td>4.8 ± 2.1h</td>
</tr>
<tr>
<td>(≥17mm)</td>
<td>(7.3 ± 3.1)</td>
<td>(6.3 ± 3.0)E</td>
<td>(5.8 ± 2.5)H</td>
<td>(4.0 ± 1.9)F</td>
<td>(3.3 ± 2.0)F</td>
</tr>
<tr>
<td>Horse preg. (all)</td>
<td>10.2 ± 1.9i</td>
<td>8.4 ± 2.4k</td>
<td>7.9 ± 3.1k</td>
<td>6.1 ± 2.0o</td>
<td>4.1 ± 2.5p</td>
</tr>
<tr>
<td>(≥17mm)</td>
<td>(6.2 ± 2.2)G</td>
<td>(5.0 ± 2.2)I</td>
<td>(5.1 ± 2.3)J</td>
<td>(3.4 ± 1.5)l</td>
<td>(2.8 ± 2.0)j</td>
</tr>
<tr>
<td>Mule preg. (all)</td>
<td>11.9 ± 1.9j</td>
<td>10.2 ± 1.7e</td>
<td>9.1 ± 1.9k</td>
<td>6.2 ± 1.9a</td>
<td>5.7 ± 1.2m</td>
</tr>
<tr>
<td>(≥17mm)</td>
<td>(8.4 ± 3.5)H</td>
<td>(7.5 ± 3.3)I</td>
<td>(6.4 ± 2.8)j</td>
<td>(4.5 ± 2.2)l</td>
<td>(3.9 ± 2.0)j</td>
</tr>
</tbody>
</table>

* Used where follicular waves of all sizes were considered to calculate the means.

** Used where only follicular waves with the diameter of the largest follicle ≥20 mm were considered to calculate the means.

* Values within the column with different superscripts are different.

** Values within columns or rows with different superscripts are different.

** Values within columns or rows with different superscripts are different (P<0.1).

** Values within columns or rows with different superscripts are different.

** Values within columns or rows with different superscripts are different (P<0.01).

** Values within columns or rows with different superscripts are different.

** Values within columns or rows with different superscripts are different.

(GLM; P<0.05).
ovary to the overall follicular profile of a mare (an indication of wave quality, also see Appendix A for individual wave profiles). All-size follicular waves and waves with the largest follicle ≥17 mm in diameter only, were considered separately for each parameter (Table 2.7).

**Number of waves identified on the right and left ovaries**

There was no difference in the number of all-size follicular waves or in the number of waves with the largest follicle ≥17 mm in diameter identified in the overall follicular profile between the three aspiration treatments (Treatments I, II and III). The number of all-size follicular waves occurring on the right ovary for the three aspiration treatments was not different. However, the number of all-size follicular waves of Treatment II on the left ovary (10.0 waves) was more (P<0.02) than the number of waves when all follicles were aspirated (7.0 waves). The difference was not significant when only follicular waves with a maximum follicular diameter ≥17 mm were considered in this analysis. Also, there was no difference between the right and left ovary in the number of all-size or number of follicular waves with the largest follicle ≥17 mm in diameter when the results were pooled across all aspiration treatments (Table 2.7).

**Number of waves contributed by the right and left ovary to the overall individual follicular profiles**

The number of all-size follicular waves that contributed to the overall follicular profile tended (P<0.10) to be more for the right (6.8 waves) than for the left (4.3 waves) ovary in the control aspiration treatment (Treatment I). Although the mean number of all-size waves contributed by the right and left ovary to the overall follicular wave profile was not different for Treatments II and III, the right ovary (6.2 waves) still tended (P<0.10) to contribute more waves than the left ovary (4.8 waves) across all
three aspiration treatment groups. A similar pattern was observed when only the number of waves with a largest follicular diameter ≥17 mm was considered. A tendency (P<0.10) was observed for more large-size waves contributed to the overall follicular wave profile by the right ovary (5.5 waves) than by the left ovary (3.0 waves) for the control aspiration treatment (Treatment I). However, this difference between the right and left ovary was not evident in Treatments II and III or in the overall contribution means across all three aspiration treatments (Table 2.7).

The right ovary of horse pregnancies contributed more (P<0.05) all-size follicular waves (6.1 waves) to the overall follicular wave patterns than the left ovary (4.1 waves). The number of all-size follicular waves contributed by the right and left ovary was not different for mule pregnancies. Furthermore, the number of all-size waves contributed by the right and left ovaries of horse and mule pregnancies was not different. Neither horse pregnancies nor mule pregnancies were different in the number of waves with a largest follicular diameter ≥17 mm that were contributed by the right and left ovary (Table 2.7).

**Total follicular diameter**

The means of the three times per week total follicular diameter values were compared between horse and mule pregnancies for days 21 to 150 of pregnancy. The mean total follicular diameters of horse and mule pregnancies were almost parallel between days 21 and 45 of pregnancy; thereafter, the means diverged. Both means started with total diameter values of ≈260 mm at day 21 of pregnancy, dropped to ≈175 mm at day 25, increased again to ≈235 mm at day 32 and dropped to maintain values of 139 to 170 mm until divergence at day 45 of gestation (Figure 2.13). At day 45 of pregnancy, the mean total follicular diameter of horse pregnancies increased sharply to a maximum at day 59 (275 mm), then decreased sharply to reach a mean
Figure 2.13. Mean total follicular diameter of horse and mule pregnancies between days 21 and 150 of pregnancy related to the mean circulating concentrations of eCG of horse pregnancies. Shaded areas between the mean total follicular diameter lines for the two pregnancy types are significantly different (GLM, P ≤ 0.10).
total follicular diameter of 106 mm at day 78, followed by a gradual decrease until day 150 of pregnancy (71 mm). In contrast, mean total follicular diameter values of mule pregnancies decreased further after divergence from the mean total diameter line of horse pregnancies at day 45 of gestation to attain a minimum value (91 mm) at day 54. The mean total follicular diameter values for mule pregnancies then gradually increased to reach a value of 125 mm at day 147 of pregnancy. When superimposed, the mean total follicular diameter lines of horse and mule pregnancies diverged approximately at day 45 of gestation (mean horse values were greater than those of the mule), converged around day 78 to run almost parallel until day 100 to then finally diverge and remain separate until the end of the 130-day experimental period (mule mean total diameter values now greater than horse mean total diameter values)(Figure 2.13). The mean total follicular diameter values for horse pregnancies were greater (P<0.02) than those of mule pregnancies between days 53 and 62 of pregnancy and, conversely, the mean total follicular diameter values for mule pregnancies were greater (P<0.1) than those of horse pregnancies between days 129 and 138 of gestation.

**Pregnancy continuation and foal births**

Up to 100 days after the last aspiration procedure, all 18 mares in this study were confirmed pregnant by the ultrasonic detection of a fetal heart beat. Five male horse, four female horse, six male mule and two female mule foals were born at term from the mares used in this study.

**Hormone profiles**

**Estradiol-17β**

Circulating concentrations of estradiol-17β were determined every third day, starting at day 40 of pregnancy and continued until the end of the experimental period (day 150). The mean estradiol levels were only compared between the pregnancy
types. There was no difference in the circulating concentrations of estradiol-17β between days 40 and 150 of pregnancy for horse and mule pregnancies (Figure 2.14). Low levels of estradiol were detected at day 40 (=5 pg per ml), started to rise around day 90 of pregnancy (> 10 pg per ml) and continued to rise sharply until the end of the experimental period (=150 pg per ml).

**Equine chorionic gonadotropin (eCG)**

Serum concentrations of eCG were measured every second day between days 30 and 40 of pregnancy followed by once weekly measurements until day 150 of pregnancy and mean circulating concentrations of eCG were only compared between horse and mule pregnancies. An obvious difference in mean eCG levels was evident between horse and mule pregnancies ($P < 0.05$)(Figure 2.15). On average, eCG was not detectable or present at very low concentrations (<1 IU per ml) between days 30 and 35 of horse and mule pregnancies. The mean circulating concentrations of eCG in horse pregnancies started to increase after day 35 of pregnancy, peaked between days 56 and 77 of pregnancy (maximum of 188 IU per ml, day 63 of pregnancy) and decreased progressively to attain values =10 IU per ml between days 140 and 150 of pregnancy.

The mean circulating concentrations of eCG in mule pregnancies also started to increase just after day 35 of pregnancy, peaked between days 39 and 70 of pregnancy (maximum of 15 IU per ml, day 56 of pregnancy) and decreased to values of $\leq 0.5$ IU after day 105 of pregnancy. The means of circulating concentrations of eCG of horse and mule pregnancies diverged at day 36 of pregnancy (Figure 2.15). However, the means of circulating concentrations of eCG were significantly different ($P < 0.05$) only between days 49 and 105 of pregnancy.
Figure 2.14. Mean circulating concentrations of estradiol-17β measured every third day between days 40 and 150 of horse and mule pregnancies. The lines representing the mean circulating estradiol-17β concentrations are not significantly different at any point between days 40 and 150 of pregnancy.
Equine Chorionic Gonadotropin: Horse vs. Mule Pregnancies

Figure 2.15. Mean circulating concentrations of eCG measured every other day between days 30 and 40 of horse and mule pregnancies followed by weekly measurements until day 150 of pregnancy. Shaded areas between the mean concentration lines of the two pregnancy types are significantly different (GLM, P≤0.05).
**Progesterone**

Progesterone concentrations were determined for alternate days between days 21 and 150 of gestation. Graphically, the mean circulating progesterone levels did not appear to be different for the three aspiration treatments until days 43 to 52 of pregnancy (Figures 2.16 and 2.17). At day 43 the line representing the mean circulating levels of progesterone when all follicles were aspirated (Treatment III) diverged from that of the control aspiration treatment (Treatment I) so that the mean concentration of mares in Treatment I were greater than those of Treatment III (Figure 2.16). These two lines converged again at day 147 of pregnancy. The same pattern was observed between the mean progesterone levels of mares in Treatment I and when only follicles with a diameter ≥20 mm in diameter were aspirated (Treatment II). The mean levels from Treatments I and II diverged at day 52 of pregnancy with Treatment I maintaining the higher circulating progesterone levels (Figure 2.17). The means converged again at day 139 of gestation. The mean concentrations of circulating progesterone were significantly greater (P<0.10) for Treatment I than for Treatment II for days 63 to 65, 67 to 75, 79 to 93, 95 to 97, 99 to 107 and 109 to 111 of pregnancy (Figure 2.17). Similarly, the mean concentrations of circulating progesterone were greater (P<0.10) for Treatment I than for Treatment III for days 63 to 79, 83 to 93, 95 to 97, and 101 to 103 of pregnancy (Figure 2.16). The mean concentrations of circulating progesterone were not different for Treatments II and III at any time during the experimental period.

Graphically, the mean circulating progesterone concentrations for females with horse and mule pregnancies diverged at day 67 of pregnancy with the mean progesterone concentrations for horse pregnancies elevated throughout the experimental period (Figure 2.18). The mean circulating progesterone concentration was significantly
Figure 2.16. Mean circulating concentrations of progesterone measured every other day between days 21 and 150 of pregnancy for the three aspiration treatments. Shaded areas between the mean concentration lines of Treatments I (Control) and III (All) are significantly different (GLM, P≤0.10).
Figure 2.17. Mean circulating concentrations of progesterone measured every other day between days 21 and 150 of pregnancy for the three aspiration treatments. Shaded areas between the mean concentration lines of Treatments I (Control) and II (>20 mm) are significantly different (GLM, P ≤ 0.10).
Figure 2.18. Mean circulating levels of progesterone measured every other day between days 21 and 150 of pregnancy for horse and mule pregnancies. Shaded areas between the mean concentration lines of the two pregnancy types are significantly different (GLM, $P \leq 0.10$).
greater (P<0.10) for horse pregnancies for days 41 to 43, 95 to 99, 105 to 107, 109 to 137, 141 to 145 and 147 to 149 of gestation.

The difference between the progesterone concentration at the morning of aspiration and 6 to 8 days later (as only alternate samples were analyzed) was used to estimate the effect of follicular aspiration on the ability of aspirated follicles to luteinize and/or produce progesterone. To allow for the possible effect of eCG on progesterone production, the change in circulating progesterone concentrations was considered separately for three relevant time periods. These time periods were days 21 to 39 (before eCG, mean levels of horse eCG between 0.4 and 38 IU per ml), 40 to 95 (high concentrations of eCG, mean levels of horse eCG between 43 and 188 IU per ml) and 96 to 150 (after eCG, mean levels of horse eCG between 7 and 59 IU per ml) of pregnancy.

There was no difference in the mean increase of circulating progesterone levels 6 to 8 days post-aspiration between mares where all follicles were aspirated and mares where only follicles with a diameter ≥20 mm were aspirated within each designated time period (days 21 to 39, 40 to 95 and 96 to 150 of pregnancy) (Figure 2.19). However, when only follicles with a diameter ≥20 mm were aspirated (Treatment II), the mean increase in circulating concentrations of progesterone 6 to 8 days post-aspiration was significantly greater between days 21 and 39 of pregnancy than between days 40 and 95 or between days 96 and 150. When all follicles were aspirated (Treatment III), the mean increase in circulating concentrations of progesterone 6 to 8 days post-aspiration was significantly higher between days 21 and 39 of pregnancy than between days 96 to 150 of pregnancy. There was no difference in the post-aspiration increase of progesterone between the 40 to 95 and 96 to 150 days of pregnancy time period within Treatments II and III. Post-aspiration changes of circulating
Figure 2.19. Increases in mean circulating progesterone concentrations 6 to 8 days post-aspiration for the three aspiration treatments between days 21 and 39, 40 and 95, and 96 and 150 of gestation.

*a,b Bars with different superscripts are significantly different (ANOVA, P < 0.05).
concentrations of progesterone for the control aspiration treatment (Treatment I) were not considered for time period comparisons, because the single sham aspiration procedure was performed between days 40 and 95 of pregnancy.

Although mean circulating progesterone concentrations were greater in mule pregnancies than for horse pregnancies between days 41 and 43 of pregnancy, the mean increase in circulating progesterone concentrations 6 to 8 days post-aspiration was not different for horse and mule pregnancies (Figure 2.20). However, the mean increase in circulating concentrations of progesterone 6 to 8 days post-aspiration tended to be higher ($P<0.09$) for horse pregnancies (1.33 ng per ml) than for mule pregnancies (0.30 ng per ml) between days 40 and 95 of pregnancy. There was no difference in the post-aspiration increase of mean circulating progesterone concentrations for horse and mule pregnancies between days 96 and 150 of pregnancy (0.38 and 0.49 ng per ml, respectively).

Mule pregnancies had a higher ($P<0.05$) post-aspiration increase in mean circulating concentrations of progesterone between days 21 and 39 of pregnancy (3.29 ng per ml) than between days 40 and 95 (0.30 ng per ml) or 96 and 150 (0.49 ng per ml). The increase in mean circulating levels of progesterone 6 to 8 days after aspiration was not different for the 40 to 95- and 96 to 150-day of pregnancy time periods of mule pregnancies. The post-aspiration increase of mean circulating concentrations of progesterone for horse pregnancies was higher ($P<0.05$) between days 21 to 39 (1.7 ng per ml) of pregnancy than between days 96 to 150 (0.5 ng per ml).

**Follicle stimulating hormone (FSH)**

The mean circulating concentrations of FSH for mares in the three aspiration treatments were characterized by multiple periodic spikes. Graphically, it seemed that the mean FSH levels of the control aspiration treatment (Treatment I) and when all
Figure 2.20. Increases in mean circulating progesterone concentrations 6 to 8 days post-aspiration for horse and mule pregnancies between days 21 and 39, 40 and 95, and 96 and 150 of gestation.
follicles were aspirated (Treatment III) were lower than when only follicles with a diameter ≥20 mm were aspirated (Treatment II) between days 46 and 105 of pregnancy (Figure 2.21). Although the pulsatile patterns of Treatments I and III crossed frequently, the mean circulating FSH concentration of Treatment III seemed higher than that of Treatment I until day 105 of pregnancy. The mean circulating concentrations of FSH were significantly different (P<0.10) for Treatments II and I for days 62 to 63, 73 and 111 of pregnancy. Also, the mean circulating concentrations of FSH were significantly different (P<0.1) for Treatments II and III for days 47, 62 to 63, 73, 103 and 111 of pregnancy. The mean circulating concentrations of FSH were not different for Treatments I and III at any time during days 21 to 150 of pregnancy.

When the graph representing the mean circulating concentrations of FSH for horse and mule pregnancies was evaluated, the mean circulating concentrations of FSH for mule pregnancies were consistently higher than those of horse pregnancies, at least between days 39 and 110 of pregnancy (Figure 2.22). The mean circulating concentrations of FSH were significantly different (P<0.09) for horse and mule pregnancies between days 47, 59, 63, 65, 72 to 76, 109, 111 and 115 of pregnancy.

Each individual mare FSH profile between days 21 and 150 of pregnancy was inspected and any visual FSH surges preceding an identified follicular wave peak was noted. The mean amplitudes of these presumptive FSH surges were different for the three aspiration treatments (Figure 2.23). The mean FSH amplitude in mares from which only follicles with a diameter ≥20 mm were aspirated (Treatment II, 42.0 ng per ml) was higher (P<0.05) than that in mares from which no follicles (Treatment I, 20.4 ng per ml) were aspirated, and tended (P<0.07) to be higher than that of mares from which all follicles (Treatment III, 26.0 ng per ml) were aspirated.
Figure 2.21. Mean daily circulating concentrations of FSH for the three aspiration treatments between days 21 and 150 of pregnancy. Multiple points, rather than areas between the mean concentration lines of the three aspiration treatments are significantly different although not indicated on the graph.
Figure 2.22. Mean daily circulating concentrations of FSH for horse and mule pregnancies between days 21 and 150 of pregnancy related to the mean circulating concentrations of eCG of horse pregnancies. Shaded area and specific points (---) between the mean concentration lines of the two pregnancy types are significantly different (GLM, P<0.10).
Figure 2.23. The mean amplitude (increase over the baseline value) of the FSH surge preceding an identified follicular wave peak for the three follicular aspiration treatments during days 21 to 150 of pregnancy.
The mean amplitude of FSH surges preceding an identified follicular wave peak was not different between horse and mule pregnancies (35.6 and 27.3 ng per ml, respectively). Also, the mean amplitude of FSH surges preceding follicular waves where a dominant follicle was identified (35.5 ng per ml) was not significantly greater than in waves where follicular dominance was not evident (29.1 ng per ml). When the mean amplitude of FSH surges preceding follicular waves with a largest follicle ≥20 mm in diameter was compared with the mean amplitude of surges preceding waves where the largest follicle did not reach 20 mm in diameter, no difference was detected (31.6 and 29.8 ng per ml, respectively).

The mean number of days between the peak of the FSH surge and the peak of the following follicular wave was different for the three aspiration treatments (Figure 2.24). The mean number of days between the peak of the FSH surge and the peak of the following follicular wave tended (P<0.08) to be higher for the control aspiration treatment (Treatment I, 6.8 days) than when only follicles with a diameter ≥20 mm (Treatment II, 6.0 days) were aspirated and was higher (P<0.05) than when all follicles (Treatment III, 5.4 days) were aspirated. The length of the FSH surge to wave peak interval was not different for Treatments II and III. The mean number of days between the peak of the FSH surge and the peak of the following follicular wave was not different for horse and mule pregnancies (6.0 and 5.9 days, respectively).

The time period between the peak of the FSH surge and the peak of the following follicular wave was significantly longer (P<0.05) for follicular waves where a dominant follicle was identified (6.5 days) when compared with waves where the expression of follicular dominance was not evident (5.7 days)(Figure 2.25). Follicular dominance was detected in 56 (30.6%) of the 183 identified follicular waves in this study. This time period was also longer (P<0.05) for follicular waves with a largest
Figure 2.24. Mean number of days between the peak of the FSH surge and the peak of the following follicular wave for the three follicular aspiration treatments between days 21 and 150 of pregnancy.
Figure 2.25. Mean number of days between the peak of the FSH surge and the peak of the following follicular wave for follicular waves with and without a dominant follicle between days 21 and 150 of pregnancy.
follicle ≥20 mm in diameter (6.4 days) when compared with waves where the largest follicle did not reach 20 mm in diameter (Figure 2.26). The overall time period between the peak of the FSH surge and the peak of the following follicular wave for all 183 waves was 5.9 days.

**Discussion**

Three size ranges were devised and used in this study to compile the frequency distribution for the maximum diameter of the largest follicle of a follicular wave. This gave an indication of the distribution of follicular wave sizes between the three follicular aspiration treatments, pregnancy types (horse and mule pregnancies) and ovary sides (right and left ovary). Recently, transrectal ultrasound studies in cycling mares (Ginther, 1990, 1993; Bergfelt & Ginther, 1993) and mares that were 0 to 50 days pregnant (Bergfelt & Ginther, 1992; Ginther & Bergfelt, 1992a) characterized major follicular waves with the diameter of the largest follicle ≥35 mm (35 to 56 mm) and minor waves with the diameter of the largest follicle <35 mm (18 to 34 mm). Based on these findings, the three follicular diameter ranges selected for this study were waves with a largest follicle < 17 mm (small), 17 to 34 mm (medium) or > 34 mm (large) in diameter.

No difference was detected in the frequency distribution of small-, medium- or large-size waves for the three aspiration treatments, indicating that the follicular aspiration treatments per se did not affect the maximum diameter that the largest follicle of a follicular wave could obtain. The distribution of wave sizes across all three follicular aspiration treatments seems to follow a similar pattern as represented by the overall wave frequency distribution. From the 204 maximum diameters of the largest follicle of a follicular wave that were measured, 32% were in the small-size category, 52% in the medium-size category and 16% were grouped in the large-size category.
Figure 2.26. Mean number of days between the peak of the FSH surge and the peak of the following follicular wave for follicular waves with a largest follicle ≥20 mm in diameter and for waves where the largest follicle did not reach 20 mm in diameter between days 21 and 150 of pregnancy.

*a,b Bars with different superscripts are significantly different (ANOVA, P < 0.05).*
For the 18 mares in this study, more than half of the follicular waves between days 21 and 150 of pregnancy had a follicle with a maximum diameter of 17 to 34 mm (equivalent to minor follicular waves), a third had a maximum follicular diameter < 17 mm and only 16% had a follicle with a maximum diameter > 34 mm (equivalent to major follicular waves). This is different from an ultrasound study on pregnant riding-type mares, where half of all the waves were classified as major waves (mean diameter of the dominant follicle of 42 mm) and the other half as minor waves (mean diameter of the largest follicle of 27 mm) (Ginther & Bergfelt, 1992a). It should be noted that only mares on days 11 to 40 of pregnancy were evaluated in this study. It is accepted, however, that low levels of FSH and follicular activity are prevalent during later stages of the equine pregnancy (Ginther, 1992).

Mule pregnancies had more medium-size follicular waves (60%) than horse pregnancies. This could be explained by looking at the frequency distribution patterns of horse and mule pregnancies (Appendix B, Figures 4 and 5). For horse pregnancies, the majority of follicular waves were large for the first two waves of pregnancy. The large waves were gradually replaced by medium-size waves and at the end of the observation period (wave numbers 7 to 14), almost all the waves were small. The same pattern could not be identified in mule pregnancies and medium-size follicular waves were most prominent almost up to day 150 of pregnancy.

This difference in wave-size distribution in horse and mule pregnancies was also evident when the number of waves with a largest follicle ≥ 17 mm in diameter during days 21 to 85 and during days 86 to 150 of gestation was compared between the two pregnancy types. Mares carrying horse pregnancies had more waves ≥ 17 mm in diameter than mares carrying mule pregnancies during days 21 to 85 of pregnancy. Conversely, mares carrying mule pregnancies had more waves ≥ 17 mm in diameter
than mares carrying horse pregnancies during days 86 to 150 of pregnancy. Horse pregnancies had fewer waves with a largest follicle ≥17 mm in diameter in the second time period than in the first time period and mule pregnancies had fewer waves with a largest follicle ≥17 mm in diameter in the first time period than during the second time period.

The distribution pattern of decreasing size and numbers of large- and medium-size follicular waves as pregnancy progressed to day 150 in horse pregnancies is in agreement with other studies that reported mare follicular, luteal and FSH activity to decrease drastically after day 100 of gestation (Allen, 1974, 1975a; Squires & Ginther, 1975; Wesson & Ginther, 1980). However, mares carrying mule pregnancies did not follow the expected pattern of decreasing follicular diameter as pregnancy progressed to day 150. Although it is accepted that eCG only has LH-like activity in the mare, it is agreed that it may act synergisticly with FSH on developing follicles of pregnancy (Squires & Ginther, 1975; Urwin & Allen, 1982b; Urwin, 1983). It is proposed that eCG by virtue of its LH activity induces a final growth spurt with resultant luteinization or ovulation of FSH-differentiated follicles (Allen, 1974, 1984).

Horse pregnancies have considerable higher circulating concentrations of eCG than mule pregnancies with a progressive decrease of serum levels of eCG after day 70 of pregnancy (Allen, 1975b, 1982, 1984; Allen et al., 1993). This may explain why horse pregnancies had more larger (largest follicle ≥17 mm in diameter) follicular waves during days 21 to 85 of pregnancy than mule pregnancies. However, it is not clear why the number of follicular waves ≥17 mm in diameter in horse pregnancies decreased so drastically after the time of peak circulating concentrations of eCG. Since the same decrease in number of larger follicular waves (largest follicle ≥17 mm in diameter) during the post-eCG period of pregnancy (days 86 to 150) was not found in
mule pregnancies (not exposed to the same high levels of eCG), it may be that the presence followed by the absence of high concentrations of eCG actively decreased the ability of mares carrying horse pregnancies to produce larger follicular waves.

The mean maximum diameter of the largest follicle of large-size waves was 40 mm, 25 mm for medium-size waves and 12 mm for small-size follicular waves. These diameter sizes are in agreement with a study on mares between days 0 and 50 of pregnancy where the mean maximum diameter was 42 mm for major follicular waves and 27 mm for minor follicular waves (Ginther & Bergfelt, 1992a). It seems that the small-size waves with a mean largest follicular diameter of 12 mm represent a third population of waves, not previously identified.

The length of the growing phase (wave length) of the ovulatory major follicular wave in cycling mares was 9 days and that of minor follicular waves was 4 days (Bergfelt & Ginther, 1993). However, only follicles ≥15 mm in diameter were studied. On average, the emergence of a follicular wave in this study (first day of identification of the largest follicle of a wave) was identified when the largest follicle was only 8 mm in diameter. Giving a typical growth rate of 3 mm per day (Ginther & Bergfelt, 1992a, 1993), one can assume that waves were detected just more than 2 days earlier in this study than in the report by Bergfelt & Ginther (1993). When a mathematical approach was used to identify wave emergence in mares 11 to 40 days pregnant, the wave lengths for major and minor waves was 11.7 and 7.8 days, respectively (Ginther & Bergfelt, 1992a). Therefore, follicular wave lengths in the present study were grouped into short (4 to 10 days), medium (11 to 15 days) and long (> 15 days) time-period ranges.

When all follicles were aspirated from pregnant mares, almost all the follicular waves (97%) were short waves. When only follicles ≥20 mm in diameter were aspirated, 73% of waves were short. Only half (52%) of the waves of the control
aspiration treatment were short waves. Therefore, when the follicle aspiration pressure was increased, (control treatment, aspirating only those ≥20 mm in diameter and aspirating all follicles), more waves from the long and medium wave-length categories adjusted to the short wave-length category (52, 73 and 97% in the short wave-length category, respectively). Under normal circumstances in cyclic and pregnant mares, follicular waves tend to overlap so that growing, static and regressing phases may be present in the same animal or even on the same ovary at the same time (Ginther et al., 1989b; Bergfelt & Ginther, 1992; Ginther & Bergfelt, 1993).

During the Spring transitional season, the time from divergence of the dominant follicle and subordinate follicles to ovulation (also the follicular wave length) was longer for the first ovulation of the year (dominant follicle from the previous anovulatory wave still present) than for the second (dominant follicle from the previous wave not present, because it ovulated)(Ginther, 1990). In heifers, the dominant follicle of any follicular wave during pregnancy did not begin to regress before the emergence of a new wave (Ginther et al., 1989b). It is proposed that the growing dominant follicle will not only suppress its own subordinate follicles, but will also prevent the emergence of a new follicular wave (Lacker et al., 1987; Bergfelt & Ginther, 1992). This may be by local as well as systemic pathways (Driancourt et al., 1991; Tucker et al., 1991; Findlay, 1994). When follicles between 30 and 34 mm in diameter were aspirated from cycling mares, ovulations have been reported to follow 3 to 8 days later (Hinrichs et al., 1991).

By aspirating all the follicles on both ovaries, the effect of follicles from a previous wave was eliminated, probably allowing for a faster growth rate so that 97% of all the waves in this treatment group were short waves, with a somewhat homogenous wave length of 7.1 days (±SD=1.9 days). By aspirating only follicles
with a diameter ≥20 mm in diameter (removing only dominant follicles), some subordinate follicles were allowed to develop further and produce long waves (e.g., Appendix A, Figures 5, 6 and 11). However, sometimes the effects of the dominant follicle on these smaller follicles was irreversible and a new wave had to develop after removal of the large follicles. These new waves probably also grew at a faster rate so that only about half of the waves normally in the long and medium wave-length categories (compared with the numbers of the control aspiration treatment) moved to the short wave-length category.

Mule pregnancies had more short follicular waves than horse pregnancies. Since there was no difference in the number of medium-length waves between the two pregnancy types and horse pregnancies had more long waves than mule pregnancies, it seems that mule pregnancies had more short waves at the expense of long waves. It is currently accepted that FSH is the primary stimulus of follicular development during pregnancy, with eCG acting synergistically (Squires & Ginther, 1975; Urwin & Allen, 1982b; Urwin, 1983). However, eCG by virtue of its LH activity is thought to induce a final growth spurt with resultant luteinization or ovulation of FSH-differentiated follicles (Allen, 1974, 1984).

Mule pregnancies resulted in considerably lower circulating concentrations of eCG than horse pregnancies and, also, eCG disappeared from the circulation in these pregnancies much earlier than what would be expected for normal intraspecies horse pregnancies (Allen, 1975b, 1982, 1984; Allen et al., 1993). Perhaps, the low levels of circulating concentrations of eCG in mule pregnancies in this study caused more short-length follicular waves due to a lack of the final growth spurt towards the end of the growing phase of the waves and reduced luteinization of the large follicles that may have lengthened the plateau phase of follicular growth.
The mean wave length of short-length, medium-length and long follicular waves across all treatments for this study was 7.1 days, 12.6 and 19.5 days, respectively. These findings are comparable with those in a study on 11 to 40 day pregnant mares, where the mean wave length for a major follicular wave was 11.2 days and that for a minor wave was 6.8 days. In the present study, however, a possible third population of long waves (19.5 days) was identified in pregnant mares.

The mean interwave interval during the anovulatory Spring transitional period was 10.8 days for 15 mixed breed mares (Ginther, 1990). During days 0 to 50 of pregnancy in Appaloosa and Quarter horse mares, the mean interwave interval of major follicular waves was 13.4 days (Bergfelt & Ginther, 1992). In 19 other mares between days 11 and 40 of pregnancy, the interwave interval for major waves was 11.7 days and that for minor waves was 7.8 days (Ginther & Bergfelt, 1992a). Therefore, the three interwave interval ranges in the present study were < 10 days (short), 10 to 14 days (medium) and > 14 days (long).

When all the follicles on both ovaries were aspirated, less short and more medium interwave intervals were detected than for the control aspiration treatment or when only large follicles (≥20 mm in diameter) were aspirated. Thus, on average the interwave interval was lengthened when all follicles were aspirated. The lengthening of the interwave interval was not found in the control aspiration treatment (where no follicles were aspirated), or when only follicles with a diameter ≥20 mm were aspirated. If it is assumed that growing, static and regressing phases of overlapping follicular waves occur in the same animal at the same time (Ginther, 1992), upcoming follicles of a new wave, large follicles of a current wave and regressing follicles of the previous wave will all be aspirated if all the available follicles are aspirated. With the
overlap effect now eliminated, a new wave has to be induced and develop with a resultant increase in the interwave interval.

The time necessary to induce a new wave after follicular aspiration is seen as gaps of no follicular activity between consecutive waves in the individual follicular wave profiles of almost all mares in this aspiration treatment (e.g., Appendix A, Figures 13, 15 and 17). This pattern is not identified in follicular wave patterns of mares in the other two follicular aspiration treatments. Typically, the time period from induction of a new follicular wave (e.g., by hemiovariectomy, exogenous steroid withdrawal, prostaglandin treatment) until ovulation in cycling mares will be 10 to 14 days long (Driancourt & Palmer, 1984; Silvia et al., 1987a; Ginther, 1992). This fits the pattern noted in the present study, where 60% of all interwave intervals were of medium length when all the follicles were aspirated with a mean interval length of 12.1 days.

In some animals where all follicles were aspirated, the interwave interval was longer than 14 days after repeated aspiration procedures and could not be explained by elimination of the overlap effect only (e.g., Appendix A, Figures 13, 14 and 15). These long interwave intervals were characterized by a long time period (>12 days) of no follicular activity towards the end of the experimental period. It is currently accepted that multiple primordial follicles are recruited independently of hormonal stimuli, to commit to growth and form a reservoir of primary follicles from where they emerge and form a follicular wave (Lacker et al., 1987; Hadley, 1988; Findlay, 1994). The number of ready follicles in this pool may become depleted after repeated aspiration of all follicles, so that no follicles were available to form new follicular waves towards the end of the experimental period.
In contrast, the long interwave intervals in the control aspiration treatment and when only follicles with a diameter ≥20 mm were aspirated were mainly due to long follicular waves (increased follicular wave length). When only follicles ≥20 mm in diameter were aspirated, it was expected that the removal of presumptive dominant follicles would allow other follicles in the same wave to continue development so that more short interwave intervals would be observed. Although the highest percentage (66%) of short interwave intervals was found when only follicles ≥20 mm in diameter were aspirated, it was not different from the 56% detected in the control aspiration treatment. In this study, follicular aspiration treatment was applied only when a follicular wave reached a peak so that the effect of the dominant follicle on the other follicles was not prevented. Many of the subordinate follicles probably would not have been rescued in time and underwent atresia instead of continuing growth into a new follicular wave. The critical timing of removal of the dominant follicle on secondary ovulation was evident in a study where preovulatory follicles were aspirated when they were between 30 to 34, 35 to 39 or 40 to 44 mm in diameter (Hinrichs et al., 1991). In the 30 to 34 mm diameter group, 55% of mares ovulated a secondary follicle 3 to 8 days later. In the other two groups, 33% and 14% of mares ovulated within 10 days post-aspiration. Follicles that did not ovulate became atretic and regressed.

It is interesting to note that when only follicles with a diameter ≥20 mm in diameter were aspirated, approximately 50% of the waves normally in the medium and long wave-length categories changed to the short wave-length category. In this case, the presumptive inhibitory effect of the dominant follicle on the growth rate of the new emerging wave has been removed successfully in 50% of the aspirations. However, when analyzing the effect of aspirating follicles ≥20 mm on interwave intervals (expected to remove the effect of the dominant follicle on follicles from the same
wave), an equivalent shortening of the interwave intervals was not evident. This may indicate that the inhibitory effect of the dominant follicle on its own subordinate follicles (same ovary) occurs earlier than its inhibitory effect on the new emerging wave. Since the new wave does not necessarily occur on the same ovary as the dominant follicle, it is tempting to speculate that the subordinate follicles are inhibited by local pathways and the growth rate and emergence of a new wave by a systemic route.

The frequency distribution pattern of interwave intervals was not different for horse and mule pregnancies in this study. This seems to be in contrast with the higher percentage of short wave lengths that were observed for mule pregnancies when compared with horse pregnancies. However, all indications are that FSH stimulates emergence and growth of follicular waves during pregnancy (Van Rensburgh & Van Niekerk, 1968; Evans & Irvine, 1975; Urwin & Allen, 1982b; Bergfelt & Ginther, 1992; Ginther & Bergfelt, 1992a) and that eCG is responsible for a final LH-like growth spurt of these follicles (Urwin, 1983; Allen, 1984). Interwave intervals are dependent on the periodic emergence of waves and, therefore, on the periodic occurrence of FSH surges (Bergfelt & Ginther, 1992; Ginther & Bergfelt, 1992a) that should be similar for mares carrying horse and mule fetuses. However, some aspect of follicular wave length may be influenced by the final eCG-induced growth spurt, so that horse and mule pregnancies with different circulating concentrations of eCG may conceivably have differences in the length of follicular waves.

The mean number of days for short interwave intervals across all treatment groups in this study was 7.1 days and is in agreement with the 7.8 day interval reported for minor follicular waves in mares during days 11 to 40 of pregnancy (Ginther & Bergfelt, 1992a). The mean length of medium interwave intervals in this study was 12
days and is equivalent to the mean interwave interval for major follicular waves of 11.7
days (Ginther & Bergfelt, 1992a) or 13.4 days (Bergfelt & Ginther, 1992) for mares in
similar studies during days 11 to 40 or days 0 to 50 of pregnancy, respectively. The
mean length of the long interwave interval group was 20 days and represented induced
long interwave intervals due to repeated aspiration of all follicles (Treatment III) as well
as long nonregressing follicular waves in the control aspiration group (Treatment I) and
when only follicles with a diameter ≥20 mm were aspirated (Treatment II).

The time interval between the peak of a follicular wave to the emergence of the
next follicular wave gives an indication of the degree of overlapping between
consecutive follicular waves with a negative value suggesting that the new wave
emerged before the largest follicle of the current wave reached a peak. The interval
between the peak of a follicular wave to the emergence of the next follicular wave was
considered to be short (< 1 day), of medium-length (1 to 5 days) or long (> 5 days).
No difference was detected in the degree of overlapping of follicular waves when no
follicles or when only follicles with a diameter ≥20 mm in diameter were aspirated.

However, when all follicles were aspirated, the number of medium-length and
long intervals between a wave peak and the emergence of the next wave were much
higher and the number of short intervals was much lower than when only follicles ≥20
mm in diameter were aspirated or for the control aspiration treatment. Thus, when all
the follicles were aspirated, the overlapping of consecutive waves was eliminated in all
but 7% of the waves in this treatment. The 7% that overlapped was due to technician
error where some small follicles were not aspirated. It was expected that aspirating all
follicles will eliminate the overlapping of consecutive waves as regressing follicles from
the previous wave, static follicles from the present wave and upcoming follicles from
the following wave were all aspirated during a single aspiration procedure.
When aspirating all follicles, approximately 60% of intervals between the peak of a follicular wave and the emergence of the next wave ranged from 1 to 5 days, with a mean of 3.3 days and 30% were longer than 5 days, with a mean of 9.4 days. The 60% of follicular waves with a mean period of no follicular activity of 3.3 days between the aspirated wave and the next wave, probably represents the time that it takes for the induction of emergence of a fresh follicular wave. The 30% of waves with a mean period of no follicular activity of 9.4 days between the aspirated wave and the next wave, probably represent waves where primary follicles were not immediately available in the reservoir pool to form a new follicular wave. It is possible that the waiting follicles in this pool became depleted after repeated aspiration of all follicles (Lacker et al., 1987; Findlay, 1994).

In general, the interval length across all observations for the short-interval group (59 to 69% of all waves in the control aspiration group and when only follicles with a diameter ≥20 mm were aspirated) was -3 days, so that a wave peak and emergence of a new wave overlapped on average with 3 days. This means that the last 3 days of the growing phase of the current wave occurred at the same time as the first 3 days of the growing phase of the next follicular wave. This agrees with studies in pregnant heifers where regression of the current dominant follicle only occurs after the emergence of another dominant follicle (Ginther et al., 1989b). Similarly, the mean interval from follicle emergence to onset of regression of the preceding anovulatory follicle of three-wave cycling heifers was ≈3 days (Ginther et al., 1989c). Since a new wave in this study frequently emerged on the opposite ovary, the inhibitory effect of the new wave 3 days after its emergence on the largest follicle of the previous wave was probably by a systemic route.
When days 21 to 150 of pregnancy were divided into the second (days 20 to 60), third (days 61 to 90), fourth (days 91 to 120) or fifth (days 121 to 150) months of pregnancy, similar follicular development patterns were noted as when follicular dynamics of early pregnancy were analyzed by grouping follicular wave parameters according to chronological wave number.

The gradual decrease of mean maximum follicular diameter as pregnancy progressed in the control aspiration treatment and when all follicles were aspirated was expected and agrees with reports that ovarian follicular and luteal activity decrease progressively to very low activity during the last half of pregnancy in the horse (Allen, 1974, 1975a; Wesson & Ginther, 1980). When all follicles with a diameter ≥20 mm were aspirated, the same pattern was not observed and the maximum diameter only started to decline in the fifth month of pregnancy. It has been demonstrated that protein (e.g., inhibin) and steroid (e.g., estradiol) hormones in large follicles may act systemically to inhibit circulating concentrations of FSH and LH, and subsequent follicular development (Miller et al., 1981; Bergfelt & Ginther, 1985; Plata-Madrid et al., 1992; Roser et al., 1994). Removing these substances by aspirating the large follicles may partially explain why follicular maximum diameters did not decline when follicles with a diameter ≥20 mm were aspirated. However, when all follicles were aspirated, the follicular fluid contents of large as well as small follicles were removed but the mean maximum follicular diameter pattern was the same as in the control treatment.

The mean maximum follicular diameter in horse pregnancies followed the expected pattern and gradually decreased as pregnancy progressed. However, the expected pattern was not detected for mule pregnancies as the mean follicular diameter was maintained at least to the end of the fourth month of pregnancy. The only
documented hormonal difference in horse and mule pregnancies is the significantly lower circulating concentrations of eCG and the shorter time period that eCG will be present in the circulation of mares carrying mule pregnancies (Allen, 1984; Allen et al., 1993). With the differences in mean maximum follicular diameter noted between horse and mule pregnancies in this study, perhaps profiles of other hormones, such as FSH or estradiol may also differ during pregnancy. The overall pattern for the mean diameter of the largest follicle of a follicular wave for all the follicular waves in this study was a gradual linear decrease from day 21 to day 150 of gestation.

The distribution pattern of follicular wave lengths over months 2 to 5 of pregnancy for the control aspiration treatment and when all follicles ≥20 mm in diameter were aspirated, showed a gradual decrease of wave lengths from the second to fifth months of pregnancy. The same pattern of decrease was not found when all follicles were aspirated and the shortest wave lengths for the other two treatments (days 121 to 150 of pregnancy) was the same as the constant but short wave lengths when all follicles were aspirated. This can be explained by the frequency distribution data of follicular wave lengths. As a result of aspirating all follicles, almost all (97%) of the waves in this treatment was already short and the effect of the ongoing pregnancy could therefore not increase this number of short waves. However, in the control aspiration treatment and when follicles with a diameter ≥20 mm were aspirated, ≈50 and ≈25% of waves were in the medium and long wave-length categories, respectively. The waves in these categories were subject to the effect of the ongoing pregnancy with a resultant decreasing mean follicular wave length as pregnancy progressed. This observation is in agreement with reports that equine ovarian follicular and luteal activity decrease progressively as pregnancy progresses (Allen, 1974, 1975a; Wesson & Ginther, 1980).
Horse pregnancies seemed to have longer mean wave lengths in the second and third months of pregnancy than mule pregnancies, however, this difference disappeared at the fourth and fifth months. Furthermore, the difference between wave lengths of horse and mule pregnancies was more striking between days 60 to 90 (third month) than between days 21 to 60 (second month) of pregnancy. Low levels of eCG are usually first detected in the maternal circulation between days 36 and 40 of pregnancy, peak between days 60 to 90 and reach low concentrations around day 120 of pregnancy (Allen, 1974; Nett et al., 1975; Allen, 1984). Horse pregnancies have much higher circulating concentrations of eCG than mule pregnancies (Allen, 1984; Allen et al., 1993). This close temporal relationship between high circulating concentrations of eCG in mares carrying horse fetuses and the longer follicular wave lengths only during this time period, indicates that eCG is responsible for the longer wave lengths between days 21 to 90 of pregnancy in mares carrying horse pregnancies.

The mean interval from a wave peak to the emergence of the next follicular wave was either negative or less than 1 day long from the second to fifth month of pregnancy for the control aspiration treatment. A low degree of overlapping (=3 days) between consecutive waves during the first 150 days of pregnancy seems to be the normal pattern. This is similar to the observation in follicular wave ultrasound studies up to day 50 of pregnancy, where a new wave emerges close to or on the day that the dominant follicle of the current wave reaches its maximum diameter (Bergfelt & Ginther, 1992; Ginther & Bergfelt, 1992a).

It was expected that overlapping of consecutive follicular waves would be eliminated for the most part when all follicles were aspirated, since growing, static and regressing follicles representing several waves were aspirated during a single aspiration period. Although not as pronounced as when all follicles were aspirated, it seems that
the interval between wave peak and emergence of the next follicular wave also increased as pregnancy progressed from the second to fifth month of pregnancy when only follicles ≥20 mm in diameter were aspirated. This may be an indication that one of the reasons for the diminishing follicular activity noted in horse pregnancies from day 21 to 150 of pregnancy may be due to an increase in the interval from a follicular wave peak to the emergence of the next follicular wave (as detected when follicles with a diameter ≥20 mm were aspirated) and that this time interval is further increased when all follicles are aspirated.

Therefore, a part of the increased time interval when all follicles were aspirated should be due to the effect of ongoing pregnancy (equivalent to that seen when follicles with a diameter ≥20 mm were aspirated) and the rest of the increase due to the aspiration procedure itself. If this assumption is correct, one would expect the interval from the peak of a follicular wave to the emergence of the next follicular wave to increase as pregnancy progresses from the second to fifth month in horse pregnancies only, since decreasing follicular activity closer to the end of the experimental period was noted in horse pregnancies only. Indeed, horse and mule pregnancies had opposite distribution patterns for the interval between wave peak and the emergence of a new follicular wave. In horse pregnancies the interval appeared to increase from months 2 to 5; whereas, in mule pregnancies the interval seemed to rather decrease.

The maximum number of days that could be covered by the growing phase (excluding static and regressing phases) was equal to the number of days in the experimental period (130 days). A distinction was made between the number of days covered by follicular waves with the diameter of the largest follicle ≥17 mm and the number of days covered by all waves, including the waves where the largest follicle does not reach 17 mm in diameter. Follicular wave size was used as an estimate of
follicular wave quality. For both follicular wave size groups, the same pattern was found in that the number of days covered by follicular wave activity decreased as the severity of the follicular aspiration treatment increased.

When only follicles ≥20 mm in diameter were aspirated, one could have expected that the number of days covered would increase rather than decrease because the suppressing effect of the dominant follicle was removed. However, follicles in this study were aspirated only when follicular waves reached a peak. If present, follicular dominance could be identified by ultrasound at least 5 to 7 days before the dominant follicle attained maximal diameter (peak of follicular wave) (Bergfelt & Ginther, 1992; Ginther & Bergfelt, 1992a; Ginther & Bergfelt, 1992b). The possibility that follicles were aspirated too late for other follicles of the same follicular wave to be rescued is supported by the lack of difference in interwave intervals between this treatment and the control aspiration treatment. However, by aspirating only follicles ≥20 mm in diameter, the wave length was shortened possibly by the timed removal of an inhibitory effect of the dominant follicle on the growth rate of the next wave. Since interwave intervals did not shorten concurrently when follicles ≥20 mm in diameter were aspirated, the shorter wave lengths created periods of no follicular activity between the peak of the shortened wave and the emergence of the following follicular wave with subsequently fewer days covered by follicular activity. The least number of days covered by follicular activity when all follicles were aspirated was expected, since early emerging waves were aspirated simultaneously with the large follicles forming the current wave peak, so that a period of no follicular activity was created after aspiration.

The results from several studies indicate that the cycling horse ovulates more frequently from the left ovary than from the right (Ginther, 1979) while others have failed to show any difference in the frequency of the side of ovulation (Ginther, 1992).
However, when reproductive status (maiden, barren, foaling) was taken into consideration, only maiden mares have been reported to ovulate more frequently from the left ovary (Ginther, 1983). It is proposed that the asymmetry of ovulation is lost after the first pregnancy for unknown reasons (Ginther, 1992). In the present study, no differences were found in the number of days covered by any follicular wave activity, the number of days covered by follicular waves with the largest follicle ≥17 mm in diameter, the total number of follicular waves or the total number of follicular waves with a largest follicular diameter of ≥17 mm between the left and right ovary. To compile the individual overall follicular wave profile for individual mares from sometimes simultaneously occurring follicular waves on both ovaries, the highest quality wave (wave of longest duration and/or largest follicle diameter) was selected. When the total number of follicular waves or the number of waves with a largest follicle ≥17 mm in diameter that contributed to the overall follicular wave profile were counted for each ovary, there was a tendency for the right ovary to contribute a greater number of follicular waves of all sizes and a greater number of higher quality waves (largest follicle at least ≥17 mm in diameter) to the overall follicular wave profile. This effect was significant when only horse pregnancies were considered. It may be speculated that maiden mares ovulate more frequently from the left, that follicular activity during pregnancy is more frequent on the right and that this contralateral activity during early pregnancy may be part of the reason why the asymmetry of side of ovulation is lost after the first pregnancy.

The mean total follicular diameter values of horse and mule pregnancies were almost parallel between days 21 and 45 of pregnancy, exhibiting at least one wave-like synchronized rise and decrease. This parallel rise and decrease in mean total follicular diameter at the beginning of the experimental period might have been induced by the
synchronized first follicular aspiration treatment on day 21 of pregnancy in all mares (except the control aspiration group), regardless of the state of the follicular population.

Clear divergence between the mean total follicular diameters of horse and mule pregnancies was detected after day 45 of pregnancy with the mean total follicular diameter of horse pregnancies increasing sharply and those of mule pregnancies decreasing. In this study, eCG was first detected in the peripheral circulation of mares carrying horse pregnancies at low levels at day 30 of pregnancy, started to rise sharply at day 35, peaked between days 56 and 77 of pregnancy and then decreased gradually to attain low mean concentrations (almost the same as peak eCG concentrations of mares carrying mule fetuses) in the circulation between days 110 and 150 of gestation. This is in close agreement with circulating concentrations of eCG reported in other studies with eCG appearing in the peripheral blood between 37 and 41 days after ovulation, peaking between days 55 and 70 and then steadily declining to disappear from the circulation between days 100 and 140 (Squires & Ginther, 1975; Urwin & Allen, 1982b; Allen et al., 1993). The circulation concentrations of eCG of mares carrying mule pregnancies were ≈18 times lower than those of mares carrying horse pregnancies, and reached very low levels at days 80 to 90 of pregnancy. The mule eCG profile also compares favorably with that reported in other studies where mares carrying mule pregnancies had considerably lower circulating concentrations of eCG (<10 IU per ml) than mares carrying horse pregnancies and where mule eCG disappeared from the circulation at day 80, much earlier than what would be expected for horse eCG from normal intraspecies horse pregnancies (Allen, 1975b, 1982, 1984; Allen et al., 1993).

The presence of high concentrations of eCG in close temporal relationship with the mean total follicular diameter peaking between days 45 and 78 in horse pregnancies and the absence of an equivalent peak of follicular activity in mule pregnancies with
corresponding low levels of circulating eCG, implicates eCG as part of the causative mechanism for the follicular activity peak found only in mares carrying horse pregnancies. However, it is currently accepted that FSH is the primary stimulus of follicular development during pregnancy with eCG only being synergistic in the process (Squires & Ginther, 1975; Urwin & Allen, 1982b; Urwin, 1983).

Equine chorionic gonadotropin expresses both FSH-like and LH-like activities in nonequine and other equid species (Stewart et al., 1976; Aggarwal et al., 1980; Urwin, 1983; Allen, 1984; Combarnous et al., 1991) but is thought to have only LH-like effects in the horse mare (Squires & Ginther, 1975; Urwin & Allen, 1982b; Manning et al., 1987). However, for the seemingly eCG-related abrupt rise of mean total follicular diameters between days 45 and 61 of pregnancy when compared with that of mule pregnancies in this study, there must have been a significant increase in the numbers of follicles and/or in the diameters of the follicles. As this rise in mean total follicular diameter did not occur in the presence of very low concentrations of eCG in mule pregnancies, it must be considered that eCG in horse pregnancies had a marked follicle-stimulating effect. The follicle-stimulating effect of eCG is further supported in this study by the pattern of increased wave lengths during days 21 to 90 detected in horse pregnancies only, and by the higher number of follicular waves noted in horse pregnancies between days 21 and 85 of pregnancy when compared with that of mule pregnancies or to that between days 86 to 150 of horse pregnancies.

Support in the scientific literature for an additional follicle-stimulating role of eCG during the early horse pregnancy includes the presence of a follicular diameter surge (up to three-fold rise) of the large follicles between days 50 to 70 of pregnancy only (Miller et al., 1980), an increase in the number of small antral follicles, mitotic index of preantral follicles and rate of antral formation associated with peak
concentrations of circulating eCG (Ginther, 1992), and an increase and decrease in total follicular diameters parallel to the increase and decrease of circulating concentrations of eCG during days 15 to 135 of pregnancy (Allen, 1974, 1975a).

Considerable follicular activity was present between days 21 and 30 of pregnancy in both mares with horse and mares with mule pregnancies, even before the first eCG was detected in the circulation. This finding is in agreement with other studies that found considerable follicular growth on the ovaries of pregnant mares between days 18 and 30 (Bain, 1967; Van Rensburgh & Van Niekerk, 1968) and days 21 to 40 (Allen, 1974, 1975a) of pregnancy. Although the wave-like shape of the graph of mean total follicular diameters might have been caused by a synchronized first aspiration, the level of follicular activity before the appearance of eCG for both horse and mule pregnancies is equivalent to the level of activity in horse pregnancies during the time of eCG dominance. The level of follicular activity (mean total follicular diameter) in horse pregnancies before the appearance of eCG (before day 35), was much higher than the level of activity in the post-eCG period (after day 100 of pregnancy). Similarly, the level of follicular activity in mule pregnancies before mule eCG appearance (before day 35) was greater than during the period of eCG presence or during the post-eCG period. Therefore, it seems that another factor(s) besides eCG is responsible for the follicular activity before and after the presence of eCG in the circulation. The other factor is believed to be the pituitary gonadotropins, especially FSH (Evans & Irvine, 1975; Squires & Ginther, 1975; Urwin & Allen, 1982b; Urwin, 1983; Nett et al., 1989).

It has been demonstrated that periodic emergence of follicular waves occurs during early pregnancy in mares in close temporal association with surges of FSH (Bergfelt & Ginther, 1992; Ginther & Bergfelt, 1992a; Ginther & Bergfelt, 1992b).
However, if FSH is the only other factor, it is expected that there will be a difference in the circulating concentrations of FSH before eCG appearance and in the post-eCG period to account for the different levels of follicular activity during these two time periods in horse pregnancies. It should be noted that a difference in mean circulating concentrations of FSH between the days 21 and 150 of pregnancy was not found for horse pregnancies in this study.

After the period of eCG presence, the mean total follicular diameter profile of horse pregnancies decreased significantly to very low values towards the end of the experimental period. This is in agreement with other studies on follicular activity in mares between days 15 to 135 of pregnancy, which also reported a significant decrease in follicular and luteal activity as pregnancy progressed (Allen, 1974, 1975a). The same pattern was not detected for the mean total follicular diameters of mule pregnancies, but rather a slight increase in diameters towards the end of the experimental period.

Since mean concentrations of FSH were not different between days 21 and 150 of pregnancy within each pregnancy type, it is proposed that the high levels of follicular activity before the appearance of eCG in the circulation of horse and mule pregnancies were due to the basal rhythmic activity of FSH, with some synergistic effect of folliculotropic factors other than FSH (probably from fetal-placental origin). The level of follicular activity detected in mule pregnancies during the equivalent period of peak concentrations of eCG in horse pregnancies, is what could be expected from periodic surges of FSH alone. However, the ovaries of mares carrying horse pregnancies were simultaneously exposed to very high concentrations of eCG that had mainly an LH-like effect but also some FSH-like action to act synergistically with surges of FSH, giving rise to the mean total follicular diameter peak found in horse pregnancies in this study. This
synergistic effect of eCG with FSH was probably due to an increase in the number of follicles recruited as well as an increase in the maximum diameter of the recruited follicles (Ginther, 1992). The levels of mule eCG were not high enough and were not present in the circulation long enough to demonstrate this synergistic effect.

After the disappearance of eCG from the circulation, the ovaries of mares carrying horse pregnancies were less responsive to the same levels of FSH that occurred before the appearance of eCG. It is possible that the constant exposure of the ovaries to such high levels of eCG desensitized the ovaries (down regulation of receptors) to the stimulating effect of circulating FSH. In contrast, the ovaries of mares carrying mule pregnancies were only exposed to very low levels of eCG for a shorter time period, subsequently were not desensitized for the follicle-stimulating effect of FSH and, therefore, maintained the same level of follicular activity until the end of the experimental period.

This difference in follicular activity after the period of circulating peak eCG concentrations between horse and mule pregnancies was also found with the lower number of follicular waves with follicles ≥17 mm in diameter during days 86 to 150 of horse pregnancies, the decreasing diameter of the largest follicle of a follicular wave towards day 150 of horse pregnancies and the increased interval between a follicular wave peak and the emergence of a next follicular wave towards the end of the experimental period in horse pregnancies.

One of the most obvious differences between the follicular dynamics of horse and mule pregnancies noted in this study was the decline in follicular activity in horse pregnancies after the time period of peak concentrations of eCG, opposed to the maintenance of the same level of follicular activity after day 45 of gestation until the end of the experimental period in mule pregnancies. Possibilities for this phenomenon
include a direct inhibitory effect of eCG on circulating concentrations of FSH, a
difference in circulating concentrations of FSH, desensitization of the ovaries at a local
level for the effect of FSH or differences in the fetal-placental estrogen profile between
horse and mule pregnancies. The estrogens of pregnancy consist of many forms of
which estradiol-17\beta is the most active, and for most resembles the distribution patterns
of other estrogens during early pregnancy (Cox, 1975; Kindahl et al., 1982; Makawiti
et al., 1983; Lasley et al., 1990).

The assay of estradiol-17\beta in this study measured free estradiol that would be
expected to be less than 1% of the total conjugated estrogens in the circulation (Ginther,
1992). Although, it has been demonstrated that the equine ovary is an additional source
of estrogens during early pregnancy (Daels et al., 1990, 1991), the purpose of
measuring estradiol in this study was to determine if there were any differences between
estrogen production by the fetal-placental units of horse and mule pregnancies.
Therefore, estradiol-17\beta was only measured from the 40th day of gestation. As
expected, the lines representing the mean circulating concentrations of estradiol-17\beta
for horse and mule pregnancies started to increase gradually between days 85 to 90 of
pregnancy to reach maximum concentrations at the end of the experimental period (day
150 of gestation). The estradiol hormone profile in this study was similar to that found
for estradiol (Ginther, 1992) and other estrogens (Kindahl et al., 1982; Lasley et al.,
1990) during equivalent stages of pregnancy in related studies. However, no
differences were detected between the mean circulating concentrations of estradiol-17\beta
between horse and mule pregnancies that could help to explain the differences in
follicular activity between the two pregnancy types after day 80 to 100 of gestation.

Since pregnant mares are also considered as oocyte donors for assisted
fertilization techniques, it is of clinical importance to observe the effect of the different
aspiration treatments and pregnancy types on circulating concentrations of progesterone. Progesterone is the only ovarian hormone that is absolutely essential for the maintenance of early pregnancy in the mare (Ginther, 1992; Knowles et al., 1994). As for estrogens, many progestagens are produced during pregnancy from which progesterone is one reliable representative (Burns & Fleeger, 1975; Holtan et al., 1975; Martin et al., 1989; Knowles et al., 1994).

The mean circulating progesterone concentrations for all three aspiration treatments increased in parallel between days 21 and 26 of pregnancy, declined until day 34 and then sharply increased again until day 43 of pregnancy after which only the mean circulating concentrations of progesterone for the control aspiration treatment continued to increase. The rise and fall of circulating concentrations of progesterone between days 21 and 34 of pregnancy in this study was attributed to the first luteal response of pregnancy, where the primary corpus luteum failed to undergo luteolysis and gradually started to produce less progesterone due to a lack of LH support during this time (Urwin & Allen, 1982b; Ginther, 1992). It was also reported in several other studies that a pronounced increase in circulating concentrations of progesterone was followed by a decrease before day 40 of pregnancy (Holtan et al., 1975; Squires & Ginther, 1975; Urwin & Allen, 1982b).

Measurable quantities of eCG were first noted in the circulation between days 35 and 40 of pregnancy (Allen, 1975b, 1984) and supplementary corpora lutea only begin to form from day 40 onwards (Squires & Ginther, 1975; Martin et al., 1989). The sharp increase in circulating concentrations of progesterone after day 34 and up to day 43 for all three follicular aspiration treatments in this study was probably due to a resurgence of the progesterone producing capability of the primary corpus luteum in response to the now appearing horse and mule eCG (Squires & Ginther, 1975; Squires,
1979; Bergfelt et al., 1989). However, the possible role of luteostatic or luteotropic substances like prostaglandin E₂ and estradiol from fetal-placental origin up to day 43 should be considered (Ginther, 1992; Brady et al., 1993; Martin & Lawrence, 1994).

The continued rise of the mean circulating concentrations of progesterone after day 43 of pregnancy for the control aspiration treatment in close association with the peak of circulating concentrations of eCG, was attributed to progesterone production by the secondary corpora lutea. The progesterone profile of the control aspiration treatment was in close agreement to those reported by other investigators for normal horse pregnancies (Burns & Fleeger, 1975; Holtan et al., 1975; Squires & Ginther, 1975). It is accepted that the formation of secondary and accessory corpora lutea and the resultant production of progesterone are mediated by eCG (Allen, 1975b; Squires & Ginther, 1975; Van Niekerk et al., 1975; Squires, 1979; Urwin & Allen, 1982b).

The mean circulating concentrations of progesterone for mares from which all follicles were aspirated and for mares from which only the follicles with a diameter ≥20 mm were aspirated, did not continue to rise after resurgence of the primary corpus luteum, but rather declined gradually after the initial rise to reach a minimum towards the end of the experimental period (day 150 of gestation). This decline after day 43 of pregnancy for these two aspiration treatments indicates that the follicular aspiration procedures did interfere with the formation of supplementary corpora lutea in this study. When preovulatory follicles of cycling mares were aspirated, the formation of normal corpora lutea and progesterone production equivalent to that after ovulation was observed (Watson & Sertich, 1990; Hinrichs et al., 1991). However, progesterone values rose more rapidly after ovulation than after aspiration (Hinrichs et al., 1991).

Abnormal luteal function after aspiration of mature preovulatory follicles in the mare has been reported, but unlike in the previous studies, these follicles have been
flushed repeatedly to maximize oocyte recovery (Carnevale et al., 1987). Abnormal luteal function was related to the amount of granulosa tissue that was removed from the follicle (Hinrichs et al., 1991).

In the present study, the follicles were also flushed repeatedly to recover the maximal number of oocytes and between 1 and 12 x 10^6 granulosa cells were recovered per aspiration procedure (several follicles flushed). Although normal looking accessory corpora lutea were frequently observed by transrectal ultrasound, it is possible that enough granulosa cells were removed from the aspirated follicles to impair progesterone production. Since there was no difference at any time during the experimental period between the circulating progesterone concentrations of mares from which all follicles were aspirated and mares from which only follicles with a diameter ≥20 mm were aspirated, it can be assumed that the unaspirated smaller follicles in the ≥20 mm-treatment did not luteinize. Thus, by aspirating only follicles ≥20 mm in diameter, the same degree of reduced progesterone production was induced than when all follicles were aspirated. However, even with the reduced production of progesterone by the corpora lutea of mares in these two follicular aspiration treatments, the mean circulating progesterone concentration never approached a level where the pregnancy was endangered during the experimental period. By day 150 of pregnancy, the mean circulating progesterone concentrations converged again and statistical differences could not be identified, so that the supplementary progesterone effect of the supplementary corpora lutea through eCG for the control aspiration treatment was no longer evident.

In the present study, mean circulating progesterone concentrations of horse and mule pregnancies during the time of the first luteal response (days 21 to 34) of pregnancy were parallel. However, during the time of resurgence of the primary corpus luteum due to the first appearance of horse and mule eCG in the peripheral
circulation (days 35 to 43), the mean circulating progesterone concentrations of mares carrying mule pregnancies was statistically greater than that of mares carrying horse pregnancies. At the time when supplementary corpora lutea were expected to start contributing to the circulating levels of progesterone (after day 43), the circulating levels converged again until day 67, after which the mean progesterone concentrations for mule pregnancies diverged to remain lower until the end of the experimental period. This difference between circulating concentrations of progesterone of horse and mule pregnancies after day 96 of gestation was significantly different. Other studies have shown that supplementary corpus luteum development and related progesterone production were equivalent in horse and mule pregnancies (Allen, 1982, 1984).

Studies on eCG, using radioreceptor assays to measure the LH-like and FSH-like biological activities of horse, donkey and horse-donkey interspecies (mule, hinny) eCG have indicated that it is the quality of the eCG rather than the quantity that causes the well described differences in supplementary luteal development and progesterone production between these different type of equid pregnancies (Stewart et al., 1977; Aggarwal et al., 1980; Manning et al., 1987). In general, horse eCG has a FSH:LH ratio of 1.23 and mule eCG a ratio of 0.7 (Stewart et al., 1977). Furthermore, it is proposed that female equids are more resistant to the stimulating effect of their own chorionic gonadotropin than to that of another equine species (Allen, 1982, 1984). This may partially explain why mule pregnancies in this study had higher mean circulating concentrations of progesterone between days 34 and 43 of pregnancy (resurgence of the primary corpus luteum) than horse pregnancies, and had equivalent circulating progesterone values between days 43 and 67 than those of horse pregnancies, even though they had much lower circulating concentrations of eCG.
The maternal leukocytic reaction against mule endometrial cups is greatly enhanced when compared with that of a normal horse pregnancy, so that the cups are rejected earlier than in an equivalent horse pregnancy, resulting in the disappearance of eCG from the circulation as early as day 70 to 80 of pregnancy (Allen, 1982, 1984; Anderson, 1988). The gradual decrease of mule eCG from day 65 of pregnancy onwards in this study, coincided with the decline of circulating progesterone concentrations detected in mule pregnancies after day 67, unlike that of horse pregnancies. The decreasing concentrations of mule eCG were probably not high enough after this time to maintain ovulation/luteinization of secondary follicles to support subsequent supplementary progesterone production.

The effect of follicular aspiration on the ability of aspirated follicles to luteinize was estimated by the mean post-aspiration rise in circulating concentrations of progesterone during the pre-eCG, peak-eCG and post-eCG periods in this study. The mean post-aspiration increase in circulating concentrations of progesterone for mares from which follicles were actually aspirated (Treatments II and II, excluding Treatment I), was higher during the pre-eCG designated time period than during the peak-eCG or post-eCG periods. The high values during days 21 to 39 of pregnancy (pre-eCG designated period) likely reflected the response of aspirated follicles to the first appearing horse and mule eCG in the peripheral circulation. The low values during days 40 to 95 of pregnancy (peak-eCG period) were partly due to the impaired luteinization and progesterone production by supplementary corpora lutea of mares from which all follicles were aspirated and mares from which only follicles ≥20 mm were aspirated.

However, for the greatest part, these low values during the peak-eCG period were probably due to the early disappearance of mule eCG from the circulation with
decreased supplementary corpora lutea formation and progesterone production well before day 95 of pregnancy. This is supported by the observed differences in the post-aspiration increase of circulating progesterone concentrations between horse and mule pregnancies. Although mule pregnancies had higher circulating concentrations of progesterone than horse pregnancies during the period of expected resurgence of the primary corpus luteum (days 34 to 43 of pregnancy), the post-aspiration increase in circulating concentrations of progesterone of mule pregnancies were not higher during days 21 to 39 of pregnancy. This was probably because days 21 to 39 of pregnancy also included a time period when the circulating progesterone concentrations of horse and mule pregnancies were equivalent. However, horse pregnancies tended to have a higher post-aspiration increase in circulating concentrations of progesterone than mule pregnancies during days 40 to 95 of pregnancy, probably due to the earlier disappearance of mule eCG from the circulation than that of horse eCG. After day 95 of pregnancy the post-aspiration increase in circulating concentrations of progesterone for the aspiration treatments as well as for the pregnancy types were low, likely due to the low and decreasing levels of eCG.

The pulsatile nature and the individual mare variation of circulating concentrations of FSH for the three aspiration treatments made it difficult to find significant differences over an extended period of time even though, multiple points were significantly different. It has been demonstrated that the daily pattern of FSH in cycling mares was highly pulsatile with mean peak durations and pulse amplitudes ranging from 4 to 10 minutes and from 0.9 to 138 ng per ml, respectively (Evans, 1990). The pulsatile nature of FSH during pregnancy has also been reported (Evans & Irvine, 1975; Allen, 1982; Urwin, 1983; Bergfelt & Ginther, 1992). When circulating concentrations of FSH were measured at hourly intervals for 51 hours from pregnant
mares in this study (Meintjes, 1995), three to eight pulses of FSH with differences up to 130 ng per ml between the low and peak values were detected. Therefore, it is likely that the daily samples in this study were collected from individual pulses, and that the variability and pulsatile nature of the mean daily circulating FSH concentrations noted were, in part, due to differential sampling of individual pulses.

However, when monitoring the mean circulating FSH concentrations, it was clear that the mean circulating FSH concentrations of mares from which follicles ≥20 mm were aspirated, fluctuated consistently at a higher level than those of the other two aspiration treatments, at least between days 48 to 117 of pregnancy. Also, the fluctuating mean circulating FSH concentrations of mares from which all follicles were aspirated appeared to be slightly higher than those of the control aspiration treatment between days 22 and 100 of pregnancy. The circulating concentrations of FSH and subsequent follicular activity during early pregnancy can be inhibited by the systemic administration of follicular fluid obtained from large equine follicles (Bergfelt & Ginther, 1986). Some of the substances in follicular fluid that may alter the course of follicular dynamics are progesterone, estradiol, androgens, growth factors, inhibin, and various activins (Miller et al., 1981; Thompson et al., 1983b, 1987b; Spicer et al., 1991; Findlay, 1994; Roser et al., 1994). The concentrations of FSH-inhibiting substances such as estradiol, progesterone and inhibin are reported to be higher in large and preovulatory follicles (Roser et al., 1994). Concentrations of estradiol-17β and progesterone exceeded 5 x 10³ and 2 x 10³ ng per ml, respectively, in the follicular fluid from follicles ≥20 mm in diameter aspirated from pregnant mares in this study (Meintjes, 1995).

The seemingly higher level of FSH in mares from which only follicles ≥20 mm in diameter were aspirated could be explained by the aspiration of substances (especially
estradiol and inhibin) in the follicular fluid that would otherwise suppress circulating concentrations of FSH. However, it was expected that when all follicles were aspirated that the lack of FSH inhibition would result in even higher levels of circulating FSH than when only follicles ≥20 mm in diameter were aspirated. Higher levels of circulating FSH when all follicles were aspirated were not observed in this study. It may be that some activin-like FSH-stimulatory substances in smaller follicles were removed together with the inhibiting substances in the large follicles so that the expected rise in FSH was not detected (Findlay, 1994).

Evaluating the mean circulating concentrations of FSH for horse and mule pregnancies, it is evident that mule pregnancies had consistently higher levels of circulating FSH, especially during the time of horse eCG peak concentrations. During days 21 to 39 of pregnancy when mean circulating FSH concentrations for horse and mule pregnancies represented the same levels, the follicular activity (as estimated by mean total follicular diameter) in horse and mule pregnancies was also similar. After day 39 of pregnancy and up to the end of the experimental period, the mean circulating FSH concentrations for the pregnancy types diverged so that mule pregnancies consistently had higher circulating FSH concentrations. However, this difference in mean circulating concentrations of FSH between horse and mule pregnancies tended to decrease towards day 150 of pregnancy.

In contrast to what would be expected with the circulating FSH concentrations between days 45 and 84 of pregnancy, horse pregnancies had significantly greater follicular activity during this time. Since days 45 to 84 of gestation in this study also coincided with the abrupt rise and maintenance of peak circulating eCG concentrations, the higher follicular activity in horse pregnancies was probably due to the high concentrations and prolonged presence of horse eCG when compared with mule eCG.
Just after day 84 of pregnancy, mule pregnancies still maintained higher circulating concentrations of FSH, while levels of horse eCG declined rapidly. It appears that the declining levels of horse eCG and lower levels of circulating FSH concentrations were not enough to maintain a higher follicular activity on the ovaries of mares carrying horse pregnancies than what was now occurring on the ovaries of mares carrying mule pregnancies. From this stage on, mule pregnancies maintained greater follicular activity and higher levels of FSH. The higher circulating FSH concentrations detected in mule pregnancies may partly explain the phenomenon of increased follicular activity in mule pregnancies during the second half of the experimental period (days 86 to 150 of gestation), as identified by several follicular dynamics parameters throughout this study.

Two possible mechanisms responsible for the lower mean circulating FSH concentrations that were detected in horse pregnancies are proposed. Since horses had higher follicular activity, between days 45 and 84 of pregnancy, horse pregnancies probably also had more large follicles with more FSH-inhibitory substances in the follicular fluid, resulting in the subsequent suppression of the mean circulating concentrations of FSH (Miller et al., 1981; Bergfelt & Ginther, 1986; Plata-Madrid et al., 1992; Roser et al., 1994). However, mule pregnancies had equivalent and higher levels of follicular activity after day 84 of pregnancy, with mean circulating FSH concentrations of horse pregnancies still remaining lower. Also, the narrowing of the difference between circulating FSH concentrations of horse and mule pregnancies towards the end of the observation period tended to coincide with the decreasing circulating concentrations of horse eCG. The decrease of the mean total follicular diameter in horse pregnancies preceded the decrease in circulating horse eCG concentrations and the period of maximum difference between the circulating FSH
concentrations of horse and mule pregnancies coincided closer to the rise and fall of eCG than to that of the mean horse total follicular diameter. Therefore, a direct negative effect of eCG on circulating concentrations of FSH must be considered.

Traditionally, it is believed that eCG has no direct negative effect on pituitary gonadotropins, because surges of FSH continue to occur in horse pregnancies even at times when eCG reaches peak concentrations in the peripheral circulation (Urwin & Allen, 1982b; Urwin, 1983; Ginther, 1992). Also, similar fluctuating FSH patterns occur in interspecies donkey in horse pregnancies (created by embryo transfer) where no eCG is being produced. The unlikeliness of a direct inhibitory effect of eCG on FSH is supported by in vitro receptor binding studies that demonstrated that the affinity of horse eCG for equine gonadal receptors is only 1 to 3% of that of equine FSH (Stewart & Allen, 1979). However, a definite decline in circulating concentrations of FSH was detected to be in close temporal relationship to the marked increase in eCG production around day 40 of pregnancy in donkeys carrying extraspecies horse pregnancies (Urwin & Allen, 1982b). It was suggested that this suppressive effect on FSH was mediated by the extremely high concentrations of progesterone (up to 800 ng per ml) from the hyperstimulated ovaries rather than by a direct inhibitory effect of eCG on circulating concentrations of FSH. However, the decline in FSH levels in donkeys carrying extraspecies horse conceptuses was temporally more closely associated with the rapid rise of eCG than with the rise in circulating levels of progesterone. The authors concluded that in this type of pregnancy only, can eCG exert a negative effect on circulating levels of FSH. Furthermore, it is well known that mares that lose their pregnancy after endometrial cup formation and not having a functional corpus luteum on their ovaries, do not cycle and ovulate until the endometrial cups have regressed completely and eCG has disappeared from the circulation (Allen,
1982, 1984). This may be an indication that FSH is not allowed to induce the emergence of a new follicular wave until eCG is no longer present.

It has been demonstrated in cycling (Evans & Irvine, 1975; Bergfelt & Ginther, 1993; Carnevale et al., 1994) and pregnant mares (Evans & Irvine, 1975; Van Niekerk et al., 1975; Urwin & Allen, 1982b; Bergfelt & Ginther, 1992) that a surge of FSH usually precedes a follicular wave by a fairly constant time period depending on the age, reproductive status and type of follicular wave under consideration.

Although only multiple points of significant difference could be demonstrated for the mean circulating FSH concentrations for the three aspiration treatments, the amplitude of the FSH surge that preceded the follicular waves in the three aspiration treatments differed significantly and displayed the same pattern as noted for the mean circulating FSH concentrations. The largest amplitude of the FSH surge that preceded a follicular wave was found when only large follicles (≥20 mm in diameter) were aspirated in the present study. The increase in FSH before the follicular wave peak of the control aspiration treatment and when all follicles were aspirated was not different from each other, but lower than when only large follicles were aspirated. As previously discussed, the highest FSH surge amplitude was expected when all follicles were aspirated, but this was not detected in this study.

In a recent study on the follicular dynamics between days 0 and 50 of the equine pregnancy, a close association between the increase in FSH concentrations and the emergence of follicular waves was found (Bergfelt & Ginther, 1992). A significant increase in the mean FSH concentrations occurred between days 4 and 5 before emergence of a major wave, reached mean maximal concentrations 3 days before emergence, and maintained these maximal concentrations for 5 days. In this study by Bergfelt & Ginther (1992), the interwave interval (only major waves considered, 35 to
52 mm in diameter) was 13 days. This implies that the mean number of days between the end of the FSH peak and the subsequent follicular wave peak was 11 days.

In the present study, the mean number of days between the FSH peak and the following follicular wave peak was 6.4 days for follicular waves with a largest follicular diameter ≥17 mm, and higher than for small waves (4.9 days) with a largest follicular diameter ≤17 mm. In a similar study on cycling mares, it was demonstrated that the mean time between significant increases in mean daily FSH concentrations and emergence of follicular waves was shorter for minor waves (a diameter of 18 to 25 mm for the largest follicle) than for major waves (a diameter of 34 to 47 mm for the largest follicle (Bergfelt & Ginther, 1993). Also in this study by Bergfelt & Ginther (1993), minor waves were considerably shorter in duration than major waves.

Since the follicular waves in the present study were smaller on average (<17 mm diameter of the largest follicle) and therefore, shorter than even the minor waves in the related studies, it can be conceived that the mean number of days between the FSH peak and the peak of the emerging wave will be lower. Similarly, the highest mean number of days between the FSH peak and subsequent follicular wave peak for the control aspiration treatment, followed by that of mares from which only follicles ≥20 mm in diameter were aspirated and then by mares from which all follicles were aspirated, could be explained by follicular wave size. The control aspiration treatment had the highest number of large and medium and also the highest number of long waves and, hence, the greatest number of days between the FSH peak and following follicular wave peak. The same pattern was found in the other two aspiration treatments, with the mares from which all follicles were aspirated having the highest number of small and short follicular waves and the lowest number of days between the FSH peak and subsequent follicular wave peak.
In a follicular dynamics-FSH study of mares 11 to 40 days pregnant (Ginther & Bergfelt, 1992a), the expression of dominance was associated with major follicular waves (mean maximal follicular diameter of 43 mm) and not with minor follicular waves (mean maximal follicular diameter of 27 mm). Therefore, follicular waves with the expression of dominance were larger than waves without the presence of a dominant follicle. The greater number of days between the FSH peak and the emerging follicular wave for waves with a dominant follicle when compared with waves that do not have a clear dominant follicle in the present study can, therefore, also be explained by differences in the follicular wave size and length.

In summary, the repeated aspiration of all follicles at the time of follicular wave peaks resulted in: 1) a decreased maximum diameter of the largest follicle of a follicular wave as pregnancy progressed; 2) a shortened follicular wave length; 3) a constant short follicular wave length as pregnancy progressed; 4) a lengthened interwave interval; 5) an increased time period between the peak of the current follicular wave and emergence of the subsequent wave; 6) an increased time period between the peak of the current follicular wave and emergence of the subsequent wave as pregnancy progressed; 7) a decrease in the total number of days covered by follicular activity; 8) reduced circulating concentrations of progesterone; and 9) an absence of the expected marked increase in the circulating concentrations of FSH after follicular aspiration.

By repeated aspiration of only follicles ≥20 mm in diameter at the time of follicular wave peaks, 1) the maximum diameter of the largest follicle of a follicular wave stayed constant as pregnancy progressed; 2) the wave length was shortened but to a lesser extent than that of mares from which all follicles were aspirated; 3) the wave length was shortened as pregnancy progressed; 4) the interwave interval was not shortened; 5) the time period between the peak of the current follicular wave and
emergence of the subsequent wave was not shortened; 6) the time period between the peak of the current follicular wave and emergence of the subsequent wave increased as pregnancy progressed; 7) the total number of days covered by follicular activity was decreased; 8) circulating concentrations of progesterone were reduced; 9) the circulating concentrations of FSH were elevated; and 10) the amplitude of the FSH surge preceding the emergence of a follicular wave was increased.

Horse pregnancies typically had reduced levels of follicular activity after day 85 of pregnancy when compared with those of mule pregnancies. The reduced levels of follicular activity after day 85 of pregnancy in mares carrying horse pregnancies were characterized by: 1) a decreasing maximum diameter of the largest follicle of a follicular wave as pregnancy progressed; 2) decreasing wavelengths as pregnancy progressed; 3) an increase in the time period between the peak of the current follicular wave and emergence of the subsequent wave; 4) fewer total number of waves for the complete experimental period; 5) fewer large follicular waves after day 85 of pregnancy compared with the number of large waves before day 85 or with mule pregnancies; 6) decreasing follicular activity towards day 150 of pregnancy as estimated by mean total follicular diameter; and 7) lower mean circulating concentrations of FSH for most of the experimental period.

Some of the reasons proposed for the phenomenon of reduced follicular activity in horse pregnancies after day 85 of pregnancy when compared with mule pregnancies include: 1) desensitization of the ovary by the extended exposure to high concentrations of horse eCG, thus, that the ovaries are less sensitive to follicular growth induction by equivalent levels of FSH (compared with concentrations before eCG appearance) after eCG disappearance from the circulation; 2) inhibition of circulating concentrations of FSH by inhibitory factors, such as estradiol and inhibin in the follicular fluid of large
follicles during the period of peak follicular activity (temporally related to peak circulating concentrations of eCG between days 45 to 84) in mares carrying horse pregnancies; and 3) a direct inhibitory effect of the high concentrations of eCG on the circulating concentrations of FSH.

The right ovary tended to be more active during pregnancy than the left when all treatments across horse and mule pregnancies were considered, as estimated by the contribution of the number of follicular waves to the overall individual mare follicular profiles. The increased activity of the right ovary during days 21 to 150 of pregnancy was significantly different when only horse pregnancies were considered. At this time, this finding can not be explained.
CHAPTER III

TRANSVAGINAL ULTRASOUND-GUIDED OOCYTE RETRIEVAL FROM CYCLIC AND PREGNANT HORSE AND PONY MARES FOR IN VITRO FERTILIZATION

Introduction

As in cattle and other domestic species, successful in vitro fertilization (IVF) in horses could provide alternative means of obtaining offspring from problem breeding females (Fulka & Okolski, 1981; McKinnon et al., 1988; Blue et al., 1989; Zhang et al., 1989; Del Campo et al., 1990). In vitro fertilization could also be used for further study of oocyte maturation, the events of fertilization (Betteridge et al., 1982; Bezard et al., 1989) and serve as a valuable research tool to evaluate the causes of reduced fertility in mares. However, the development of a consistent and practical IVF procedure for the horse has been limited, in part, by the lack of techniques for successful and repeatable in vivo oocyte recovery.

In recent years, efforts have been made to develop in situ oocyte recovery methods in the mare. Various approaches to oocyte recovery include: 1) paralumbar laparotomy (Betteridge et al., 1982; McKinnon et al., 1988; Vogelsang et al., 1988; Bezard et al., 1989); 2) paralumbar needle puncture of follicles with manipulation of the ovary through a colpotomy incision (Hinrichs et al., 1990) or per rectum (Hinrichs & Kenny, 1987; Palmer et al., 1987; McKinnon et al., 1988; Vogelsang et al., 1988; Hinrichs et al., 1991); and 3) transvaginal ultrasound-guided oocyte recovery procedures (Brück et al., 1992; Cook et al., 1992). Recovery rates have been highly variable with different methods, ranging from as low as 10% (Vogelsang et al., 1988) to as high as 73% with paralumbar aspiration procedures (McKinnon et al., 1988; Hinrichs et al., 1990). With the exception of the transvaginal approach to oocyte
aspiration, these methods are considered to be invasive, often cumbersome and with limited repeatability due to adhesion and scar tissue formation.

In the latter reports, mostly large preovulatory follicles were aspirated to recover oocytes during estrus, thus reducing the oocyte recovery potential for cyclic mares. Besides oocytes that can be obtained from cycling females, supplementary follicles produced during early pregnancy in the mare provide an additional potential source of oocytes, especially with the limited success of current ovarian superstimulation methods. The possibility exists that oocytes harvested from these follicles may outnumber those that could be obtained from a cycling donor female during a restricted time period. Since supplementary follicles are usually found on the ovaries of early pregnant mares, even outside the traditional breeding season, additional offspring could potentially be produced year round by IVF of oocytes recovered from a valuable donor mare with an established pregnancy. This could be done without risking a barren season, uterine infection and damage related to transcervical embryo recovery procedures or an ongoing pregnancy. Recently, offspring have been produced by IVF of oocytes recovered from hormone-treated pregnant beef cattle, using a transvaginal ultrasound-guided oocyte aspiration procedure (Meintjes et al., 1995c). The ability to produce oocytes similarly from valuable pregnant mares in a safe repeatable manner would be of great value, if retrieved oocytes could be used for IVF procedures.

The objectives of this study were: 1) to develop an effective and repeatable ultrasound-guided, transvaginal oocyte recovery procedure for developing follicles of different sizes in cycling pony and horse mares; 2) to determine the safety and feasibility of this approach for harvesting oocytes from early gestating mares; 3) to retrieve adequate numbers of viable oocytes from pregnant mares during the first half
of gestation by repeated transvaginal ultrasound-guided aspiration and (4) to confirm the viability of oocytes obtained from pregnant donors by IVF.

Materials and Methods

Experimental design

Fourteen mixed breed horses (379 to 500 kg) and 19 Welsh and Shetland pony mares (168 to 363 kg) all in good body condition and with normal-length estrous cycles were considered for follicular aspiration in the first part of Experiment I. Potential donor animals were observed for estrual behavior over a 32-day period from mid-July to mid-August of 1992. A total of 54 mare estrous cycles were monitored during the experimental period. Follicles were aspirated transvaginally from these mares during estrus. When the dominant follicle of the pony mares was 32 to 34 mm in diameter, they were given 1500 IU of hCG (Follutein®, Solvay Veterinary, Princeton, NJ) intravenously, and aspiration of the preovulatory and all other follicles > 8 mm in diameter (present on either ovary) was performed as close as possible to the predicted time of ovulation. Alternatively, when the dominant follicle of horse mares was 35 to 38 mm in diameter, these mares were given 2500 IU of hCG intravenously, and then similarly in pony mares the preovulatory and all other follicles > 8 mm in diameter were aspirated as close as possible to the predicted time of ovulation. Preovulatory follicles were identified and measured (diameter) by daily palpation per rectum and ultrasonography, using the basic ultrasound guidelines previously reported by Pierson & Ginther (1985). A follicle was identified as the preovulatory follicle during transrectal ultrasonic examination when it became larger than the other follicles in the ovary and/or when it exhibited a pronounced change in shape from spherical to conical, associated with a softened consistency as determined by palpation per rectum. Ovulation was estimated by palpation per rectum at 2-hour
intervals, starting 24 hours after hCG administration. The estrous cycles of mares were shortened by the intramuscular injection of 5 mg (pony mares) or 10 mg (horse mares) of prostaglandin F$_2$α (Lutalyse®, Upjohn, Kalamazoo, MI) during early diestrus, with a follicular aspiration procedure performed during each induced estrus. This allowed each cycling horse or pony mare to be aspirated one to three times during the 32-day experimental period.

In the second part of Experiment I, the follicles of five naturally-mated pregnant mares from the same animal pool were aspirated between days 22 and 66 of gestation in a preliminary trial to assess the safety of this procedure on early pregnant mares. Follicular development on the ovaries of these mares was monitored by transrectal ultrasound twice per week and the follicles aspirated when the largest follicle on either ovary reached a diameter ≥25 mm, or if three or more follicles >15 mm in diameter were detected. Aspiration of all follicles >8 mm were attempted at that time when these criteria were met and mares subjected to follicular aspiration either one, two or three times between days 22 and 66 of pregnancy. Pregnant mares were not administered hCG prior to aspiration.

In Experiment II, 14 pregnant mares of mixed breeds (372 to 569 kg) were used as oocyte donors between days 21 and 150 of gestation from April 1993 through March 1994 to evaluate the number and viability of oocytes that could be harvested from these mares during the first half of gestation. These pregnant oocyte donor animals were considered for aspiration only when a follicular wave on either ovary reached a growth peak as estimated by transrectal ultrasound. The follicular wave was considered to peak when the diameter of individual growing follicles of the wave began to plateau across days of ultrasound monitoring. A plateau was most often reached when the diameter of the largest follicle of the follicular wave maintained the
same diameter or decreased in diameter. Similarly, this plateau could be reached when \( \geq 80\% \) of the smaller follicles in the follicular wave maintained the same diameter or decreased in diameter (even in the presence of a large follicle increasing in diameter) between two subsequent transrectal ultrasonic examinations. Follicular aspiration was performed whenever a follicular wave could be identified by performing transrectal ultrasonography on alternate days. Two aspiration treatments were applied to this group of pregnant mares. Only follicles with a diameter \( \geq 20 \) mm were aspirated in seven of these mares (Treatment I); whereas, all follicles \( \geq 4 \) mm were aspirated in the other seven oocyte donor mares (Treatment II). During a single aspiration procedure, aspiration was attempted on all follicles on both ovaries that met the criteria for the specific treatment group.

**Animal preparation**

All mares were premedicated with 0.01 mg per kg of detomidine (Dormosedan®, Smith Kline Beecham, West Chester, PA), 0.05 mg per kg of atropine (Anpro Pharmaceutical, Arcadia, CA) and 0.1 mg per kg of butorphanol (Torbugesic®, Fort Dodge Laboratories, Fort Dodge, IA) prior to the aspiration procedure. Pregnant mares were administered an additional prophylactic dose of 20,000 IU per kg of penicillin-G (Smith Kline Beecham, West Chester, PA) and 1 mg per kg of flunixin meglumine (Banamine®, Schering Corporation, Kenilworth, NJ) prior to aspiration to protect from premature luteolysis and pregnancy loss (Elsworth-Swihart et al., 1985).

Once sedated, the mare was restrained in a modified holding chute (padded cattle squeeze chute) to prevent excess movement. The rectum of the mare was manually evacuated, a tail bandage applied and the perineal area aseptically prepared prior to the aspiration procedure.
Follicle aspiration procedure

The aspiration procedure used in this study was modified from the basic method described by Cook et al. (1992). A modified 5 MHz sector transducer attached to an Aloka 500-V ultrasound unit (Corometrics Medical Systems, Wallingford, CT) was used for follicle aspiration. The transducer was fitted at a fixed angle on the end of an extended handle (500 mm in length). A sterile, latex probe cover (Cone Instruments, Solon, OH) filled with sterile, ultrasound-conduction gel was pulled tightly over the transducer. Once the transducer (with the aspiration needle) was inserted into the vagina, a technician manipulated the ovary rectally against the distal end of the transducer, so that the puncture line (displayed on the monitor screen) transected the hypoechoic follicle through its longest axis. A 12-gauge, single-lumen needle (500 mm in length) was advanced through the anterior vaginal wall by a second technician to aspirate each follicle. A third technician flushed the follicles three to ten times with oocyte flushing medium by alternate filling and emptying. The oocyte flushing medium consisted of Dulbecco’s phosphate-buffered saline (Gibco, Grand Island, NY) with 1% calf serum, 100 IU of penicillin-G, 100 μg of streptomycin and 2 IU of heparin per ml of medium. A calibrated plastic hand pump (a modified sheep oral dosing gun) was used to fill follicles and ≈90 mm Hg vacuum pressure to recover the flushing fluid.

Depending on the size of the follicle, 10 to 250 ml of medium were flushed through the follicle. A sterile silicon stopper with two separate tubes passing through it (one tube connected to the suction device and the other to the aspiration needle) was fitted tightly on a 500 ml glass collection bottle to harvest the flushing fluid (Figure 3.1). Where the collection line attached to the hub of the aspiration needle, a plastic polymer Y-shaped adapter allowed an inflow port for the flushing medium. Follicular
Figure 3.1. Using the Aloka 500-V ultrasound unit with a plastic hand pump and negative pressure to harvest oocytes from developing follicles in both small and large mares.
fluid from each follicle was collected into a separate collection bottle. Mares were closely monitored for 24 to 48 hours following the aspiration procedure. Donor mares were observed for signs of colic, and then evaluated by ultrasonography up to 100 days after the last aspiration to verify pregnancy status.

Oocyte isolation and evaluation

Shortly after aspiration, the contents of each collection bottle were filtered through a standard bovine embryo filter (Pets Inc., Tyler, TX) with a 75μm mesh screen. The flushing medium retained in the filter was agitated with a suction pipette 20 to 30 times against the mesh screen to resuspend the oocytes. Contents of the filter were then poured into a 100 mm² square plastic collection dish (Becton-Dickenson Labware, Lincoln Park, NJ) and the medium carefully scanned for oocytes under a Nikon stereomicroscope (10 to 40X magnification).

In this study, the size of pre-aspirated follicles from cycling mares was classified into three categories: 1) small (8 to 25 mm in diameter); 2) medium (25 to 35 mm in diameter); and 3) large (>35 mm diameter) for subsequent oocyte data analysis. A fourth group of follicles (4), selected from size categories two and three, was further identified and noted as dominant and/or preovulatory follicles, using ultrasonic guidelines previously described by Pierson & Ginther (1985).

Following isolation, oocytes of cycling and pregnant mares were evaluated under inverted light microscopy at 40, 100 and 400X magnification for ooplasm consistency and cumulus cell morphology, as previously outlined for equine oocytes by Hinrichs (1991). The 25 oocytes recovered from pregnant mares on the preliminary field trial (days 22 to 66 of pregnancy) were not cultured after evaluation, but rather stained to determine the stage of oocyte maturation at the time of collection. The oocytes were stained with 2% aceto-orcein and evaluated under phase contrast
microscopy for nuclear maturation after a fixation period of 48 hours in a solution of acetic acid and ethanol (1:3 ratio). The oocytes collected from the second group of pregnant mares (days 21 to 150 of gestation, Experiment II) were divided into three categories before they were exposed to \textit{in vitro} maturation procedures: 1) degenerate (broken oolemma, oval shape of the zona pellucida); 2) poor quality (intact oolemma heterogenous or patchy ooplasm, oval shape of the zona, sparse cumulus support); and 3) good to excellent quality (intact shiny oolemma, homogenous ooplasm and ample cumulus support).

\textbf{\textit{In vitro} maturation/\textit{in vitro} fertilization procedure}

A randomly selected group of 47 oocytes collected from the second group of pregnant mares (Experiment II) were exposed to \textit{in vitro} maturation and \textit{in vitro} fertilization procedures as described by Li \textit{et al.} (1995). Oocytes were matured \textit{in vitro} for 48 hours in 100 $\mu$l of maturation medium under a mineral oil cover at 37°C in 5% CO$_2$ in air. The maturation medium consisted of Tissue Culture Medium-199 (TCM-199) supplemented with 10% fetal bovine serum (FBS), 2.5$\mu$g of FSH (Sigma Chemical Co., St. Louis, MO), 2.5$\mu$g of LH (Sigma Chemical Co., St. Louis, MO) and 1 $\mu$g Estradiol-17$\beta$ (Sigma Chemical Co., St. Louis, MO) per ml. These oocytes were exposed to the maturation medium for a 30-h interval.

After this 30 hours of \textit{in vitro} maturation, oocytes were stripped of cumulus cells by gentle pipetting of the oocytes for $\approx$10 minutes in a phosphate-buffered saline solution containing 0.25% hyaluronidase. This allowed visualization of the zona pellucida for zona drilling, using an acidic Tyrode’s solution (pH = 2.5). A small hole (2 to 3 $\mu$m in diameter) was created in the exposed zona pellucida by repeated, pulsatile administration of microvolumes of acidic Tyrode’s solution onto the targeted surface area of the equine oocyte. The zona drilling procedure was conducted in a 10
μl microdrop of PBS supplemented with 10% FBS under mineral oil on a warmed
glass slide. After this procedure, oocytes were removed from the manipulation
droplet, washed and transferred to an equilibrated 50 μl fertilization microdroplet of
Bracket-Oliphant medium (B-O medium) supplemented with 1% bovine serum albumin
(BSA, Fraction-V, Sigma Chemical Co., St. Louis, MO).

Fresh extended ejaculated semen from a mature, fertile stallion was washed
twice with B-O medium, supplemented with 10 mM caffeine (Sigma Chemical Co.,
St. Louis, MO), by centrifugation at 500 g for 7 minutes. The sperm pellet was
resuspended in ≈2 ml fresh B-O medium and then treated with a 1 to 3 μM
concentration of Ca2+ ionophore A23187 for 6 minutes. Then, 25 μl of this sperm
suspension (5x10^6 sperm cells per ml) was added to the fertilization droplets of 50 μl
of B-O medium and co-incubated with the micromanipulated oocytes at 39°C in 5%
CO2 in air for 2 hours. After fertilization, oocytes were co-cultured on a bovine
oviduct cell monolayer together with ten 8-cell mouse embryos in 100 μl droplets of
TCM-199 with 10% FBS at 39°C in an atmosphere of 5% CO2 in air for 192 hours
as recently outlined by Li et al. (1994).

Results

Cyclic mares

Of 54 estrous cycles monitored in 33 mixed breed mares (14 horse and 19 pony
mares) in Experiment I, oocyte aspirations were attempted in 36 cycles, involving 25
(11 horse and 14 pony mares) of these 33 mares. Six of the 54 cycles were
considered abnormal as judged by transrectal ultrasonic examination or palpation
(hemorrhagic preovulatory follicles, lack of normal pre-ovulatory follicular
development, or those classified as anovulatory follicles), and were not considered for
aspiration in this study. Additionally, follicles developing during 12 of the 48 normal
cycles were not subjected to aspiration due to unanticipated ovulations less than 24 hours after hCG administration.

The mean number of follicles >8 mm in diameter at the time of attempted aspiration was 2.28 (range 1 to 7), for a total of 82 follicles. In seven cycles with only one follicle >8 mm in diameter at the time of aspiration, the follicle could not be flushed successfully (being able to flush the follicle at least three but up to ten times with oocyte flushing medium, recovering 80% and more of the flushing medium). These follicles were punctured but could not be flushed due to medium leakage or inability to maintain the tip of the needle in the follicle (mare movement, technician error). Thus, at least one follicle could be flushed successfully in 29 of the 36 attempted cycles (81%). In the remaining 29 cycles involving 21 mares (nine horse and 12 pony mares), 62 follicles (1.72 follicles per cycle) were successfully aspirated (75.6%). A total of 29 oocytes were recovered for a recovery rate of 100% per cycle, 46.8% per successfully aspirated follicle, or 35% per follicle attempted for aspiration.

Oocyte recovery rates from different size follicles based on oocytes recovered per successfully aspirated follicles are shown in Table 3.1. The greatest recovery rate was noted in follicles 25 to 35 mm in diameter (68.8%), which was not significantly different from the recovery rate for follicles 8 to 25 mm in diameter (43.5%). However, the recovery rate from follicles measuring 25 to 35 mm in diameter was significantly greater than that of follicles >35 mm in diameter (34.8%). Aspiration technique complications often mentioned, such as pockets of medium left behind in large follicles or follicle rupture and medium leakage from smaller follicles were not as apparent when aspirating medium size follicles. This may partially explain this trend towards a higher oocyte recovery rate when aspirating medium-size follicles.
Table 3.1. Recovery rates from different size follicles and from oocyte donor mares

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<tr>
<th>Follicle diameter, mm</th>
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<td>Follicles</td>
<td>Oocytes</td>
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<td></td>
<td>aspirated</td>
<td>recovered</td>
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<td>8-25</td>
<td>23</td>
<td>10</td>
<td>43.5%</td>
<td>46.8%</td>
<td>75.8%</td>
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<tr>
<td>25-35</td>
<td>16</td>
<td>11</td>
<td>68.8%</td>
<td>42.9%</td>
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<tr>
<td>&gt; 35</td>
<td>23</td>
<td>8</td>
<td>34.8%</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>62</td>
<td>29</td>
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<tr>
<td>Preovulatory</td>
<td>28</td>
<td>12</td>
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<td>Pregnant</td>
<td>33</td>
<td>25</td>
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*Means within rows with different superscripts are significantly different ($\chi^2$; $P < 0.05$).*
When follicles were group classified as dominant and/or preovulatory follicles across all mares, the overall oocyte recovery rate was 42.9%.

There was no difference in oocyte recovery rate per cycle between horse mares and pony mares (13/13 and 16/16, respectively). Also, there was no difference in the number of follicles aspirated between horse and pony mares. The number of oocytes recovered per cycle ranged from 0 to 3. The most oocytes recovered from any mare over the 32 day experimental interval was 4. Seven cyclic mares were subjected to aspiration two times and the follicles of two mares aspirated three times. No adhesions or scar tissue was detected by palpation per rectum or ultrasonography 30 days following these aspiration procedures.

**Pregnant mares (preliminary study)**

Of the five pregnant oocyte donors in the preliminary aspiration trial, 33 of 38 follicles ≥8 mm identified by ultrasound could be aspirated (86.8%). Eight aspiration procedures from these five mares yielded 25 oocytes, resulting in a 75.8% recovery rate (oocytes recovered/follicle aspirated). This oocyte recovery rate was significantly greater than that of cyclic mares from the same experimental herd (46.8% vs. 75.8%). One pregnant mare was aspirated twice and a second mare three times. The mean number of oocytes recovered per mare across aspiration procedures was 5, with a minimum of 1 and a maximum of 11. The most oocytes recovered from a single aspiration procedure was 7. Furthermore, the oocyte donor mares in this preliminary study were able to maintain adequate luteal function to support their pregnancies, evidenced by these donor mares all giving birth to normal-appearing, healthy foals at term.
Pregnant mares

The results of the follicular aspirations for the third group of mixed breed mares (21 to 150 days of gestation) aspirated the following season (Experiment II) are summarized in Table 3.2. The diameter of follicles aspirated from this group of gestating mares ranged from 4 to 50 mm. The mean number of aspirations performed per donor female across all these mares was 7.6 (range 1 to 11), the mean number of follicles aspirated per mare was 44.9 (range 7 to 98) and the mean number of oocytes recovered per mare was 18.9 (range 5 to 37). The total number of oocytes recovered from these 14 pregnant donor mares was 266, with a mean number of oocytes recovered during a single aspiration procedure of 2.5 (range 0 to 13). The average inter-aspiration interval was 12.1±4.2 days for the 106 aspiration procedures performed on this group of pregnant mares. Of all the follicles identified for aspiration only 8% could not be aspirated due to various mechanical difficulties, such as animal movement and/or unintentional follicle puncture. The percentage of good to excellent quality oocytes retrieved (56.6% vs. 55.7%), as well as, the overall oocyte recovery rate per mare (40.2% vs. 43.3%) was not different between the two follicular aspiration treatments. However, more follicles were aspirated and more oocytes recovered per donor when all follicles (≥4 mm in diameter) were aspirated compared with the number of follicles aspirated and oocytes recovered when only those follicles ≥20 mm in diameter were considered for aspiration. There was no difference in oocyte recovery rates or in the number of good/excellent quality oocytes recovered between treatment groups. Also, none of the pregnant mares lost their pregnancies due to the repeated (up to 11 times) transvaginal follicular aspiration procedures, and all aspirated mares gave birth to normal, healthy foals.
Table 3.2. Mean oocyte recovery rate (±SE) and quality recovered from follicular aspiration of pregnant mares between 21 to 150 days of gestation

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Good/excellent quality oocytes</th>
<th>No. procedures</th>
<th>No. FOL(^a) aspirated</th>
<th>No. oocytes recovered</th>
<th>Recovery rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment 1</td>
<td>56.6%</td>
<td>7.3±3.7</td>
<td>28.4(^b) ±18.9</td>
<td>11.4(^b) ±6.1</td>
<td>40.2%</td>
</tr>
<tr>
<td>(FOL ≥ 20 mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment 2</td>
<td>55.7%</td>
<td>7.9±2.1</td>
<td>61.3(^c) ±25.6</td>
<td>26.6(^c) ±10.4</td>
<td>43.3%</td>
</tr>
<tr>
<td>(FOL ≥ 4 mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)FOL = follicles.  
\(^b\)\(^c\) Means within columns with different superscripts are significantly different (\(\chi^2\); P < 0.05 or paired t-test; P < 0.02).
Oocyte viability

Following microscopic evaluation, six of 29 oocytes (21%) collected from cyclic donors were covered by only what appeared to be corona cells, with no additional cumulus cells. Two of these oocytes (7%) were classified as being morphologically degenerate. Also, 11 of the 29 oocytes (38%) were surrounded by four or more layers of tightly packed cumulus (Figure 3.2). The cumulus cells of 12 oocytes (41%) were fully expanded and were embedded loosely around the oocyte in a gelatinous mass, creating a characteristic sunburst effect (Figure 3.3), as previously described by Van Niekerk & Gerneke (1966). The oocytes of the latter two groups had normal appearing organelles and ooplasm when evaluated by light microscopy.

Less than 20% of the oocytes retrieved from the pregnant donors in the preliminary study showed mucification and cumulus cell expansion. Most often the oocytes harvested were surrounded either by three to four layers of tightly packed cumulus cells or by the corona radiata alone (Figure 3.4). The 25 oocytes retrieved from the pregnant mares in this preliminary trial, that were stained immediately following collection, had variable morphological development. The majority of these oocytes (68%) was found to be at the GV stage, with only three oocytes (12%) having undergone GV breakdown. The remaining five oocytes were classified as being morphologically degenerate (20%).

The results obtained from in vitro maturation (IVM) and in vitro fertilization of a randomly selected group of 47 oocytes collected from the second group of pregnant mares (21 to 150 days of gestation) are summarized in Table 3.3. A total of 47 oocytes were classified into three oocyte quality groups at the time of collection. Polar bodies were observed in 50% to 60% of good to excellent quality oocytes. The 20 oocytes that were classified as good to excellent quality oocytes resulted in a
Figure 3.2. An example of oocytes harvested from cyclic mares that were surrounded by four or more layers of cumulus cells arranged in a compact fashion. The cumulus cells of these oocytes were arranged in concentric layers around the zona pellucida.
Figure 3.3. Another example of follicular oocytes recovered from cyclic mares. The ooplasm and zona pellucida detail are difficult to visualize because of the three-dimensional nature of the cumulus cell network. The cumulus cells are loosely arranged around the oocyte in a radial pattern creating a characteristic sunburst effect. The oocyte is embedded in a gelatinous mass providing structural support to loosely aggregated cumulus cells.
Figure 3.4. Five oocytes harvested from the follicles of a pregnant mare during an aspiration procedure. Ooplasm consistency and oocyte morphology were similar to that of cyclic mares. Oocytes retrieved from pregnant mares, however, often lacked the cumulus cell support.
Table 3.3. Development of *in vitro*-fertilized oocytes collected from pregnant mares

<table>
<thead>
<tr>
<th>Oocyte quality</th>
<th>(n)</th>
<th>Cleavage(%)</th>
<th>2-Cell</th>
<th>4 to 8-Cell</th>
<th>16-Cell</th>
<th>Morula/BLST*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Good to excellent</td>
<td>20</td>
<td>65b</td>
<td>13</td>
<td>10</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Poor</td>
<td>15</td>
<td>20c</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Degenerate</td>
<td>12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*BLST = blastocyst.
*b,cDifferent superscripts within the same column are significantly different ($\chi^2 < 0.05$).
cleavage rate of 65% and 38.4% of those cleaving developed to morula and blastocyst stages after 192 hours of co-culture with bovine oviduct cells and eight-cell mouse embryos. In contrast, only 20% of the poor quality oocytes cleaved during co-culture and none of the oocytes that were considered as degenerate cleaved.

Discussion

More difficulty was experienced in aspirating follicles of small ponies than those of horse mares, due to the lack of arm space in the rectum and/or space for the vaginal probe in the pelvic cavity. Even with the physical restraints for small mares, however, there were no difference in oocyte recovery rates between horse and pony mares. The transvaginal oocyte recovery rates for cyclic mares in the present study was similar to those recently reported for mares in other laboratories (Brück et al., 1992; Cook et al., 1992; Dippert et al., 1994). Interestingly, the pattern of the highest oocyte recovery originating from medium size follicles (25 to 35 mm in diameter) of cyclic mares in this study has also been noted by Vogelsang et al. (1988) (highest recovery from follicles 21 to 30 mm in diameter) and Dippert et al. (1994) (highest recovery from follicles ≤ 27 mm in diameter).

On microscopic examination, oocytes harvested from both cyclic and pregnant mares appeared normal (intact shiny oolemma, homogenous ooplasm, round shape of the zona pellucida, cumulus support) under light microscopy. However, a fully expanded cumulus was seldomly observed in oocytes harvested from pregnant mares. Also, the oocytes of pregnant mares occasionally demonstrated a total lack of cumulus cell support, however, the majority (68%) of chromatin was found to be at the GV-stage when evaluated post-staining. This percentage of immature oocytes obtained just after aspiration from the pregnant horses were similar to those reported for oocytes obtained from slaughterhouse ovaries before in vitro maturation. Fulka & Okolski
(1981) have reported 69.6% of oocytes harvested from mares to be at the GV stage of nuclear maturation just after they were recovered from 5 to 30 mm follicles at slaughter. Similarly, Zhang et al., (1989) found that 70% of oocytes (with a fully compact cumulus investment) also collected from mares at slaughter were at the germinal vesicle and germinal vesicle breakdown stages.

Polar bodies were observed in 50% to 60% of good to excellent quality oocytes obtained from the pregnant mares in Experiment II by the time zona drilling was performed for IVF. Previously, Webel et al., (1977) have found only one half of the in vivo matured, ovulated equine oocytes in their study to have a visible first polar body. These findings suggest that all oocytes with first polar bodies were not necessarily detected when evaluated by light microscopy, and that staining would be necessary to more accurately determine the maturation rate of oocytes in studies of this nature. As efforts to detect oocytes with first polar bodies were made only after 30 to 32 hours of in vitro maturation in the present study, the possibility also exists that more polar bodies would have been detected if more time was allowed for the in vitro maturation process. This is supported by Zhang et al. (1989) and Hinrichs et al. (1993), who reported that 61% to 63% of oocytes with a fully compacted cumulus investment after 30 hours and 72% of the oocytes with a fully compacted cumulus investment after 32 hours of in vitro maturation were at the metaphase-II stage of nuclear maturation. Unexpectedly in this study, a high percentage of oocytes cleaved (65% for those of excellent to good quality) following in vitro maturation and IVF of immature oocytes harvested from pregnant donors. Thus, it seems that the in vitro maturation protocol used during this trial was sufficient for the maturation and post-fertilization development of equine oocytes harvested at the immature stage from pregnant donors.
There was no indication of parthenogenic cell divisions found in this laboratory in oocytes aspirated from cyclic mares and oocytes aspirated from pregnant mares during the *in vitro* maturation period, as has previously been reported for *in vivo* collected oocytes (Van Niekerk & Gemeke, 1966). Li *et al.* (1995), detected no parthenogenic activity within a small group of sperm-exposed, zona-drilled oocytes aspirated from the follicles of pregnant mares. Further studies are needed to verify these findings.

The results from the IVF of the oocytes recovered from early pregnant donors indicate that these oocytes were viable, and do have the potential to develop to the morula and blastocyst stages *in vitro*. To our knowledge, this is the first report of successful *in vitro* fertilization of *in vitro* matured equine oocytes collected from pregnant donors. When *in vitro* fertilization and cleavage are used as an indicator of oocyte viability, indications are that ≥34% of oocytes (16 of the 47 sperm-exposed oocytes cleaving) harvested from early pregnant mares were viable. Since we were unable to monitor the status of aging follicles, it is assumed that an unknown number of oocytes from atretic follicles were included in this study.

It should be noted that up to 39% of oocytes obtained from atretic follicles may be of acceptable quality (evaluated by ooplasm morphology only) when compared with oocytes obtained from viable follicles (Hinrichs, 1991). Depending on follicle size, 79% (follicles <10 mm in diameter), 58% (follicles 10 to 19 mm in diameter) or 17% (follicles >20 mm in diameter) of the follicles can be atretic from a group of randomly collected ovaries. Furthermore, germinal vesicle breakdown is often an indication of degenerate oocytes obtained from atretic follicles; however, these oocytes may also be obtained from nonatretic follicles (Fulka & Okolski, 1981). Of the oocytes obtained from mares in Experiment II, 32% might have been degenerate (12%
with germinal vesicle breakdown and 20% classified as morphologically degenerate). In a previous study also conducted in this laboratory, Meintjes et al. (1994) found that ≈40% of oocytes harvested from pregnant donors were likely degenerate at the time of collection (based on the same criteria; germinal vesicle breakdown and oocytes classified as morphologically degenerate). With the majority of follicles aspirated from pregnant mares being between 15 and 25 mm in diameter in this laboratory and an overall IVF cleavage rate of 34% of oocytes obtained from mares in Experiment II, it is likely that between 40% and 60% of follicles aspirated from pregnant horses in this study were atretic.

The ease of follicle aspiration, respectable oocyte recovery rates and the increased number of oocytes aspirated per mare (>30 oocytes) that were obtained from several pregnant mares in this study were unexpected when compared with the results from the same aspiration procedure used on cyclic mares. It should be noted that several oocytes have been collected previously from pregnant mares using a paralumbar approach (Palmer et al. 1987).

Based on the findings of the present study, the recovery of at least one oocyte could be expected, if one to two follicles were aspirated from a cyclic mare during a single follicular aspiration procedure. In contrast, 2.5 oocytes could be expected from any single follicular aspiration procedure from a pregnant donor. From results obtained at this laboratory over the last two breeding seasons, an oocyte aspiration procedure could be performed every 7 to 10 days (utilizing the follicular waves of early gestation) on pregnant donors without any hormonal treatment compared with a 21-day oocyte aspiration schedule for cyclic, estrual mares. Thus, it is likely that more oocytes could be harvested from mares during the early months of gestation (Meintjes et al., 1994) than would normally be expected from cyclic, gonadotropin-
treated mares (Cook et al., 1993a; Dippert et al., 1994). It is estimated that an average of 19 oocytes could be retrieved from a mare between 21 and 150 days of gestation compared with 12 oocytes collected during a similar 130 day interval in cyclic mares.

If taken into consideration that significantly more oocytes were recovered per donor when all follicles ≥4 mm in diameter were aspirated and that the percentage of good to excellent quality oocytes retrieved and the oocyte recovery rate per mare were not different between the two follicular aspiration treatments of Experiment II, it seems that Treatment II (aspirating all follicles available >4 mm in diameter) should be the most practical approach to obtain supplementary oocytes from valuable pregnant donor mares for in vitro fertilization.

This approach to obtain additional offspring from a valuable donor animal may be an alternative to conventional embryo transfer practices to produce offspring outside the breeding season, since supplementary follicles are usually found on the ovaries of early pregnant mares. Successful IVF of oocytes recovered from a pregnant mare may yield additional foals without risking a barren season, uterine infection, damage related to transcervical embryo recovery procedures or an ongoing pregnancy.

Transvaginal oocyte aspiration proved to be a safe, repeatable and reliable means of obtaining intact cumulus-oocyte complexes from cyclic as well as pregnant mares. Furthermore, transvaginal aspiration does not disrupt the luteal function during early pregnancy, as evidenced by no pregnancy loss to date attributed to this aspiration procedure. On the contrary, evidence does exist that removal of follicular fluid actually enhances luteinization and progesterone production by the subsequent luteal tissue in the mare (Hinrichs et al., 1991).
Superovulation methodology for routine use in equine embryo transfer programs has not yet been fully developed. With the potential for success with GIFT and similar procedures in horses (McKinnon et al., 1988; Zhang et al., 1989) and the recent success with IVF procedures in the horse (Bezard et al., 1989; Del Campo et al., 1989; Zhang et al., 1990; Palmer et al., 1991; Li et al., 1994), oocytes obtained from foal heat, cyclic and gestating mares may expand the economic value of genetically-important mares. Since it was found that pregnant mares could be aspirated and viable oocytes recovered continuously during the period April 1993 through March 1994 in this study, the traditional breeding season of horses could potentially be extended into the otherwise nonreproductive winter season. Oocytes can now be easily harvested using transvaginal ultrasound during early stages of gestation in the mare, without disrupting the ongoing pregnancy. Correspondingly, transvaginal follicular aspiration of cyclic and pregnant mares should not be overlooked in the future as an alternative to superovulation treatments and embryo collection in the horse.
CHAPTER IV

IN VITRO FERTILIZATION AND DEVELOPMENT OF IN VITRO MATURED OOCYTES ASPIRATED FROM PREGNANT MARES

Introduction

Attempts at maturing and fertilizing equine oocytes in vitro has only resulted in limited progress (Zhang et al., 1989; Del Campo et al., 1990; Zhang & Boyle 1990; Palmer et al., 1991). This is surprising because successful in vitro maturation of immature equine oocytes (Willis et al., 1990; Del Campo et al., 1992; Hinrichs et al., 1993) and capacitation with subsequent acrosome reaction of equine sperm cells have been reported (Varner et al., 1987; Samper et al., 1989; Zhang et al., 1990). Furthermore, it has also been reported that capacitated and acrosome-reacted equine sperm cells have the capacity to penetrate zona-free hamster oocytes in vitro (Brackett et al., 1982a; Samper et al., 1989). In vitro-matured equine oocytes were capable of in vivo fertilization, development in utero and establishing a pregnancy after transfer into the oviducts of a recipient mare (Zhang et al., 1989). However, in vitro fertilization rates of the equine oocytes and subsequent embryo development have been less than anticipated (Del Campo et al., 1990; Zhang et al., 1990; Palmer et al., 1991), with the most advanced embryos derived from in vitro maturation and in vitro fertilization reaching the four- to six-cell developmental stages under in vitro culture conditions (Zhang et al., 1990).

The reason(s) for poor in vitro fertilization and subsequent development rates of equine oocytes remains unclear. Since equine oocytes usually have a thick zona pellucida, we suspected that the zona pellucida of the in vitro-matured oocytes is, in part, a barrier to in vitro-prepared sperm cells. The potentially altered zona pellucida of in vitro-matured oocytes, in addition to less than adequate sperm cell preparation for
in vitro fertilization, likely contributes to poorer than expected in vitro fertilization rates. Following preliminary studies evaluating different chemical and mechanical approaches to make an opening in the equine zona pellucida, we proposed that pre-fertilization zona drilling of the equine oocyte may enable motile sperm cells to quickly overcome the zona barrier, thus increasing the opportunity for in vitro fertilization.

In an initial report, Gordon & Talansky (1986) noted that a simple zona drilling procedure using acidic Tyrode's solution enabled the sperm cells to enhance fertilization in laboratory animals. Since then, live offspring have been produced in mice (Gordon & Talansky, 1986) and humans (Ng et al., 1988; Cohen et al., 1991; Cohen et al., 1992) following the fertilization of zona-drilled oocytes. Zona-drilling is now being used in human IVF programs to treat patients with oligospermia, teratozoospermia and unexplained male infertility (Cohen et al., 1990; Cohen et al., 1991). Making an opening in the zona pellucida using either an acidic Tyrode's solution, mechanical massage or zona renting procedures has been reviewed for human oocytes by Cohen et al. (1992).

A pilot experiment at this laboratory showed that the zona pellucida of in vitro-matured equine oocytes is very sensitive to acidic Tyrode's solution, with this solution usually dissolving through the thickness of the equine zona pellucida wall within 30 seconds. Furthermore, mechanical cutting or massage of the equine zona pellucida was found to be very difficult without damaging the oocyte. Based on these preliminary findings, acidic Tyrode's solution was chosen for pre-fertilization zona drilling of the equine oocytes in this experiment. The objectives of this study were to increase the in vitro fertilization rate of equine oocytes by altering the concentration of Ca++ ionophore used for sperm cell preparation, incorporating pre-fertilization zona
drilling and to increase the in vitro development of these IVF-derived embryos with a novel embryo co-culture system using bovine oviduct cells along with 8-cell mouse embryos.

**Materials and Methods**

**Oocyte collection**

During the summer, fall and winter of 1993, the follicular waves of a group of mixed breed pregnant mares were monitored by ultrasonography during the first 150 days of gestation. Oocytes were collected via transvaginal ultrasound-guided aspiration procedures (n = 14) from 12 of these mares between days 33 and 146 of gestation. Ultrasound-guided aspirations were conducted at the peak of follicular waves identified during pregnancy, as previously reported by this laboratory (Meintjes et al., 1995a). The aspirated oocytes were washed three times with Tissue Culture Medium-199 (TCM-199) supplemented with 10% fetal bovine serum (FBS) and then transferred to maturation medium.

**Maturation of oocytes**

The medium for oocyte maturation consisted of TCM-199 supplemented with 2 μg equine LH (Sigma Chemical, St. Louis, MO), 2 μg ovine FSH (Sigma Chemical), 1 mg estradiol (Sigma Chemical) and 10% FBS per ml. Oocyte maturation was conducted in 1 ml maturation medium covered with medical-grade mineral oil in a 35 mm petri dish at 37°C in an atmosphere of 5% CO₂ in air for 32 to 48 hours. The cumulus cells of the in vitro-matured equine oocytes were then removed by exposure to 0.25% of hyaluronidase in phosphate-buffered saline (PBS) at 37°C for 10 minutes and by repeated pipetting with a small bore pipette. The oocytes free of cumulus cells were evaluated under a Nikon inverted microscope (400X) and classified to be of good or poor quality based on the shape of the oocytes, the transparency of the ooplasm and
the distribution of the pigment and lipid granules within the ooplasm. The oocytes with a regular shape, transparent ooplasm and even distribution of pigment and lipid granules were classified as good quality oocytes (Hinrichs, 1991), and only these oocytes were used for this experiment. The oocytes with irregular shape, dark ooplasm or uneven distribution of granules were classified as poor quality oocytes and was not used in this study.

**Zona drilling**

The oocytes free of cumulus cells were washed and then transferred into a microdroplet of 10 µl PBS medium supplemented with 10% FBS covered with mineral oil on a warmed, sterile glass slide. Zona drilling was conducted within the microdroplet of PBS medium with the use of Leitz manipulator units, while viewing the oocytes under an inverted Nikon microscope (200X). A glass pipette (90 µm outside diameter) was used to hold the oocyte via negative pressure, and a second glass pipette (2 to 3 µm in diameter) filled with acidic Tyrode's solution was brought into contact with the zona pellucida of the oocyte (Figure 4.1). Pulses of acidic Tyrode's solution (pH=2.5) were expelled from the pipette (total volume of 2 to 4 µl) to dissolve a 3 to 4 µm opening in the zona pellucida. Quick withdrawal of the pipette was necessary with this procedure to prevent damage to the internal components of the oocyte. The oocytes were carefully washed with fresh PBS medium and then transferred to the *in vitro* fertilization droplets.

**Sperm cell preparation**

Fresh equine sperm cells from a mature, fertile stallion were separated from the seminal plasma by washing with freshly prepared Brackett-Oliphant (B-O) medium (Brackett & Oliphant, 1975) supplemented with 10 mM caffeine (Sigma Chemical). Sperm cells were concentrated by centrifugation at 500 g for 5 minutes and then the
Figure 4.1. The procedure for making a small opening (2 to 3 μm) in the zona pellucida of cumulus-free, *in vitro*-matured equine oocytes using acidic Tyrode's solution.
sperm pellet was resuspended in B-O medium supplemented with 10 mM caffeine. The sperm cells were then treated with Ca++ ionophore A23187 (Sigma Chemical) for 6 minutes and recovered by centrifugation at 500 g for 5 minutes. The sperm pellet was resuspended a second time in 5 ml of fresh B-O medium without caffeine. The sperm cell concentration was adjusted to 5x10⁶ spermatozoa per ml by adding an appropriate amount of B-O medium. In a preliminary trial, the sperm parameters of this stallion were evaluated after treatment with Ca++ ionophore A23187 at concentrations of 0, 0.1, 1.0, 3.0, 5.0 or 7.0 μM in B-O medium supplemented with 10 mM caffeine for 6 minutes.

**Sperm motility and acrosome reaction**

A small 10 μl microdroplet of the sperm cell/B-O medium mixture was transferred to a warmed glass slide and the number of the immotile sperm cells was immediately counted under high magnification (400X). After air drying for 15 minutes, the total number of sperm cells in the droplet was then counted. Sperm motility (stationary and progressive motility) was calculated by the difference from the total number of sperm cells minus the number of immotile sperm cells. To estimate the percentage of acrosome-reacted sperm cells, the glass slide with the dried sperm cell smear was fixed with a methanol/galactic acid solution (3:1) and then stained with 0.1% Giemsa. The sperm cells without a visible acrosome (a light color in the region of the acrosome) were considered as acrosome-reacted and those with a dark color in the acrosomal region were considered as acrosome-intact sperm cells in this study.

**Experimental design**

After the preliminary trial to evaluate the effect of different concentrations of Ca++ ionophore A23187 on stallion sperm-cell parameters, it was decided that both the 5.0 and 7.0 μM concentrations of the Ca++ ionophore would least likely produce viable
embryos in this in vitro fertilization experiment. Thus, freshly collected sperm cells from the same stallion were allotted to one of four treatment groups. Sperm cells in Treatment I were exposed to B-O medium supplemented with 10 mM caffeine and no Ca\textsuperscript{2+} ionophore (control), and those in Treatments II, III and IV were exposed to 0.1, 1.0 or 3.0 \(\mu\text{M}\) concentrations of Ca\textsuperscript{2+} ionophore, respectively, in B-O medium supplemented with 10 mM caffeine. All treatment groups were incubated in their respective treatment solutions at room temperature for 6 minutes. To increase the chance of fertilization only good quality oocytes, as previously described by Hinrichs (1991), were selected and subjected to the zona drilling procedure with acidic Tyrode's solution before co-incubated in the fertilization droplets with sperm cells treated with varying concentrations of Ca\textsuperscript{2+} ionophore in Treatments I through IV. After the fertilization procedure, all oocytes were exposed to the same co-culture conditions for 192 hours.

**In vitro fertilization**

In vitro fertilization was conducted in 50 \(\mu\text{l}\) droplets of B-O medium supplemented with 1\% bovine serum albumin (BSA, Fraction-V; Sigma Chemical) under a covering of mineral oil using a procedure similar to that routinely used for bovine IVF in this laboratory (Zhang et al., 1992). The zona-drilled oocytes were transferred to the fertilization droplets and 25 \(\mu\text{l}\) of sperm cell/B-O medium mixture (5\times10^6 sperm cells per ml) was then added to each fertilization droplet (a total of 75 \(\mu\text{l}\) per droplet). The oocytes and the sperm cells were co-incubated for 30 minutes to 2 hours at 39\textdegree C in an atmosphere of 5\% CO\textsubscript{2} in air. The co-incubation interval was adjusted for each group of oocytes after visualization of the first sperm cell in the perivitelline space.
A supplemental *in vitro* fertilization trial

Since only a limited number of mares were available for transvaginal oocyte aspiration procedures in this study, additional treatment groups were used to test for parthenogenetic activity using abattoir oocytes. In the first treatment group, zona-drilled oocytes (n=16) were exposed to 1 μM Ca++ ionophore-treated sperm during co-incubation in a 75 μl fertilization microdroplet as described previously (same as Treatment III). In the second treatment group, intact oocytes (not zona drilled) (n=20) were similarly exposed to 1 μM Ca++ ionophore-treated sperm during co-incubation in a 75 μl microdroplet. Oocytes in the third treatment group (n=20) were zona drilled and exposed to the same medium used to prepare the sperm for IVF (BO medium exposed to a 1 μM Ca++ ionophore concentration for 6 minutes, but without sperm cells) during co-incubation in a 75 μl microdroplet.

Embryos obtained from the sperm-exposed oocytes in the first treatment group, as well as, any oocytes from the remaining two treatment groups displaying cytoplasmic division/fragmentation were fixed with a drop of a 1:1 solution of methanol:acetic acid on a clean glass slide, and after drying, placed overnight in a clearing solution (methanol:acetic acid, 3:1). These embryos/oocytes were then stained with a 10% Giemsa solution to verify the presence or absence of cell nuclei.

**Mouse embryo preparation**

ICR female mice (4- to 8-weeks old) were treated with 5 IU of eCG and 5 IU hCG 48 hours later to induce superovulation. The females were paired with ICR males immediately following the hCG treatment and mating was determined by the presence of a vaginal plug the next morning (day 0). Embryos were collected on day 2 (at the eight-cell stage) by flushing the oviducts of the females with PBS medium.
**Bovine oviduct cell preparation**

Bovine oviducts were collected from a local abattoir and transported (within 6 hours) to the embryo laboratory in PBS medium supplemented with 5% FBS. The oviducts were washed with PBS medium and then luminal epithelial cells were collected by expressing the oviducts with a pair of sterile forceps into 5 ml of TCM-199 supplemented with 10% FBS. The harvested cells were washed and recovered by centrifugation at 500 g for 5 minutes. The cells were resuspended in 5 ml of TCM-199 with 10% FBS and then seeded into 75 µl droplets of TCM-199 supplemented with 10% FBS. Cells were incubated in these microdroplets at 39°C in an atmosphere of 5% CO₂ in air, usually forming confluent monolayers within 3 days.

**In vitro culture**

The sperm-exposed equine oocytes were removed from the fertilization droplet and washed three times with TCM-199 supplemented with 5% FBS. These oocytes (one to two oocytes per microdroplet) were then co-cultured on a bovine oviduct cell monolayer with ten mouse eight-cell embryos in 100 µl droplets of TCM-199 with 10% FBS under equilibrated mineral oil at 39°C in an atmosphere of 5% CO₂ in air as previously described (Li et al., 1993). The culture medium was changed every other day and at this time fresh eight-cell mouse embryos were also added to the culture droplets. Embryo development was evaluated under a Nikon inverted microscope (200 and 400X) at 48-hour intervals during a 192-hour culture period.

**Results**

**Preliminary trial: Effect of Ca++ ionophore on sperm motility and acrosome reaction**

Semen from one mature, fertile stallion was used in the IVF experiment with transvaginally harvested oocytes to minimize the variation in the response of the sperm
cells to the Ca++ ionophore. The motility of the sperm cells from this stallion was evaluated in a preliminary trial after treatment with Ca++ ionophore A23187 at concentrations of either 0, 0.1, 1.0, 3.0, 5.0 or 7.0 μM in B-O medium supplemented with 10 mM caffeine. Calcium ionophore did not noticeably affect the motility of the sperm cells at concentrations of up to 3 μM. However, progressive motility dropped dramatically from 79% to 40% when sperm were exposed to Ca++ ionophore at the 5 μM concentration, and further declined to 1% when sperm were exposed to a concentration of 7 μM (Figure 4.2). In contrast, the percentage of acrosome reactions in the sperm samples increased with increasing concentrations of Ca++ ionophore, and reached 64% when sperm cells were incubated at a concentration of 7 μM from a low of 5% at the 0.1 μM concentration.

**The effect of Ca++ ionophore on cleavage rate and embryo development**

Based on the analysis of the motility and acrosome reaction of the sperm cells after treatment with Ca++ ionophore in the preliminary trial, concentrations of 0, 0.1, 1.0 and 3.0 μM Ca++ ionophore were selected as concentrations for use in Treatments I, II, III and IV, respectively, for the present IVF experiment. Only good quality oocytes were selected (n=32) and allotted, after zona drilling, to one of the four treatment groups. No oocytes cleaved in the control group (Treatment I) without the addition of Ca++ ionophore (Table 4.1). None of the oocytes cleaved in the treatment group where sperm cells were treated with a 0.1 μM concentration of the ionophore (Treatment II). However, 11 of 14 sperm-exposed oocytes (79%) cleaved in the treatment group fertilized with sperm cells treated with a 1 μM concentration of the ionophore (Treatment III). Significantly more sperm-exposed oocytes cleaved in Treatment III during culture than in Treatments I, II and IV. Only two of six oocytes (33%) exposed to sperm cells treated with the 3 μM concentration of Ca++ ionophore
Figure 4.2. Sperm motility and acrosome reaction of fresh sperm cells from one stallion treated for 6 minutes with different concentrations of Ca\textsuperscript{++} ionophore A23187 in B-O medium supplemented with 10 mM caffeine.
Table 4.1. *In vitro* development of equine embryos fertilized by sperm cells treated with either 0, 0.1, 1.0, or 3 μM concentrations of Ca\(^{++}\)ionophore A23187

<table>
<thead>
<tr>
<th>Trt</th>
<th>Ca(^{++}) ionophore</th>
<th>n</th>
<th>Cleavage(%)</th>
<th>2-Cell</th>
<th>4-Cell</th>
<th>8-Cell</th>
<th>16-Cell</th>
<th>BLST/ Morula</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>0 μM</td>
<td>4</td>
<td>0(^c)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>0.1 μM</td>
<td>8</td>
<td>0(^c)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>1.0 μM</td>
<td>14</td>
<td>79(^b)</td>
<td>11</td>
<td>9</td>
<td>8</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>IV</td>
<td>3.0 μM</td>
<td>6</td>
<td>33(^c)</td>
<td>2</td>
<td>0</td>
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</tbody>
</table>

\(^a\) BLST=blastocyst

\(^b\) Mean values in the same column with different superscripts are significantly different (χ\(^2\); P<0.05)
cleaved (Treatment IV), however, both of these embryos ceased development prior to the four-cell stage.

As incubation continued, five of the 11 cleaved embryos (45.5%) in Treatment III developed to the morula stage and 18.2% of those embryos that originally cleaved reached the blastocyst stage by the end of the culture interval. In this study, there was no indication that the range of time for oocyte maturation (37 to 48 hours) altered in vitro fertilization and subsequent embryo development. All embryos developing to later stages were observed to be in close synchrony with the chronology of normal in vivo development that has been previously reported for equine embryos (Betteridge et al., 1982; Bezard et al., 1989).

In the supplemental IVF trial, a cleavage rate of 44% was obtained in the first treatment group, where zona-drilled oocytes were exposed to sperm cells treated with a 1 μM concentration of ionophore. However, none of the intact, sperm-exposed oocytes in the second treatment group cleaved. Furthermore, no signs of cleavage were detected in the third treatment group, where zona-drilled oocytes were exposed to Ca++ ionophore, but with no sperm cells. After staining with Giemsa, the blastomeres of all the embryos obtained from the first treatment group were found to contain clearly definable cell nuclei. The ooplasm of some oocytes (≈10%) formed cell-like structures near the end of the in vitro maturation period, and thus were not assigned to respective treatment groups. When these oocytes were stained, however, no nuclei were detected, indicating cytoplasmic fragmentation rather than parthenogenetic activity. None of the IVF-derived embryos obtained from the aspirated oocytes or those derived from abattoir ovaries that developed beyond the cleavage stage in this study gave any indication of parthenogenetic activity or abnormal embryo development in vitro.
Discussion

The reason(s) for low in vitro fertilization rates and subsequent poor in vitro embryo development of in vitro-matured equine oocytes is presently not clearly understood. Sperm cell capacitation, oocyte maturation and the quality of oocyte investment have all been offered as possible reasons for the less than adequate laboratory results (Zhang et al., 1990). The results of the present study indicate a possible role of the oocyte investment and poor sperm cell preparation in the low in vitro fertilization rates obtained for in vitro-matured equine oocytes. The cleavage rate of the zona-drilled equine oocytes in the present study was 79%, which is greater than the cleavage rate reported for the intact oocytes in other laboratories (Del Campo et al., 1990; Zhang et al., 1990; Palmer et al., 1991). A primary modification in the IVF procedure in the present study was incorporation of zona drilling using acidic Tyrode’s solution. This approach is simple (∼30 seconds per oocyte) and has a procedure survival rate of >98%. A zona-intact control was not attempted in this study because of the limited number of donor mares available for aspiration. In a preliminary study (data not presented), oocytes harvested from cyclic, estrual mares in the same experimental herd were exposed to sperm cells treated with Ca++ ionophore A23187 at various concentrations (without zona drilling), however, no cleavage was detected from any of these zona-intact oocytes.

The mammalian fertilization process involves the penetration of the oocyte investment by active sperm cells, as well as, the interaction and fusion of the sperm cells with oocytes (Saling, 1988). It has been shown that the investment abnormalities of mouse oocytes may prevent the sperm cells from penetrating the zona pellucida and thus, hinder the fertilization (Downs et al., 1986; Saling, 1988). It is unclear at the present time why the cumulus cells and/or the zona pellucida poses a barrier for in vitro...
fertilization of the equine oocytes. The zona pellucida of the equine oocytes may be altered during the in vitro maturation process and thus, making it more difficult for individual sperm cells prior to and during zona penetration. In addition to the changes in the zona pellucida that may occur during in vitro maturation and fertilization, less than adequate in vitro sperm cell preparation may also play a role in the low success rate in equine in vitro fertilization programs and subsequent embryo development.

The most encouraging method for the induction of capacitation and acrosome reaction of equine sperm cells, at present, is the use of Ca$$^{++}$$ ionophore. Zhang et al. (1990) used 7.14 μM Ca$$^{++}$$ ionophore to treat equine sperm cells for 5 to 10 minutes, and after 5 minutes of sperm pre-incubation a 33% fertilization rate resulted from these in vitro-matured oocytes. Although the initial sperm motility rate was 89%, there was still 75% sperm motility remaining after the Ca$$^{++}$$ ionophore treatment. After 30 minutes of sperm pre-treatment equilibration, Palmer et al., (1991) used 6 μM Ca$$^{++}$$ ionophore to treat equine sperm cells for 10 minutes prior to oocyte exposure. After insemination, there was only an 18% fertilization rate from in vivo-matured oocytes, however, the sperm motility after the Ca$$^{++}$$ ionophore treatment was not reported.

It has been established that some sperm cells will undergo a spontaneous acrosome reaction when cultured in ionophore-free medium alone. In a study reported by Varner et al. (1987), this percentage of acrosome-reacted sperm (not treated) increased significantly over time of incubation. After 6 hours of incubation, progressive sperm motility decreased from 80% to 35%, however, the percentage of acrosome-reacted sperm cells remained only at »11%. When Ca$$^{++}$$ ionophore was added either 3 hours or 6 hours after the start of incubation, the percentage of acrosome reacted-sperm cells increased from »8% to >90%, and was not different between either time interval to treatment. It seems that although pre-equilibration of semen has some
beneficial effect on the percentage of spontaneous acrosome-reacted sperm, the quantity of reacted sperm obtained by this approach does not justify the overall loss in sperm motility. Furthermore, it appears that a higher percentage of acrosome reacted-sperm cells having a greater progressive motility can be obtained without an extended pre-incubation interval. Results from the present study indicate that sperm cells treated with Ca++ ionophore using only a minimum equilibration time are capable of fertilizing equine oocytes. This finding is in agreement with results using Ca++ ionophore on stallion sperm obtained by Zhang et al. (1990) and Palmer et al. (1991).

The media used in different experiments may be partially responsible for the differences reported in post-treatment sperm motility. While treating the sperm cells with Ca++ ionophore in the present study, BSA was not added to the medium to reduce the chance of neutralizing the Ca++ ionophore effect. In this study, neither 1.0 or 3.0 μM concentrations of the ionophore gave any indication of a negative effect on sperm motility. However, when sperm cells were exposed to Ca++ ionophore at a 5 μM concentration for 6 minutes, it reduced sperm motility and at a 7 μM concentration for the same interval the sperm cells were rendered immobile.

There was not a significant difference in the acrosome reaction rates between sperm cell populations treated with either 1.0 or 3.0 μM concentration of the Ca++ ionophore. However, there was a significant difference in the fertilization rate and subsequent embryo development between the 1.0 and 3.0 μM ionophore treatments. More zona-drilled oocytes were fertilized and reached morula stage in the treatment of oocytes fertilized by sperm cells treated with 1.0 μM concentration of Ca++ ionophore than in the treatment of oocytes fertilized by sperm cells treated with the 3.0 μM concentration. This finding may indicate that a high concentration of the Ca++
ionophore may reduce the fertilization capacity of the sperm cells even before this level reduces the sperm motility.

From the present study, it should be noted that a 6-minute exposure of the 0.1 μM concentration of the Ca++ ionophore was unable to prepare the sperm cells to complete the fertilization process, even though the sperm cells had access to the perivitelline space via the zona opening. This finding is in agreement with the results reported by Del Campo et al. (1990), who found that a 0.1 μM concentration of Ca++ ionophore was unable to increase the in vitro fertilization rate in equine oocytes. The high concentration of Ca++ ionophore may actually stimulate the sperm cells to reach a state of supercapacitation (Zhang et al., 1990), which allows sperm to penetrate zona-intact equine oocytes, but may damage the fertilization capacity of the equine sperm cells. In contrast, a low concentration of Ca++ ionophore may be unable to enhance capacitation and the subsequent acrosome reaction that are necessary for fertilization (Bedford, 1977). More than likely, however, a threshold level of Ca++ ionophore is needed for equine sperm cells to obtain the optimal fertilizing capacity. Thus, the goal is to find the lowest Ca++ ionophore concentration for optimal acrosome reaction, while maintaining an optimal sperm motility.

Although parthenogenetic activation of manipulated oocytes was a valid concern in this study, embryos derived from the zona-drilled aspirated and abattoir-derived oocytes gave no indication of parthenogenetic activity. No zona-drilled oocytes in the aspirated oocyte control group (Treatment I), that were exposed to untreated sperm cells, cleaved during the fertilization interval or the subsequent 196-hour in vitro culture interval. Also during the supplemental in vitro fertilization trial, no intact oocytes that were fertilized with treated sperm cells or zona-drilled oocytes exposed to Ca++ ionophore alone cleaved during the corresponding 196-hour in vitro culture interval.
Only a few oocytes (»10%) formed cell-like structures during the in vitro maturation period, before any fertilization treatment was attempted. However, after staining these oocytes no nuclei were found, indicating that this was cytoplasmic fragmentation and not parthenogenetic activity. It should be noted that these oocytes were usually obtained after 6-hour of transport enroute to the laboratory and thus, had a longer interval than usual prior to the start of the in vitro maturation procedure.

In contrast, the blastomeres of all the embryos obtained from the first treatment group in this supplementary in vitro fertilization trial were found to contain stained cell nuclei. The development of the IVF-derived embryos obtained from the aspirated oocytes, as well as those derived from abattoir ovaries, were in close synchrony with those of in vivo produced embryos (Betteridge et al., 1982; Bezard et al., 1989). There was no indication that the IVF-derived embryos in this study displayed parthenogenetic activity or developed abnormally during in vitro culture. Using the same zona-drilling IVF procedure, Meintjes et al. (1995b) obtained up to a 61% cleavage rate, and then subsequent embryo development to the blastocyst stage, when inseminating oocytes that were harvested from culled, free-ranging zebra.

Zona-drilling does tend to increase the polyspermy rate in human IVF programs, and in some cases even to the extent of 25% (Cohen et al., 1991). Although oocytes would have to have been sacrificed in the present study to determine the percentage of polyspermic fertilizations by staining, the possibility does exist that some of the embryos obtained might indeed have been polyspermic. In a recent report, Choi et al. (1994) used partial zona removal (PZR) and partial zona dissection (PZD) in an attempt to fertilize equine oocytes in vitro. Sperm penetration rates evaluated by staining were 52% for PZR oocytes and 12% for PZD oocytes. Although polyspermy was evident in some of these oocytes with a large rent in the zona pellucida, monospermic
penetration rates were found to be between 57% and 58%. Further study is needed to evaluate the role of polyspermy in the development of equine IVF embryos derived from zona-drilled oocytes.

Equine research groups have recently been encouraged with the successful birth of an IVF-derived foal in France (Palmer et al., 1991). To obtain this pregnancy, eight fertilized embryos were transferred to the ampulla of eight recipients, resulting in one pregnancy. It may be beneficial to culture the IVF-derived equine embryos to the late morula or early blastocyst stage when the embryos can be nonsurgically transferred into the uterus of a recipient mare. Previously, equine oviduct cell monolayers have been developed and found to be successful for culturing early stage equine embryos in vitro (Battut et al., 1989; Ball & Miller, 1992). Equine oviductal explants and trophoblastic vesicles have also been found to enhance in vitro development of day-2 equine embryos (Ball et al., 1991). Furthermore, bovine fetal uterine fibroblast monolayers (Wiemer et al., 1989) and a bovine granulosa cell co-culture system (Rodriguez et al., 1991) have also been shown to be beneficial for the in vitro culture of pre- and post-compacted equine embryos. From our previous experience, equine uterine and oviduct cell populations for monolayer culture systems have been more difficult to maintain than those of other farm animal species. Thus, a bovine oviduct cell monolayer and mouse embryo co-culture system was used to incubate the equine IVF-derived embryos in this study. A similar culture system using bovine granulosa cells and 8-cell mouse embryos has been shown to be beneficial to the development of IVF-derived bovine embryos in vitro (Li et al., 1993).

In this study, oocyte cleavage rate following sperm exposure was 79% in the 1.0 \( \mu \text{M} \) concentration \( \text{Ca}^{++} \) ionophore treatment. In addition, 36% of the oocytes exposed to sperm cells and 45.5% of the oocytes that cleaved in this treatment group developed
to morula and blastocyst stages. Although the *in vitro* development of equine IVF-derived embryos is less than optimal when compared with those of other domestic animals (Trounson *et al.*, 1994), the results from the present IVF experiment were encouraging. Additional studies are needed to verify these findings and to produce viable offspring from IVF-derived equine embryos using this methodology.
CHAPTER V

IN VITRO FERTILIZATION OF IN VITRO MATURERED OOCYTES RECOVERED FROM FREE RANGING BURCHELL'S (Equus burchelli) AND HARTMANN'S (Equus zebra hartmannae) ZEBRA

Introduction

Many wild equids are in danger of becoming extinct due to a continued decrease in natural habitat, restricted distribution of free-ranging animal island populations (Penzhorn, 1985; Penzhorn & van der Merwe, 1988; Westlin-van Aarde et al., 1988) or interbreeding with domestic equine species (Klingel, 1975, Smithers, 1986). As in cattle (Parrish et al., 1986) and other domestic animals (Nagai, 1994; Trounson et al., 1994; Keskintepe, 1994), successful in vitro fertilization techniques have the potential to increase the number of potential offspring in endangered equids, especially if combined with interspecies embryo transfer (Bennett & Foster, 1985; Kydd et al., 1985; Summers et al., 1987). Successful in vitro fertilization and embryo production of exotic species could: 1) enhance the needed importation of valuable genetic material from free-ranging populations (Loskutoff et al., 1995); 2) allow genetically superior females to become pregnant and still donate oocytes for assisted reproduction; and 3) recapture the genetic material of valuable physically impaired or clinically infertile animals.

Although several laboratories have had some success with equine in vitro fertilization (Zhang et al., 1990; Del Campo et al., 1990), in vitro fertilization rates and subsequent embryo development have been less than anticipated, with only one foal reported being born after oviductal transfer of very early stage IVF-derived equine embryos (Palmer et al., 1991). Recently, higher fertilization rates have been obtained consistently by applying a simple prefertilization zona drilling procedure, with a high...
percentage of IVF-derived embryos developing to morula and blastocyst stages \emph{in vitro} (Li \emph{et al.}, 1995; Meintjes \emph{et al.}, 1995a).

However, the development of a consistent and practical \emph{in vitro} fertilization procedure for the horse has been limited, in part, by the lack of techniques for successful and repeatable \emph{in vivo} oocyte recovery. A safe, repeatable, noninvasive, transvaginal ultrasound-guided oocyte recovery procedure has been applied effectively to harvest oocytes for \emph{in vitro} fertilization from both cycling (Cook \emph{et al.}, 1992, 1993b) and pregnant (Meintjes \emph{et al.}, 1994; 1995b) horse mares. If oocytes could be similarly harvested from wild equids and successfully fertilized \emph{in vitro}, \emph{in vitro} fertilization procedures could have an impact on the conservation of endangered equine species. Therefore, the present study was undertaken to: 1) test the applicability of \emph{in vitro} maturation and \emph{in vitro} fertilization procedures recently developed for horse oocytes on free-ranging zebra in South-Africa; and 2) to evaluate the use of the transvaginal ultrasound-guided oocyte recovery procedure, originally developed for horse and pony mares, on captive cycling and pregnant zebra mares.

\textbf{Materials and Methods}

\textbf{Experimental design}

Oocytes obtained from the ovaries of six Burchell’s and six Hartmann’s zebra mares were used for the \emph{in vitro} maturation and \emph{in vitro} fertilization study. All oocytes obtained with an intact oolemma and at least one cumulus cell layer were entered into the \emph{in vitro} fertilization experiment. Oocytes from Burchell’s as well as from Hartmann’s zebra mares were fertilized with epididymal sperm cells obtained from two culled Burchell’s zebra stallions. Two treatments were applied to the sperm utilized to fertilize the oocytes. Half of the oocytes were fertilized with Ca\textsuperscript{++} ionophore treated sperm cells and half with sperm cells not exposed to the ionophore. Since more oocytes
were obtained from Hartmann's zebra ovaries, an additional co-culture (bovine oviductal cells vs. zebra cumulus cells) and no zona drilling control treatment were assigned to oocytes from this species.

Donor animals

During a 48-hour time period in May, freeranging zebra were culled from a helicopter on the Mdala nature reserve in the Northern Transvaal of South Africa as part of an annual pasture managing program. Oocytes obtained from these animals were used for the *in vitro* maturation and *in vitro* fertilization study. The ages of the culled animals were estimated to be between 3.5 and 15 years, judged by incisor wear as previously described for Cape mountain zebra by Penzhorn, (1987). Furthermore, five of the six Burchell's zebras were found to be 6 to 8 months pregnant at the time of the cull as assessed by the crown-rump measurements of the fetuses (330 to 550 mm). One of the six Hartmann's zebra mares was ≈4 months pregnant (crown-rump measurement of 210 mm) (Westlin-van Aarde *et al*., 1988). The pregnant Hartmann's zebra mare and two of the pregnant Burchell’s zebra mares were also lactating. When a set of ovaries had a follicle >20 mm in diameter with most of the other follicles much smaller, these animals were considered to be in pro-estrus/estrus (Westlin-van Aarde *et al*., 1988). Three of the Hartmann’s zebra mares and one Burchell’s zebra mare were in pro-estrus/estrus. Two Hartmann’s zebra mares had one well developed corpora lutea on one of the ovaries with several (13 and 15 follicles each) similar size (6 to 17 mm in diameter) small and medium follicles and were considered to be cycling but in diestrus.

Three captive Burchell’s zebra mares from the Pretoria Zoo in South Africa and two semi-captive Burchell’s zebra mares from the Rondebuilt Romevite research farm just outside Pretoria (grazing on ≈100 ha of natural pasture and supplemented with hay
and concentrates) were used as donors for oocytes recovered by transvaginal ultrasound-guided aspiration. These donor mares were of known age (3.5 to 12 years) and chosen for aspiration at the time of immobilization.

**Postmortal oocyte collection**

Ovaries were aseptically removed from the zebra carcasses at a central processing facility on the reserve where they were culled within 15 to 110 minutes of culling. The ovaries were transported to the *in vitro* fertilization laboratory in warm (37.5°C) holding medium (PBS supplemented with 1% calf serum and antibiotics). At the laboratory, all ovaries were trimmed from excess peri-ovarian tissue, weighed and ultrasonically scanned for follicles with a diameter ≥4 mm by placing them in a container filled with sterile holding medium. A 5 MHz linear transducer was used to scan the ovaries in the fluid filled container. Ovaries were then individually sectioned in 100 mm² square plastic search dishes (Beckton-Dickenson Labware, Lincoln Park, NJ) into ≈5 mm slices and the inside lining of all visible follicles were carefully scraped with a scalpel blade. The sliced ovary was placed in a 200 ml plastic container that contained 50 ml of holding medium. A lid was placed on the container, the medium agitated and the ovary washed to liberate any remaining oocytes. The contents of the search dish and the 200 ml container were pooled and filtered through a standard bovine embryo filter (Pets Inc., Tyler, TX) with a 75 μm mesh screen. The filter was rinsed into a clean search dish and the medium scanned for oocytes under a Nikon stereoscope (10 to 40X magnification). Using inverted light microscopy, the isolated oocytes were evaluated at 40, 100 and 400X magnification and assigned to five classes according to cumulus morphology (≥4 layers of compact cumulus cells, <4 layers of compact cumulus cells, corona radiata alone, denuded and expanded pyknotic cumulus) and to three classes according to ooplasm consistency (granular and evenly distributed,
irregularly clumped and mishappen) as previously outlined for horse oocytes by Hinrichs, (1991). Furthermore, a subjective judgement was made of the quality of individual oocytes based on the general appearance of the oocyte, zona pellucida shape and a combination of the cytoplasmic and cumulus classes assigned to the oocyte. According to this final evaluation, oocytes were divided into excellent quality, good quality, poor quality and nonviable groups. After evaluation, all oocytes with intact oolemmas and at least one layer of cumulus cells were washed and placed into maturation medium.

In vitro maturation

After oocyte isolation, all oocytes with an intact oolemma were washed and matured in vitro in 50 μl microdrops for 27 to 34 hours at 37.5°C in an atmosphere of 5% CO₂ in air. In vitro maturation medium consisted of TCM-199 with 10% fetal bovine serum (FBS), 2.5 μg of ovine FSH, 2.5 μg of equine LH and 1 μg of estradiol per ml of medium.

Zona drilling

Before in vitro fertilization, oocytes were stripped from most cumulus cells by gentle pipetting in PBS medium containing 0.25% of hyaluronidase and 0.025% of trypsin to allow adequate visualization of the zona pellucida for prefertilization zona drilling. Zona drilling was performed in a 10 μl PBS microdroplet supplemented with 10% FBS covered with warm mineral oil on a warmed sterile glass slide with the use of two Leitz micromanipulators under 400X magnification. A small hole (2-3 μm) was created in the zona by repeated, pulsatile administration of microvolumes of an acidic PBS (pH 2.2) while stabilizing the oocyte with a holding pipette (90 μm outside diameter) as previously described for equine oocytes by Li et al. (1994). After successful zona drilling, oocytes were removed from the manipulation droplet, washed
and transferred to an equilibrated 50 µl fertilization droplet consisting of Brackett-Oliphant medium with 3% BSA and free floating bovine oviduct cells. After zona drilling, all remaining oocytes with an intact oolemma were pooled within each species and randomly assigned to the different in vitro fertilization treatment groups.

**Sperm cell preparation and in vitro fertilization**

Epididymal sperm cells, collected from the epididymi of two culled Burchell’s zebra stallions after 6 hours of direct refrigeration of the whole testes at 4°C (Bezuidenhout *et al.*, 1995) were used to inseminate the oocytes from both Burchell’s and Hartmann’s zebra. The caput epididymi were dissected free from connective tissue and blood vessels on a 4°C ice pad. Four 3 mm-long sections of the ductus epididymis were dissected from the caput epididymi of one testis each of the two Burchell’s stallions using sterile instruments. The contents of each piece of dissected epididymal duct was expressed in a common 25 mm² plastic petri dish containing 1 ml of Hams F-10 medium at 4°C. The pooled epididymal sperm cells were gradually warmed up from the handling temperature of 4°C by incubation for 20 minutes at 39°C in an atmosphere of 5% CO₂ in air. The sperm sample was then divided in two petri dishes and the sperm number simultaneously adjusted to 9 x 10⁶ cells per ml by dilution with Ham’s F-10 medium. One sperm sample was exposed to a 1 µM concentration of Ca²⁺ ionophore A23187 for 6 minutes before being used for in vitro fertilization and the other served as a control (no Ca²⁺ ionophore exposure). A 50 µl Ham’s F-10 sperm cell suspension (9 x 10⁶ cells per ml) from the appropriate sperm treatment was added to each fertilization droplet so that the final fertilization droplet consisted of 100 µl of a 50:50 mixture of Ham’s F-10 and B-O medium with 1.5% BSA. Oocytes, sperm cells and bovine oviduct cells were co-incubated for 6 to 13 hours at 37.5°C.
Embryo co-culture and staining

After IVF, the sperm-exposed oocytes were washed and incubated on either a primary zebra cumulus cell or bovine oviduct cell monolayer in 50 μl microdrops of 10% FBS supplemented Ham's F-10 medium at 37.5°C in an atmosphere of 5% CO₂ in air. The cleavage rate was assessed 72 hours after *in vitro* fertilization, and all oocytes/embryos were stained after 8 days of co-culture. Embryos with visible blastomeres were placed in a hypotonic 1% sodium citrate solution for 3 to 5 minutes and then carefully placed on a clean glass slide in a minimum amount of this solution. A drop of fixative (methanol:acetic acid, 1:1) was dropped onto the embryo and then allowed to air dry. Fixed embryos were placed in a clearing solution (methanol:acetic acid, 3:1) for 12 hours and then stained with 5% giemsa for 15 minutes. Oocytes/embryos where no clear blastomeres could be visualized under light microscopy were fixed under a coverslip in a methanol:acetic acid (3:1) solution for 48 hours and then stained with 2% aceto-orcein. Cell nuclei were counted and fertilization status evaluated where possible under an inverted light microscope at 400X magnification.

Immobilization

For transvaginal oocyte collection procedures, Burchell's zebra mares were immobilized with an intramuscular injection of a combination of 4 to 8 mg of etorphine (M99®, Krüger-Med, Motortown, South Africa) and 6 to 8 mg of detomidine (Domosedan®, Ciba-Geigy, Isando, South Africa). The drug combination was loaded in a 3 ml plastic dart (pressurized by atmospheric air) and delivered to the targeted animal with a Telinject® (Telinject S.A., Randburg, South Africa) blowgun. The mares became recumbent 7 to 13 minutes after successful drug delivery. The immobilized oocyte donor mares were blindfolded, lifted onto a cushioned transport trolley and
moved to a darkened enclosure where the aspiration procedure was performed. The rectum of the mare was manually evacuated and the perineal area and tail base aseptically prepared prior to the aspiration procedure. Transvaginal oocyte aspiration was performed with the whole animal elevated and in sternal recumbency. After follicular aspiration and oocyte recovery, general anaesthesia was reversed with 5 mg of diprenorphine (M5050®, Krüger-Med, Motortown, South Africa) intravenously.

Transvaginal ultrasound-guided oocyte aspiration and oocyte isolation

Oocyte donor mares were chosen for aspiration at the time of immobilization if at least one follicle ≥ 10 mm in diameter was detected on either ovary by transvaginal ultrasound. Repeated aspirations were performed whenever possible ≈ 14 days apart without consideration of reproductive state. A modified 5 MHz sector transducer fitted on a 500-mm long extended handle and attached to an Aloka 500-V® realtime ultrasound unit (Corometrics Medical Systems, Wallingford, CT) was used to transvaginally harvest the oocytes as previously described for cycling and pregnant mares by Meintjes et al. (1995b). The transducer was placed in the anterior vagina and the ovary manipulated per rectum so that the puncture line, displayed on the monitor screen, transected the hypoechoic follicle through its longest axis. Follicles were punctured by a second technician with a 12-gauge, 550-mm single lumen needle through a needle guide that was attached to the handle at a fixed angle. When the follicular fluid was aspirated, a third technician introduced warm (35 to 37°C) flushing medium into the follicle with a hand pump after which it was removed by suction (≈ 90 mm Hg). Follicles were flushed in this way three to 10 times. The oocyte flushing medium consisted of PBS supplemented with 1% calf serum, 2 IU of heparin, 100 μg of streptomycin and 100 IU of penicillin-G per ml of medium. Recovered fluid was trapped in a 500 ml collection vessel and kept at 37.5°C until transport to the in vitro
fertilization laboratory. The oocytes were isolated from the 500 ml collection vessel by filtering the collected follicular fluid/flushing medium through a standard bovine embryo filter as described for oocytes obtained from culled zebra mares. Also, the filter was rinsed, the oocytes identified and then classified into four quality groups according to general appearance, cumulus morphology and ooplasm consistency as for culled animals.

Statistical analyses

The percentage of oocytes assigned to the different cumulus morphology classes, ooplasm consistency classes and quality grades were compared between zebra species by using the Chi-Square Analysis. The Chi-Square Analysis was also used to compare the cleavage rates and the percentage of oocytes that developed to the four-cell stage in the eight treatment groups of the epididymal in vitro fertilization experiment. Transvaginal ultrasound-guided oocyte aspiration recovery rates and the percentage of viable oocytes recovered from each mare were similarly tested for significance. Differences in ovarian weights between the two zebra species were analyzed with Student's t-test (Montgomery, 1991). The number of follicles detected by ultrasound on each ovary and the number of oocytes recovered per ovary for Burchell's and Hartmann's zebra mares were similarly compared using the t-test.

Results

Postmortem oocyte recovery

The mean ovarian weight (±SE) of ovaries collected from Hartmann's zebra mares was 31.11 ±2.23 g and different (P < .001) from that of Burchell's zebra mares with a mean weight of 15.28 ±1.94 g. One to three corpora lutea were found on each set of ovaries (pregnant and nonpregnant) except for one nonpregnant Hartmann's zebra mare where no luteal structures could be identified. The number of follicles per ovary
(±SE) that could be detected by ultrasound was 6.75±.39 (4 to 27 mm in diameter) for Hartmann's zebra mares. This tended to be higher (P < .08) than the 4.75±1.21 (4 to 20 mm in diameter) follicles detected per ovary for Burchell's zebra mares. After the slicing procedure, 6.92±1.32 oocytes per ovary were recovered from Hartmann's zebra mares and 5.08±.95 oocytes per ovary from the Burchell's zebra mares.

The resultant classification of recovered oocytes from each species according to cumulus investment and ooplasm morphology is summarized in Table 5.1. More (p< .05) oocytes recovered from the ovaries of Hartmann's zebra were surrounded by four or more cumulus cell layers than oocytes recovered from the ovaries of Burchell's zebra. Conversely, more oocytes from Hartmann's zebra were found to have irregularly clumped ooplasm than those of Burchell's zebra.

Of the 61 oocytes recovered from the six Burchell's zebra mares, 34% were considered to be of excellent quality and similarly 37% of the 83 oocytes recovered from the six Hartmann's zebra mares were of excellent quality (Table 5.2). Poor quality and nonviable oocytes accounted for 35% of oocytes recovered from Burchell's zebra mares and for 23% of oocytes from Hartmann's zebra mares. No significant differences in oocyte quality were found between the two species.

**In vitro fertilization**

After *in vitro* insemination with ionophore-treated epididymal sperm cells, 38% of sperm exposed oocytes cleaved and after insemination with the control epididymal sperm, 44% of the oocytes cleaved. Cross fertilization of Hartmann's zebra oocytes with Burchell's zebra epididymal sperm resulted in a 38% cleavage rate and 45% of Burchell's zebra oocytes cleaved when similarly fertilized with the Burchell's sperm cells. More (P < .05) zona-drilled oocytes from Hartmann's zebra, fertilized with ionophore-treated sperm cells and cultured on bovine oviductal cells, developed to the
Table 5.1. Classification of oocytes recovered from the ovaries of culled Hartmann's and Burchell's zebra according to cumulus morphology and ooplasm consistency

<table>
<thead>
<tr>
<th>Cumulus morphology</th>
<th>Ooplasm consistency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hartmann's</td>
</tr>
<tr>
<td></td>
<td>≥4 layers</td>
</tr>
<tr>
<td></td>
<td>&lt;4 layers</td>
</tr>
<tr>
<td></td>
<td>Corona only</td>
</tr>
<tr>
<td></td>
<td>Nude</td>
</tr>
<tr>
<td></td>
<td>Exp.†</td>
</tr>
<tr>
<td></td>
<td>Even</td>
</tr>
<tr>
<td></td>
<td>Clumped</td>
</tr>
<tr>
<td></td>
<td>Mishappen</td>
</tr>
<tr>
<td>Hartmann's</td>
<td>41(49%)</td>
</tr>
<tr>
<td></td>
<td>29(35%)</td>
</tr>
<tr>
<td></td>
<td>2(3%)</td>
</tr>
<tr>
<td></td>
<td>5(6%)</td>
</tr>
<tr>
<td></td>
<td>6(7%)</td>
</tr>
<tr>
<td></td>
<td>55(66%)</td>
</tr>
<tr>
<td></td>
<td>24(29%)</td>
</tr>
<tr>
<td></td>
<td>4(5%)</td>
</tr>
</tbody>
</table>

† Expanded pyknotic cumulus investment.

* Percentages within columns with different superscripts are significantly different ($\chi^2$; P < 0.05)
Table 5.2. Quality grades of oocytes recovered from the ovaries of culled Hartmann’s and Burchell’s zebra based on general appearance of the oocytes, zona pellucida shape, cumulus morphology and ooplasm consistency

<table>
<thead>
<tr>
<th></th>
<th>Excellent</th>
<th>Good</th>
<th>Poor</th>
<th>Degenerate</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hartmann’s</td>
<td>31 (37%)</td>
<td>33 (40%)</td>
<td>15 (18%)</td>
<td>4 (5%)</td>
<td>83</td>
</tr>
<tr>
<td>Burchell’s</td>
<td>21 (34%)</td>
<td>19 (31%)</td>
<td>18 (30%)</td>
<td>3 (5%)</td>
<td>61</td>
</tr>
</tbody>
</table>

No significant differences between species detected.
four-cell stage when compared with the nondrilled control oocytes. Also, more
Burchell's zebra oocytes, inseminated with untreated sperm cells, developed to the four-
cell stage if compared with the nondrilled control oocytes (Table 5.3). Furthermore,
more ($P < .05$) of the sperm-exposed oocytes that were cultured on primary bovine
oviduct cell monolayers developed to at least the morula stage (22%), than those
cultured on zebra cumulus cell monolayers (0%).

Overall, a 38% cleavage rate was obtained with 16% of sperm-exposed oocytes
developing to morula or blastocyst stages (Table 5.3). When only prefertilization zona-
drilled oocytes were considered, a cleavage rate of 41% was obtained. Only one of the
10 non-drilled oocytes cleaved and failed to develop to later stages.

**Oocyte aspiration**

Ten transvaginal ultrasound-guided oocyte aspiration procedures were performed
on five captive Burchell's zebra mares (one =45 day lactating pregnant mare) during
a period of 28 days (Table 5.4). Oocyte aspiration procedures were performed one to
three times per animal with an interaspiration interval of 12 to 13 days without any
noticeable adverse effects on the donor mares that could be related to the aspiration
procedure. However, one mare was lost due to adverse reactions to the immobilization
drugs. A total of 33 oocytes were recovered (52% recovery rate) with 6.6 (range 5 to
8) oocytes recovered per female. Recovery rates between individual mares were not
different. A minimum of 1 and a maximum of 5 oocytes were recovered per aspiration
procedure. From the 33 oocytes collected, 36% (12 oocytes) was considered to be of
excellent quality, 34% (11 oocytes) was of good quality, 24% (8 oocytes) was of poor
quality and only 6% (2 oocytes) were classified as degenerate. Quality grades between
mares were not different.
Table 5.3. Results of IVM, IVF and co-culture of zebra oocytes fertilized with epididymal sperm cells

<table>
<thead>
<tr>
<th>Species</th>
<th>Co-culture treatment</th>
<th>Semen treatment</th>
<th>(n)</th>
<th>2-cell</th>
<th>4-cell</th>
<th>8 to 16 cell</th>
<th>MORL</th>
<th>BLST</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>BOC</td>
<td>Ca++</td>
<td>11</td>
<td>6\textsuperscript{bc}</td>
<td>6\textsuperscript{bc}</td>
<td>5</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>H</td>
<td>BOC</td>
<td>No Ca++</td>
<td>11</td>
<td>3\textsuperscript{ae}</td>
<td>2\textsuperscript{ae}</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>H</td>
<td>CUM</td>
<td>Ca++</td>
<td>8</td>
<td>3\textsuperscript{ae}</td>
<td>3\textsuperscript{ae}</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H</td>
<td>CUM</td>
<td>No Ca++</td>
<td>7</td>
<td>2\textsuperscript{ae}</td>
<td>2\textsuperscript{ae}</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H</td>
<td>BOC (No Dril)</td>
<td>Ca++</td>
<td>5</td>
<td>1\textsuperscript{ae}</td>
<td>0\textsuperscript{a}</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H</td>
<td>BOC (No Dril)</td>
<td>No Ca++</td>
<td>5</td>
<td>0\textsuperscript{a}</td>
<td>0\textsuperscript{a}</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>BOC</td>
<td>Ca++</td>
<td>20</td>
<td>6\textsuperscript{ae}</td>
<td>6\textsuperscript{ae}</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>BOC</td>
<td>No Ca++</td>
<td>18</td>
<td>11\textsuperscript{bc}</td>
<td>9\textsuperscript{bc}</td>
<td>6</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

Total | 85 | 32 | 28 | 23 | 13 | 1 |

MORL = morula, BLST = blastocyst, H = Hartmann's zebra, B = Burchell's zebra, BOC = bovine oviduct cells, CUM = zebra cumulus cells, No Dril = no zona pellucida drilling.

\textsuperscript{a,b}Different superscripts within columns indicate significant differences ($\chi^2$; $P<0.05$)
Table 5.4. Results obtained from transvaginal ultrasound-guided oocyte aspiration of captive Burchell's zebra mares

<table>
<thead>
<tr>
<th>Mare number</th>
<th>Number of procedures*</th>
<th>Follicles aspirated</th>
<th>Oocytes recovered</th>
<th>Recovery rate (%)*</th>
<th>Viable oocytes (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>11</td>
<td>8</td>
<td>73%</td>
<td>100%</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>18</td>
<td>7</td>
<td>39%</td>
<td>100%</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>12</td>
<td>8</td>
<td>67%</td>
<td>100%</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>13</td>
<td>5</td>
<td>38%</td>
<td>100%</td>
</tr>
<tr>
<td>5*</td>
<td>1</td>
<td>9</td>
<td>5</td>
<td>56%</td>
<td>60%</td>
</tr>
<tr>
<td>Total =</td>
<td>10</td>
<td>63</td>
<td>33</td>
<td>52%</td>
<td>94%</td>
</tr>
</tbody>
</table>

*90 days pregnant.

*No significant differences in oocyte recovery rates and percentage viable oocytes detected.

*All aspiration procedures were performed during a period of 28 days.
Discussion

The finding in this study that the ovaries of Hartmann's zebra mares were larger than those of Burchell's zebra mares are in agreement with similar studies on culled animals in Southern Africa (Smuts, 1976; Westlin-van Aarde et al., 1988). The larger ovaries of Hartmann's zebra seemed to allow the development of more follicles and subsequently the recovery of a higher number of oocytes at postmortem. More oocytes were recovered after slicing the ovaries than the number of follicles that could be detected by ultrasound. This can be accounted for by follicles smaller than 4 mm in diameter that contributed oocytes, since the resolution of the 5 MHz linear ultrasound probe allowed only the accurate identification of follicles ≥4 mm.

The 49% of oocytes recovered from Hartmann's zebra mares that had a complete (≥4 cumulus cell layers) compact cumulus investment after slicing and scraping of visible follicles was very similar to the 47% of oocytes with complete cumulus that was recovered from slaughterhouse ovaries of domestic horses (Hinrichs & DiGiorgio, 1991). It is not clear if the lower percentage of oocytes (31%) with similar cumulus morphology obtained from Burchell's zebra mares was due to species differences or due to differences in animal physiological state since five of the six Burchell's zebra mares were pregnant and only one of six Hartmann's zebra mares. Fewer oocytes obtained from the ovaries of the two zebra species were surrounded by expanded pyknotic cumuli (7% and 16% for Hartmann's and Burchell's zebra respectively) when compared with oocytes similarly harvested from horse ovaries at slaughter (~40%) (Hinrichs, 1991; Hinrichs & DiGiorgio, 1991). In these studies, oocytes were harvested from horses in the peak of the breeding season, whereas oocytes from the zebra mares in this study were harvested in May just after the peak breeding season (December to January for Burchell's zebra and November to April for
Hartmann's zebra) (Smithers, 1986; Westlin-van Aarde et al., 1988). This may partially explain the fewer mature looking oocytes obtained from the zebra mares than from horses.

More oocytes (29%) from Hartmann's zebra mares had irregular clumped ooplasms than oocytes from Burchell's mares (15%). For both species, this was lower than what could be expected from horse oocytes recovered from slaughterhouse ovaries (35%) (Hinrichs, 1991). There are indications in the horse that a higher proportion of oocytes from viable follicles would have an even granular ooplasm and that oocytes from atretic follicles would be more likely to have an irregular clumped ooplasm (Hinrichs, 1991). Hinrichs (1991) found 58% of follicles on horse ovaries from a slaughterhouse to be atretic. Although at least 50% of follicles from the zebra ovaries were expected to be atretic in this study, 65% and 77% of all the oocytes recovered from the culled Burchell's and Hartmann's zebra mares, respectively were judged to be of good to excellent quality. As shown in the horse, the number of atretic follicles present on the ovary is not a reliable indicator of the number of viable oocytes that can be expected from an ovary, since the frequent recovery of apparently normal oocytes from atretic follicles indicates that morphological follicular atresia may occur before oocyte degeneration begins (Hinrichs, 1991).

The in vitro maturation and prefertilization zona-drilling procedure previously described for horse oocytes in this laboratory (Li et al., 1995) seemed to be equally effective to assist in vitro fertilization in the zebra, as later stage zebra embryos were similarly produced in this study. The 41% cleavage rate obtained from the insemination of all zona-drilled oocytes (including poor quality oocytes) was encouraging when compared with the 46% cleavage rate of oocytes that were similarly zona drilled after transvaginal recovery from pregnant horse mares (Meintjes et al., 1995b). Only one
of the oocytes that were not zona-drilled cleaved and it failed to develop further than the two-cell stage. This is in agreement with other *in vitro* fertilization studies in the horse where only limited fertilization and development to later stages were obtained when intact oocytes were inseminated with Ca$$^{++}$$ ionophore treated sperm. Therefore, indications are that zebra oocytes behave similarly than horse oocytes when exposed to this *in vitro* fertilization procedure. All morulae and blastocysts were obtained from the bovine oviduct cell co-cultures, which indicate that in this experiment, bovine oviductal cells were superior to zebra cumulus cells for co-culturing zona-drilled *in vitro* fertilized zebra oocytes.

Cleavage rates for oocytes inseminated with Ca$$^{++}$$ ionophore treated epididymal sperm and for oocytes inseminated with epididymal sperm not treated with the ionophore were not different. Results obtained from this study therefore suggest that Ca$$^{++}$$ ionophore treatment of sperm cells may not be necessary if epididymal sperm cells are used for *in vitro* fertilization in the zebra. This is in agreement with similar *in vitro* fertilization studies in other wild ungulate species where epididymal sperm was used successfully for *in vitro* fertilization with minimal capacitation induction treatment (Loskutoff *et al*., 1995). However, when ejaculated semen is used for *in vitro* fertilization in the horse, Ca$$^{++}$$ ionophore pretreatment of sperm cells seems to be a prerequisite for successful *in vitro* fertilization (Zhang *et al*., 1990; Del Campo *et al*., 1990, Palmer *et al*., 1991).

It is known that Burchell’s zebra mate freely with donkeys (Smithers, 1986) and that most zebra species can interbreed in captivity (Klingel, 1975). Free ranging Burchell’s zebra and Hartmann’s zebra populations exhibit similar social organization types comprising of long-lasting non-territorial family groups (Klingel, 1975, Pentzborn, 1988, Smithers, 1986). The natural distribution of these two species only
overlaps in a small area in northern Namibia. The growing game farming industry in Southern Africa and the increased value of the Hartmann’s zebra (because of its restricted availability and confined distribution in Namibia) have lead to the introduction of Hartmann’s zebra to traditional Burchell’s zebra territory in South Africa. This poses a concern to conservation agencies as an increased possibility of hybridization now exists. Since the purity of Hartmann’s zebra populations residing on the same reserves as Burchell’s zebra are questioned, culling rather than capturing and relocation of Hartmann’s zebra in these areas are currently advocated. The six Hartmann’s zebra mares obtained for this project were part of such a culling operation. Although the in vitro fertilization results suggest that there were no differences in the in vitro fertilization rates between Hartmann’s zebra and Burchell’s zebra (38% vs. 45%) when inseminated with Burchell’s zebra sperm cells, the viability of these in vitro fertilized embryos were not confirmed by embryo transfer. Furthermore, karyotyping of skin and blood samples from all culled mares, stallions and fetuses confirmed that all animals on this project were genetically pure (32 chromosomes for Hartmann’s zebra and 44 chromosomes for Burchell’s zebra). Thus, although these two species shared the same reserve for several years, no evidence of interbreeding could be found.

Cleavage of inseminated oocytes was only assessed 72 hours after fertilization and oocyte/embryo staining was carried out at the end of the 8 day co-culture period to allow optimum conditions for embryo development to later morula and blastocyst stages. Since most oocytes were zona drilled and many uncleaved oocytes displayed marked degeneration after 8 days in co-culture, a large proportion of the uncleaved oocytes broke and were lost during the fixing and staining procedure or displaced marked fragmentation when evaluated. Therefore, an accurate assessment of in vitro maturation, in vitro fertilization and polyspermy rates could not be made for the
uncleaved oocytes. However, some oocytes still displayed metaphase plates and evidence of polyspermy was seen in one zona-drilled oocyte (five pronuclei).

For *in vitro* fertilization to be useful as a tool for the conservation of endangered equine species, a safe noninvasive method must be available to obtain oocytes from valuable live animals. With the encouraging *in vitro* fertilization results of zebra oocytes recovered from culled animals in this study, it was considered important to test an *in vivo* transvaginal ultrasound-guided oocyte retrieval procedure on available captive Burchell's zebra mares. The procedure tested was similar to the procedure used to recover oocytes from cycling (Meintjes *et al.*, 1995b) and pregnant horse mares (Meintjes *et al.*, 1994) by this research group. The overall oocyte recovery rate of 52% from 10 aspiration procedures on the five zebra mares compared well with the recovery rates reported for preovulatory follicles of cycling (51%) (Cook *et al.*, 1993b) or pregnant horse mares (59%) (Meintjes *et al.*, 1994) when flushing the follicles with a similar 12 gauge single lumen needle. As for oocytes harvested from the ovaries of culled animals, the high percentage (70%) of excellent and good quality oocytes obtained from the live animals was surprising. The aspiration procedure proved to be equally reliable, safe and repeatable as in horse mares, since individual zebra mares were aspirated up to three times during a 28 day interval and the pregnant mare that were aspirated maintained her pregnancy. However, the mare that was lost during immobilization proved that nondomestic equids are much more susceptible to stress than the domestic horse and that repeated immobilization and handling of these animals poses a greater threat to the animal's well being than the oocyte aspiration procedure itself.

Results from this study indicate that the oocytes of Burchell's and Hartmann's zebra mares responded favorably when exposed to *in vitro* maturation and *in vitro* fertilization procedures recently developed for horse oocytes in this laboratory.
Furthermore, the transvaginal ultrasound-guided oocyte aspiration procedure, successfully used in horse and pony mares, was equally effective, safe and repeatable in pregnant as well as in nonpregnant zebra mares. This approach may be an attractive alternative to produce gametes for assisted reproduction in endangered equine species.
SUMMARY AND CONCLUSIONS

It has been well documented over the years that considerable follicular activity occurs during early pregnancy in the mare. In large, the follicular activity of pregnancy has been related to the presence of eCG in the peripheral circulation. However, the presence of marked follicular activity before the first appearance of eCG in the peripheral circulation (prior to day 35 of pregnancy), the presence of follicular activity in interspecies equid pregnancies where little or no eCG is produced and the identification of rhythmical surges of FSH during pregnancy have questioned the role of eCG as the principal agent responsible for the follicular development observed during early pregnancy. The involvement of pituitary gonadotropins in follicular development patterns of pregnancy in the mare has been suggested as early as 1968. Detailed studies on the associations between FSH surges and follicular development patterns up to day 50 of the equine pregnancy were only made possible recently, when ultrasound scanners became available to the animal research community. However, multiple research studies concluded that peak follicular activity in the mare occurred only after day 50 of pregnancy.

One of the primary objectives of the present study was to determine the feasibility of using pregnant mares as oocyte donors for assisted reproductive technologies as one alternative to the current ineffectiveness of superovulation in the mare or the reduced success with conventional embryo transfer procedures in older or subfertile mares. If in vitro fertilization and GIFT procedures could be performed successfully on oocytes obtained from pregnant mares, supplementary offspring could potentially be produced from valuable pregnant mares without jeopardizing an ongoing pregnancy. Furthermore, this approach of collecting viable oocytes for in vitro or surrogate in vivo fertilization may provide an opportunity to recapture the genetic
potential of prized infertile mares. Successful in vitro fertilization and embryo production in exotic equids could also enhance the needed importation of valuable genetic material from free-ranging populations, similarly allow genetic superior females to become pregnant and still donate oocytes for assisted reproduction, and enable the production of offspring from valuable physically impaired or clinically infertile animals.

The effective harvesting of multiple oocytes from a valuable pregnant donor mare will require multiple oocyte collection procedures during an optimal time period without endangering the established pregnancy. Therefore, the effect of repeated follicular aspiration procedures on the follicular dynamics of early pregnancy, the associated endocrinological changes and the viability of the pregnancy were evaluated during days 21 to 150 of gestation in this study. Also, the oocytes collected from the pregnant donor mares were subjected to a novel in vitro fertilization procedure for the equine species to confirm their viability and developmental competency. The transvaginal ultrasound-guided oocyte collection procedure developed for pregnant horse mares and the successful equine assisted in vitro fertilization approach described in this study, were also applied to free-ranging and semi-captive Burchell's zebra mares.

Follicular waves were identified up to day 150 of horse and mule pregnancies. Although the follicular wave size and other follicular wave characteristics over the first 50 days of pregnancy were similar to that reported in previous studies, it was clear that the ongoing pregnancy had a pronounced modulating effect on follicular development. Furthermore, the repeated aspiration of follicles (up to 11 times) also had marked influences on the follicular wave parameters and circulating hormonal profiles of pregnancy. Progesterone is an example of a hormonal profile that has been negatively affected by the follicular aspiration procedures. As far as could be detected by
ultrasonic monitoring and circulating hormonal concentrations, the ongoing pregnancy was not endangered by the aspiration procedures despite the noted changes in follicular development and circulating endocrinological profiles.

The most striking difference in follicular development patterns was not seen in the follicular aspiration treatments in this study, but between mule and horse pregnancies. Mares carrying horse pregnancies had a peak of follicular activity closely associated with that of circulating horse eCG concentrations. After the disappearance of horse eCG from the peripheral circulation of mares carrying horse conceptuses, the follicular activity on the ovaries was reduced to very low levels. In contrast, mares carrying mule conceptuses did not have a peak of follicular activity associated with peak circulating concentrations of mule eCG. However, after the disappearance of mule eCG from the circulation, mule pregnancies maintained a similar level of follicular activity as before the appearance or during the presence of mule eCG in the circulation.

Some of the reasons proposed for the phenomenon of reduced follicular activity in horse pregnancies in the post-eCG period when compared with the peak-eCG or pre-eCG periods or with the post eCG-period of mule pregnancies include: 1) desensitization of the ovary by the extended exposure to high concentrations of horse eCG, thus, that the ovaries are less sensitive to follicular growth induction by equivalent levels of FSH (compared with concentrations before eCG appearance) after disappearance of eCG from the circulation; 2) inhibition of circulating concentrations of FSH by inhibitory factors, such as estradiol and inhibin in the follicular fluid of large follicles during the period of peak follicular activity (temporally related to peak circulating concentrations of eCG, days 45 to 84) in mares carrying horse pregnancies; and 3) a direct inhibitory effect of the high concentrations of eCG on the circulating concentrations of FSH.
If pregnant mares are to be used as oocyte donors for assisted reproduction techniques, it will be important to know which type of pregnancy (horse vs. mule) will yield the greatest number of viable oocytes. Slightly more oocytes were collected from mares carrying mule fetuses when compared with mares pregnant with horse fetuses (21 and 17 oocytes per mare, respectively) over the 130 day experimental period in this study. For mares carrying mule pregnancies, 46% of the total number of oocytes were collected before day 85 and 54% after day 85 of pregnancy. In contrast, 80% of the total number of oocytes were collected before day 85 of pregnancy in mares carrying normal intraspecies horse pregnancies and only 20% after day 85. Therefore, the presence of the follicular activity peak associated with peak circulating horse eCG concentrations in mares carrying horse pregnancies made it possible to collect 80% of the oocytes over a shorter time period. A greater number of oocytes were recovered per aspiration procedure during this time period and fewer potentially risky collection procedures were performed for 80% of the oocytes when compared with mares carrying mule pregnancies. It is concluded that mares pregnant with normal horse pregnancies should be the most desirable pregnant oocyte donor, because more oocytes can be collected over a shorter time period with reduced handling and risk to the donor mare.

The results from the IVF of the oocytes recovered from early pregnant donors indicate that these oocytes were viable, and had the potential to develop to the morula and blastocyst stages in vitro. To our knowledge, this is the first report of successful in vitro fertilization of in vitro matured equine oocytes collected from pregnant donors. When in vitro fertilization and cleavage rates are used as indicators of oocyte viability, indications are that ≥34% of oocyte harvested from early pregnant mares are viable.

Results from this study indicate that the oocytes of Burchell's and Hartmann's zebra mares responded favorably when exposed to in vitro maturation and in vitro
fertilization procedures developed for horse oocytes. Furthermore, the transvaginal ultrasound-guided oocyte aspiration procedure, successfully used in horse and pony mares, was equally effective, safe and repeatable in pregnant as well as in nonpregnant zebra mares.

Since no pregnancies were yet obtained from zona-drilled *in vitro* fertilized oocytes, the possibility of parthenogenic and/or polyspermic embryos was a valid concern in this study. To help solve some of these questions, normospermic and polyspermic embryos were recently produced by intracytoplasmic (ICSI) and subzonal sperm injection of oocytes similarly obtained from pregnant mares (Meintjes, 1995). Appropriate parthenogenic control groups were included in the experiment. None of the polyspermic embryos developed further than the two-cell stage and almost 10% of intracytoplasmic-injected oocytes developed to the morula or blastocyst stages. After the surgical intraoviductal transfer of a two- and four-cell stage ICSI-derived embryo, an ultrasonically detectable embryonic vesicle was detected. However, this pregnancy was lost between days 14 and 16 post-transfer.

The simultaneous occurrence of follicular waves of pregnancy on both ovaries was a common finding in this study and, therefore, the interpretation of the follicular developmental data was sometimes complicated. In future studies of this nature, the use of hemiovariectomized experimental animals should be considered to gain more accurate and explicit data on the follicular dynamics of early pregnancy in the mare. The results of the *in vitro* fertilization of oocytes obtained from pregnant donor mares are encouraging, but the viability of these embryos need to be confirmed by the production of offspring. Although the recent sperm-injection procedures seem to be a step in the right direction, much work still needs to be done for this approach to become commercially acceptable. Equids are a group of animals that are most receptive for
interspecies embryo transfer. Once successful in horses, the transvaginal noninvasive ultrasound-guided oocyte collection procedure combined with interspecies in vitro-fertilized embryo transfer may have far reaching implications in the maintaining of genetic diversity and the conservation of endangered equid species.
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APPENDIX A

FOLLICULAR WAVE PROFILES FOR INDIVIDUAL MARES

Appendix A represents the follicular growth patterns for both ovaries and the combined follicular growth profile for each individual mare between days 21 and 148 of pregnancy. Each block in the diagram represents one day of pregnancy.

= Follicular waves where the largest follicle attains a diameter ≥17 mm in diameter

= Follicular waves where the diameter of the largest follicle is < 17 mm
Animal No. 1

Pregnancy Type: Horse

Aspiration Treatment: Control

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Right Ovary

Left Ovary

Combined

Figure 1. Follicular waves during days 21 to 150 of pregnancy (Horse no. 85).
Animal No. 2  Pregnancy Type: Horse  Aspiration Treatment: Control

20 30 40 50 60 70 80
1234567890123456789012345678901234567890123456789012345678901234

Right Ovary

Left Ovary

Combined

90 100 110 120 130 140
56789012345678901234567890123456789012345678901234567890123456789012345678

Right Ovary

Left Ovary

Combined

Figure 2. Follicular waves during days 21 to 150 of pregnancy (Horse no. 111).
Figure 3. Follicular waves during days 21 to 150 of pregnancy (Horse no. 86).
Animal No. 4  Pregnancy Type: Mule  Aspiration Treatment: Control

20  30  40  50  60  70  80
123456789012345678901234567890123456789012345678901234567890123

Right Ovary

Left Ovary

Combined

90  100  110  120  130  140
567890123456789012345678901234567890123456789012345678901234567890123

Right Ovary

Left Ovary

Combined

Figure 4. Follicular waves during days 21 to 150 of pregnancy (Horse no. 2-8).
**Animal No. 5**  
**Pregnancy Type:** Horse  
**Aspiration Treatment:** Aspirate follicles ≥20 mm

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**Right Ovary**

**Left Ovary**

**Combined**

**Figure 5.** Follicular waves during days 21 to 150 of pregnancy (Horse no. 3721).
Figure 6. Follicular waves during days 21 to 150 of pregnancy (Horse no. 428).
Animal No. 7  Pregnancy Type: Horse  Aspiration Treatment: Aspirate Follicles ≥20 mm

Right Ovary

Left Ovary

Combined

90 100 110 120 130 140

Right Ovary

Left Ovary

Combined

Figure 7. Follicular waves during days 21 to 150 of pregnancy (Horse no. 11).
Animal No. 8  Pregnancy Type: Mule  Aspiration Treatment: Aspirate Follicles ≥20 mm

Right Ovary

Left Ovary

Combined

Figure 8. Follicular waves during days 21 to 150 of pregnancy (Horse no. 456).
Animal No. 9  

Pregnancy Type: Mule  

Aspiration Treatment: Aspirate Follicles ≥20 mm

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Right Ovary

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Combined

90 100 110 120 130 140

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Right Ovary

Left Ovary

Combined

Figure 9. Follicular waves during days 21 to 150 of pregnancy (Horse no. 66).
Animal No. 10: **Pregnancy Type:** Mule  
**Aspiration Treatment:** Aspirate Follicles ≥20 mm

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Figure 10. Follicular waves during days 21 to 150 of pregnancy (Horse no. 48).
Figure 11. Follicular waves during days 21 to 150 of pregnancy (Horse no. 409).
Figure 12. Follicular waves during days 21 to 150 of pregnancy (Horse no. 72).
Figure 13. Follicular waves during days 21 to 150 of pregnancy (Horse no. 2).
Animal No. 14  Pregnancy Type: Horse  Aspiration Treatment: Aspirate all Follicles

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Right Ovary

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Right Ovary

Left Ovary

Combined

Figure 14. Follicular waves during days 21 to 150 of pregnancy (Horse no. 64).
Animal No. 15  Pregnancy type: Horse  Aspiration Treatment: Aspirate all Follicles

Right Ovary

Left Ovary

Combined

90 100 110 120 130 140

Right Ovary

Left Ovary

Combined

Figure 15. Follicular waves during days 21 to 150 of pregnancy (Horse no. 82).
Animal No. 16
Pregnancy Type: Mule
Aspiration Treatment: Aspirate all Follicles

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Right Ovary

Left Ovary

Combined

Figure 16. Follicular waves during days 21 to 150 of pregnancy (Horse no. 95).
Animal No. 17  

Pregnancy Type: Mule  

Aspiration Treatment: Aspirate all Follicles

![Diagram of follicular waves during days 21 to 150 of pregnancy (Horse no. 97).]

Figure 17. Follicular waves during days 21 to 150 of pregnancy (Horse no. 97).
Figure 18. Follicular waves during days 21 to 150 of pregnancy (Horse no. 445).
APPENDIX B

FREQUENCY DISTRIBUTION GRAPHS FOR
FOLLICULAR WAVE PARAMETERS

Appendix B represents frequency distribution graphs for four follicular wave parameters between days 21 and 150 of pregnancy. The four parameters are 1) the maximum follicular diameter of the largest follicle on a follicular wave; 2) wave length (number of days from the retrospective identification of the largest follicle of a wave to the time when this follicle attain maximum diameter); 3) interwave interval (number of days between two consecutive wave peaks); and 4) the interval in days from wave peak to the emergence of a next wave. Three diameter (mm) or period (days) ranges (each displayed by a separate graph) were assigned to each parameter and the number of follicular waves that fit in each range (Y-axis) was further grouped according to the wave number (X-axis) in chronological order as they occurred from day 21 to 150 of pregnancy. The mean for all the waves grouped under a wave number is indicated above each bar. The follicular wave parameters are compared between the left and right ovaries, the 3 aspiration treatments and pregnancy types.
Figure 1. Frequency distribution of wave maximum diameters for the control aspiration treatment where no follicles were aspirated.
Figure 2. Frequency distribution of wave maximum diameters when all follicles >20 mm in diameter were aspirated.
Figure 3. Frequency distribution of wave maximum diameters when all follicles were aspirated.
Figure 4. Frequency distribution of wave maximum diameters for horse pregnancies.
Figure 5. Frequency distribution of wave maximum diameters for mule pregnancies.
Figure 6. Frequency distribution of wave maximum diameters for the right ovary.
Figure 7. Frequency distribution of wave maximum diameters for the left ovary.
Figure 8. Frequency distribution of wave lengths for the control aspiration treatment where no follicles were aspirated.
Figure 9. Frequency distribution of wave lengths when all follicles > 20 mm in diameter were aspirated.
Figure 10. Frequency distribution of wave lengths when all follicles were aspirated.
Figure 11. Frequency distribution of wave lengths for horse pregnancies.
Figure 12. Frequency distribution of wave lengths for mule pregnancies.
Figure 13. Frequency distribution of wave lengths for the right ovary.
Figure 14. Frequency distribution of wave lengths for the left ovary.
Figure 15. Frequency distribution of interwave intervals for the control aspiration treatment where no follicles were aspirated.
Figure 16. Frequency distribution of interwave intervals when all follicles > 20 mm in diameter were aspirated.
INTERWAVE INTERVALS
Aspirate All Follicles

Figure 17. Frequency distribution of interwave intervals when all follicles were aspirated.
Figure 18. Frequency distribution of interwave intervals for horse pregnancies.
Figure 19. Frequency distribution of interwave intervals for mule pregnancies.
Figure 20. Frequency distribution of interwave intervals for the right ovary.
Figure 21. Frequency distribution of interwave intervals for the left ovary.
Figure 22. Frequency distribution of intervals from wave peak to start of next wave for the control aspiration treatment.
Figure 23. Frequency distribution of intervals from wave peak to start of next wave when aspirating all follicles >20 mm.
Figure 24. Frequency distribution of intervals from wave peak to start of next wave when all follicles were aspirated.
Figure 25. Frequency distribution of intervals from the wave peak to the start of the next wave for horse pregnancies.
Figure 26. Frequency distribution of intervals from the wave peak to the start of the next wave for mule pregnancies.
VITA

Marius Meintjes was born on February 28, 1965 in Johannesburg, South Africa, the second of four sons of Justus and Sarie Meintjes. At thirteen years of age, his parents moved from the city of Johannesburg to a citrus and grape farm just outside Naboomspruit, a small rural town located in the Northern Transvaal bushveldt. He graduated from Nylstroom High School in Nylstroom, a neighboring town of Naboomspruit in 1982. In 1983 he attended the University of Pretoria to pursue a five and a half year veterinary degree at the Faculty of Veterinary Science located at Onderstepoort, just north of Pretoria. After receiving his veterinary degree in 1988, he served for two years in the South African Medical Services as a lieutenant. During this time he acted as a state veterinarian for the South African Defense Force and was responsible for all veterinary wildlife management in the area. He also established and managed a large animal private practice and provided relief work for veterinarians of small animal practices. At the end of 1990, he accepted a temporary position as lecturer and ambulatory veterinarian in the Department of Large Animal Medicine at the University of Pretoria. At the beginning of 1991, he received a graduate degree in veterinary physiology, pharmacology and clinical pathology from the University of Pretoria. In the summer of 1991 he and his wife, Elmarie Meintjes, traveled to the United States where he enrolled in a joint PhD graduate program in the Department of Veterinary Physiology, Pharmacology and Toxicology and the Department of Animal Science at Louisiana State University. Currently he is a candidate for this degree in reproductive physiology and plans to continue his research into human and equine assisted reproduction.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Marius Meintjes

Major Field: Veterinary Medical Sciences

Title of Dissertation: Follicular Development Patterns of Pregnant Mares During the First Half of Gestation

Approved:

Robert A. Dozier
Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Steven F. Kenneally

L. Wolfer

William Hamel

Date of Examination:

October 12, 1995