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Follicle dynamics and selection in mares

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Abstract

The interovulatory intervals of mares contain various combinations of minor follicular waves (largest follicle does not become dominant) and major waves (largest follicle becomes dominant). After emergence of an ovulatory follicular wave, the follicles grow in a common-growth phase until the beginning of deviation. At deviation, the dominant follicle continues to grow and the subordinate follicles begin to regress. Deviation begins when the future dominant follicle is about 22.5 mm. The capacity for follicle dominance is similar among the four largest follicles at the beginning of deviation, as indicated by a study involving ablation of one, two, or three largest follicles. The ovulatory waves, as well as major anovulatory waves and minor waves, originate from the stimulation of an FSH surge, which reaches a peak when the largest follicle is about 13 mm. The initial decline in the FSH surge appears to be a function of inhibin; circulating estradiol does not begin to increase until about 2 days after the FSH peak or about 1 day before the beginning of deviation. Concentrations of LH of the ovulatory LH surge reach a transient plateau encompassing deviation of the ovulatory wave. The intrafollicular concentrations of estradiol, IGF-1, inhibin-A, and activin-A increase differentially in the future dominant follicle versus the future subordinate follicles about 1 day before the beginning of diameter deviation. These factors may be enablers for differentially enhancing the FSH and LH responsiveness of the future dominant follicle, based on the results of *in vitro* studies in nonequine species. Injection of a physiologic dose of IGF-1 into the second-largest follicle of mares at the expected beginning of deviation increased the concentrations of inhibin-A and activin-A, but not estradiol, within 24 hours. Injection of an IGF binding protein into the largest follicle at the expected beginning of deviation resulted in decreases within 24 hours in several follicular-fluid factors in the largest follicle and ovulation from the second-largest follicle. Ablation of the largest follicle at the expected beginning of deviation resulted in experimental deviation between the two largest remaining follicles beginning 24 hours after ablation; concentrations of IGF-1 increased differentially in the converting future dominant follicle 12 hours before the beginning of experimental deviation, whereas inhibin-A, activin-A, and estradiol did not begin to increase differentially until 24–48 hours after the beginning of deviation. Results of these three experimental approaches indicated that the IGF-1 system is critical for the initiation of deviation in mares.

Keywords: follicle selection, follicular waves, mares

Introduction

The most dynamic, continually changing, macroscopic system in the body of a mare involves the cohorts of antral follicles that grow and regress in waves during the estrous cycle. During a specific wave, one of the follicles is empowered to become the ovulatory follicle. This phenomenon is known as follicle selection. Follicle selection has aroused the interest of biologists since at least the 1960s and can be considered one of the most enduring mysteries in reproductive biology of the monovular species. In recent years, the mare has become an increasingly productive model in this research area.

The multitude of ever-changing follicles in mares challenges breeding-farm veterinarians who use them for guidance and equine biologists who strive to characterize their dynamics and underlying hormonal and molecular controls. Moreover, the striking similari-

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ties between mares and women in follicle dynamics during the interovulatory interval and the ovulatory follicular wave favor the mare as an experimental model for study of folliculogenesis in women. The diameter of follicles in mares is more than twice the diameter of follicles in women throughout a follicular wave, providing superb experimental access for *in vivo* experimental procedures, such as intrafollicular injection, sequential sampling of follicular fluid, and monitoring vascular changes by color Doppler ultrasonography. Thus, equine follicle dynamics is a salient topic for many seemingly divergent groups, including horse owners and managers, veterinarians and physicians, and reproductive scientists in both the animal and human areas.

This review will consider the complex morphologic interrelationships among the follicles during an interovulatory interval and the ovulatory follicular wave. The current status of the follicle selection mechanism in mares will be reviewed at the morphologic, hormonal, and molecular levels. Previous reviews are available on comprehensive equine follicle biology (Ginther, 1992), the selection mechanism in monovular species (Fortune *et al*., 2001; Ginther *et al*., 2001; 2003; Mihm and Austin, 2002; Mihm *et al*., 2002; Monget *et al*., 2002), and on the breakthrough technology of equine follicle ultrasonography (Ginther, 1995).

A. Interovulatory Interval

A1. ***Definition***

The interovulatory interval begins at an ovulation associated with an estrus and ends at the ovulation of the next estrus. The days between ovulations are a far better reference for most follicle research purposes than days of the estrous cycle. This approach eliminates ambiguity associated with variations in methods of detection and definitions of estrus and minimizes error due, for example, to silent estrus. The mean length of the interovulatory interval is 21 or 22 days in horses and 24 days in ponies (Ginther, 1992). In a study of 61 intervals in Quarter Horses, the mean and standard deviation were 21.0 ± 2.3 days (range, 16–25 days); eight additional intervals were statistical outliers (Ginther and Pierson, 1989).

A2. Follicular waves

Follicular wave refers to several follicles that emerge and initially grow in synchrony. Various numbers and types of follicular waves develop during an interovulatory interval. In a major wave, the largest follicle of the wave attains the diameter of a dominant follicle (≥ 28 mm). In minor waves, the largest follicle does not become dominant. Mean maximum diameter of the largest follicle for minor waves has been reported as 22 or 23 mm (Ginther, 1993; Ginther *et al*, 2004d**)**. Examples of types of waves are illustrated by using the changing diameters of the largest follicle of each wave type (Fig. 1). The follicles of major waves eventually dissociate or deviate (Section B2). Deviation is characterized by continued and preferential growth of one, occasionally two, members of the wave. The favored follicle is termed the dominant follicle. The physiologically selected dominant follicle grows to a large diameter (≥ 28 mm) and then either regresses (anovulatory major wave) or ovulates (ovulatory wave). The remaining follicles (subordinate follicles) undergo atresia. Thus, a selection or deviation mechanism is part of the definition of a major wave. There are exceptions in that other follicles may not be detected during the development of the dominant-sized follicle. This occurred in 25% of major anovulatory waves in one study (Ginther, 1993). In this regard, development of only the dominant-sized follicle during an ovulatory wave and delayed emergence of the follicle are common occurrences in old mares (> 20 years; Ginther *et al*., 1993). The selection or deviation phenomenon for the ovulatory wave is discussed in Sections B and C.

Major anovulatory waves precede the wave that originates the ovulation associated with estrus or the follicular phase (primary ovulation). The incidence of major anovulatory waves has been reported as 24% in Quarter Horses (Ginther, 1993) and 25% in Brazilian Bretons (Ginther *et al*, 2004d). In other monovular species, a similar incidence of major anovulatory waves occurs in women (Baerwald *et al*., 2003; Ginther *et al*., 2004d), whereas cattle have two or three major anovulatory waves preceding the ovulatory wave (reviewed in Ginther *et al*., 2003). Maximum diameter of the dominant follicle is smaller for major anovulatory waves than for ovulatory waves (e.g., means of 37 and 46 mm; Ginther, 1993). During anovulatory major waves, the uterus remains ultrasonically characteristic of diestrus (Ginther, 1993). Most major anovulatory waves (e.g., 75%; Ginther, 1993) emerge before the last ovulation of the previous interovulatory interval (Fig. 1; Ginther *et al*., 2004d) at a time of an increase in the concentration of FSH in some mares (Ginther, 1992). A paradox in mares is the occurrence of ovulation from the dominant follicle of what was expected to be an anovulatory wave. These ovulations have been called secondary or diestrus ovulations and occur during high progesterone concentrations. This phenomenon has not been reported in other monovular nonequine species, but has been reported in horses (Hughes *et al*., 1972). The incidence of secondary ovulations varies widely among breeds from 18% to 21% of estrous cycles in Standardbreds and Thoroughbreds to 4% in Trotters and close to 0% in Quarter Horses, ponies (reviewed in Ginther 1992), and Bretons (Ginther *et al*., 2004d**)**. The oocytes from diestrous or secondary ovulations are fertilizable (Hughes and Stabenfeldt, 1977).

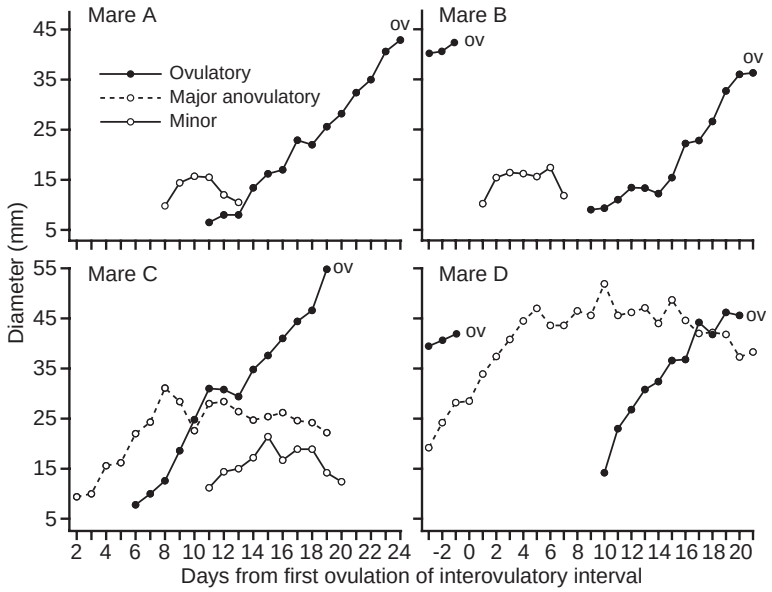

Figure 1. Types of follicular waves during the interovulatory interval, as illustrated by the changing diameters of the largest follicle after removal of other follicles.

A3. Multiple dominant follicles and ovulations

The incidence of multiple (usually double) ovulations varies considerably among breeds, extending from 2% in ponies to 25% in Thoroughbreds (reviewed in Ginther, 1992). The incidence of double dominant follicles is greater than the incidence of double ovulations; one follicle of the pair may regress (Ginther *et al*, 2004d). Diameters of double follicles on the day before ovulation were 4 mm smaller when on the same ovary and 9 mm smaller when on opposite ovaries than for single follicles (Ginther, 1995). An aspect of double ovulations that has not often been considered is that the two ovulations can originate from separate follicular waves. In one study (Ginther, 1993), origins of the second ovulation were as follows: 1) a second dominant follicle of the ovulatory wave, 2) a previous diestrus or secondary wave, and 3) a second dominant follicle of the previous ovulatory wave. Double ovulations may occur synchronously or asynchronously and unilaterally or bilaterally. These aspects of double ovulations have been reviewed (Ginther, 1992). For this review, however, it is noted that asynchronous ovulations may befuddle the definition of an interovulatory interval. In our studies, the first ovulation of the asynchronous pair is used to denote the beginning and end of the interovulatory interval or the mare may be considered separately.

B. Follicle Dynamics during the Ovulatory Follicular Wave

Data profiles of groups of follicles in various diameter categories (Pierson and Ginther, 1987) and tracking of individual follicles (Sirois *et al*., 1989) have established that the ovulatory follicle of mares originates from a follicular wave. The emergence of the follicles of the wave refers to the examination when an indicated follicle is first detected at a diameter that should be defined for each report. The beginning of observed deviation is indicated by the day a dominant follicle continues to grow at an apparently constant rate and the largest subordinate follicle begins to grow at a comparatively reduced rate or begins to regress as indicated by a decrease in diameter. Thus, the two follicles are designated after their destiny becomes clear, and the identity is extended, retrospectively, to emergence. The common-growth phase extends from follicle emergence to the beginning of deviation. That is, the end of the common-growth phase and beginning of deviation are synonyms. A follicular wave may be defined as encompassing all follicles that emerge before the beginning of deviation. In addition, some studies further restrict the definition of a wave by allowing a lapse of no more than 1 day between successive emergence of follicles.

B1. Emergence

In the initial studies which used follicle tracking, follicle emergence was based on the day the future dominant or ovulatory follicle was about 15 mm (Sirois *et al*., 1989; Ginther, 1990; Bergfelt and Ginther, 1992; 1993a), owing to the difficulty in retrospectively tracking smaller follicles. Subsequently, 6 mm was used to indicate emergence. This was attained by ab-

lating all follicles ≥ 5 mm and thereby eliminating follicles from a previous wave that would have intermingled with the ovulatory wave (Gastal *et al*., 1997). An operator with considerable experience may be able to monitor most follicles in most mares beginning at 6 mm without previous ablation (Ginther *et al*., 2004d).

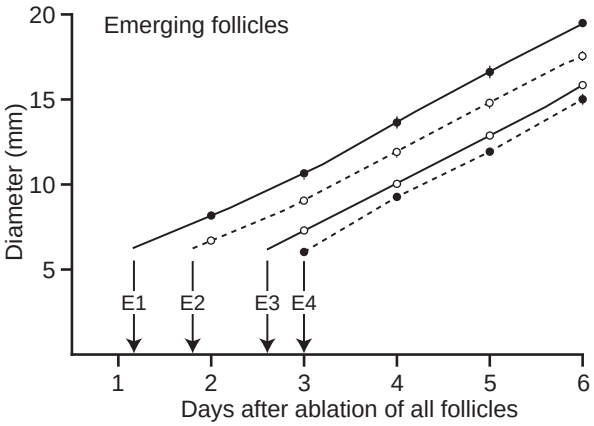

Figure 2. Mean ± SEM diameters of four largest follicles (E1, E2, E3, and E4) during 1 to 6 days after ablation of all follicles ≥ 6.0 mm (n = 59 mares). The four follicles were ranked on the day of emergence (E1 = first to emerge). E = mean day of emergence for each follicle. From Gastal *et al*., 2004.

In an initial study (Gastal *et al*., 1997), a two-follicle model was developed in which all but the two largest follicles of a new wave were ablated periodically to facilitate characterizing emergence and development of the two retained follicles. Following ablation of all follicles 10 days after ovulation, the future dominant and subordinate follicles emerged in means of 1.8 and 2.6 days—a significant difference. In a recent study (Gastal *et al*., 2004), all follicles ≥ 6.0 mm were ablated 10 days after ovulation and all follicles of the new wave were left intact (all-follicle model). The four largest follicles of the postablation wave were ranked at the expected beginning of deviation (largest follicle, ≥ 20.0 mm), according to descending diameter. The same four follicles were also ranked independently, according to order of emergence at 6.0–6.9 mm as E1 (first to emerge), E2, E3, and E4. The four follicles emerged during a mean of 1.3 to 3.1 days after ablation (Fig. 2). Emergence was well dispersed over days with mean intervals of 0.4 to 0.7 days between consecutive emergence of follicles. A mean of 12.4 follicles emerged between ablation and the expected beginning of deviation and an additional 4.5 follicles, not considered part of the wave, emerged during the interval between deviation and ovulation (Fig. 3). In a study in which previous follicle ablations were not done (Ginther *et al*., 2004), the future dominant follicle emerged at 13 mm 7 days after ovulation and 0.8 days before emergence of the future largest subordinate follicle.

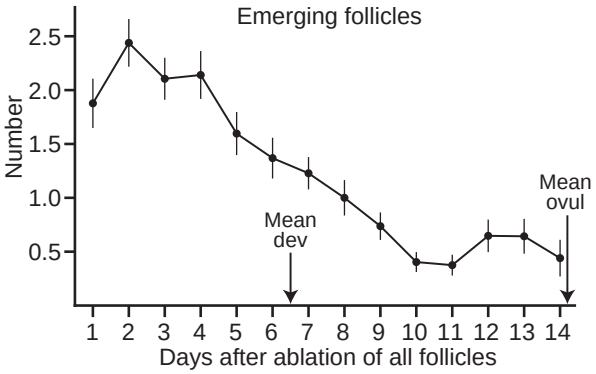

Figure 3. Mean ± SEM number of follicles emerging on each day between ablation of all follicles ≥ 6.0 mm and ovulation (n = 59 mares). Mean dev = day of the expected beginning of deviation (largest follicle ≥ 20 mm); mean ovul = day of ovulation. From Gastal *et al*., 2004.

B2. Common-growth phase and deviation

Observed deviation for individuals is determined in retrospect by inspection of the sequential changes in diameters of the dominant and subordinate follicles. Although only the two largest follicles may be used to detect deviation, other follicles may begin to decrease in growth rate simultaneously with the second-largest follicle (Ginther *et al*., 2004d) and can be considered in estimating the time of deviation. Deviation was not apparent (10% of waves) when all of the future nondominant follicles remained small or were nondetectable throughout the common-growth phase. When follicles are grouped into diameter ranges with each mare used only once, the beginning of deviation is indicated statistically by the diameter group that precedes the first group with a significant increase in the differences in diameter between the two largest follicles. Mean diameter of the largest follicle at the beginning of observed deviation is generally considered to be 22.5 mm (Ginther *et al*., 2001; 2003). Means for individual studies have ranged from 22.2–24.8 mm (Gastal *et al*., 1997; 1999d; 1999c; 2000; Ginther *et al*., 2004d). The protocol of an experiment may preclude the occurrence of deviation and expected deviation can be used, based on previous reports for observed deviation.

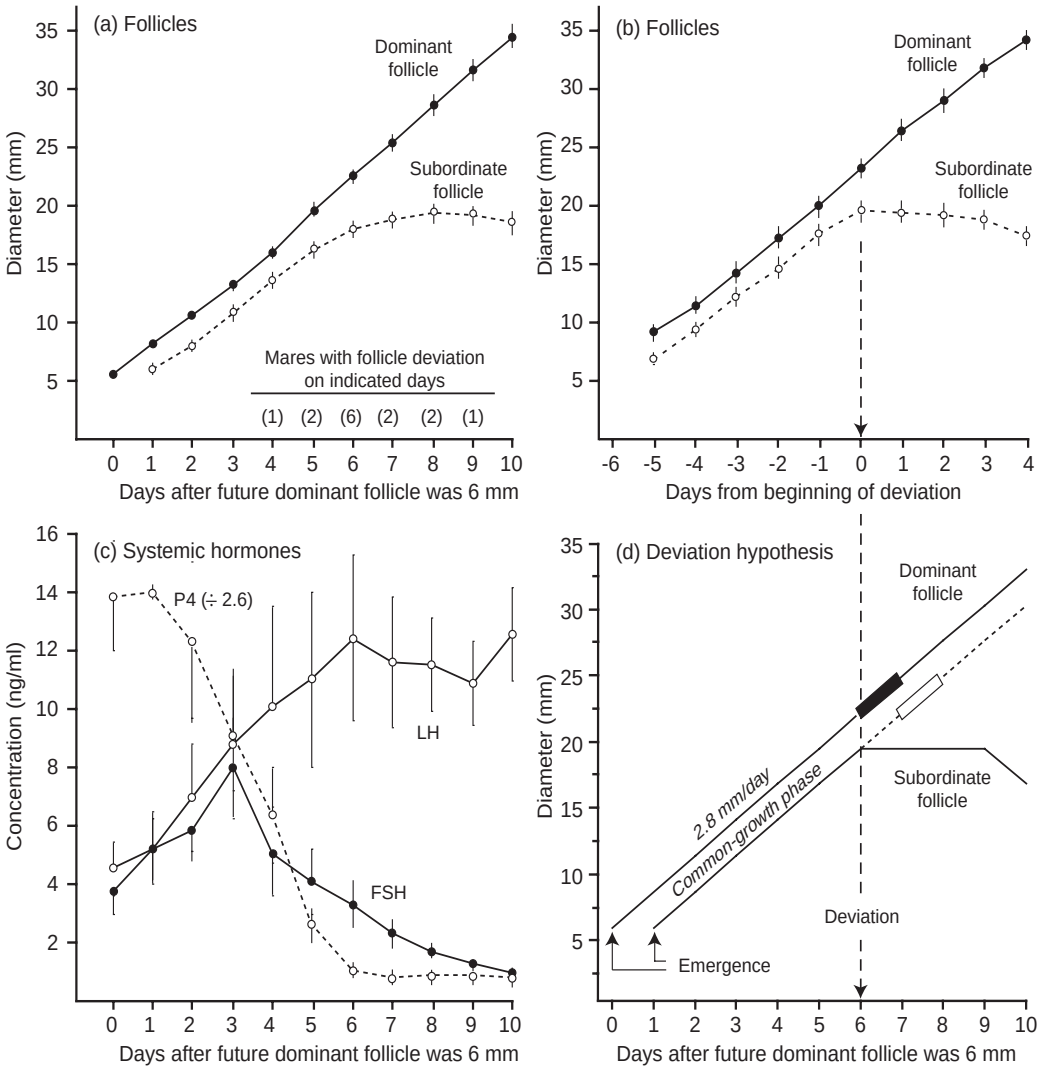

Figure 4. Mean ± SEM diameter of the dominant and subordinate follicles (two-follicle model) and hormone concentrations centralized to emergence (ac) and the same follicle data centralized to observed deviation (b), and depiction of a deviation hypothesis (d). The numbers in parentheses for the follicle data centralized to emergence (a) are for number of mares with deviation on the indicated days. Centralizing the data to the day of deviation (b) produced a sharper diameter departure between the two follicles. For the deviation hypothesis (d), the larger follicle (future dominant) has a size advantage and is the first to reach a critical stage (black bar) associated with a mechanism that inhibits the smaller follicle (future subordinate) before it can reach a similar stage (white bar). Adapted from Gastal *et al*., 1997.

In the two-follicle model, the follicles grew approximately in parallel during the common-growth phase (Fig. 4; Gastal et al., 1997). When the two follicles were profiled beginning at their emergence, the means diverged gradually for a few days beginning 4 days post emergence. Observed deviation began on various days among individuals, as shown (Fig. 4). However, when the follicles were centralized to the day of observed deviation, the means were in parallel (no difference in growth rates) during the common-growth phase. On average, the future dominant follicle emerged first, maintained its ranking during the common-growth phase, and was the first to reach a critical stage associated with deviation. The mean difference in diameter between the two follicles at the beginning of deviation (about 3 mm) was equivalent to approximately 1 day. Presumably, this time advantage allowed the destiny of the future dominant follicle to become established before the next largest follicle reached a similar diameter (Fig. 4).

This interpretation was compatible with the results of a subsequent study which used ablation at

day 10 after ovulation without restricting the number of follicles in the wave (Gastal *et al*., 2004); the differences in diameers between the first two follicles to emerge were similar during the common-growth phase between 3 days (2.7 mm) and 6 days (2.9 mm) after ablation (Fig. 2). When data were examined retrospectively beginning at the end of the common-growth phase, the constant separation of the follicles as depicted in the means was not a feature in some individual mares, as shown (Fig. 5). In a study in which no follicles were ablated (Ginther *et al*., 2004d), the growth rate of the two largest follicles (2.8 mm/day) was similar between emergence at 13 mm and the expected beginning of deviation at ≥ 20 mm. The intervals from the emergence at 6 mm to deviation was about 6 days for the two-follicle model and the all-follicle model.

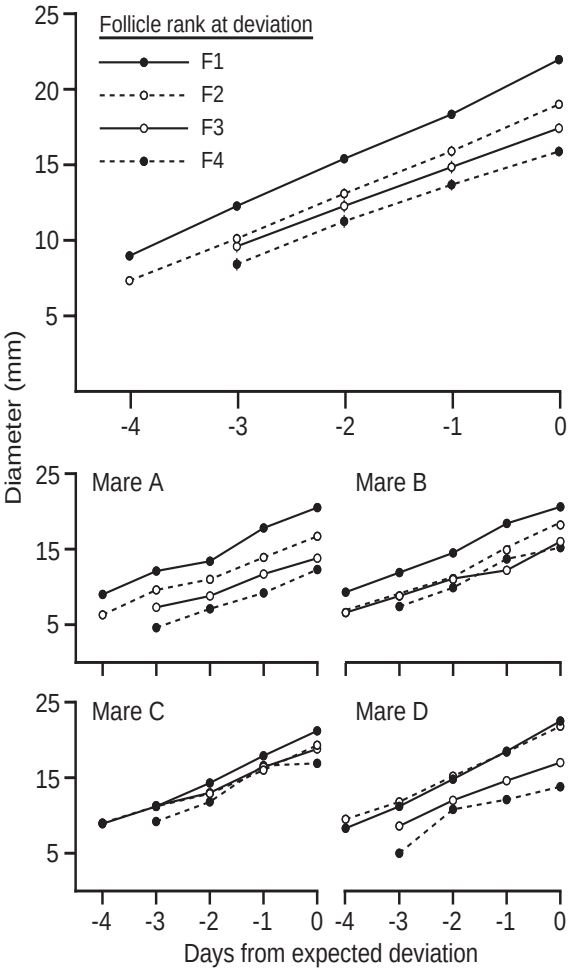

Figure 5. Mean ± SEM diameters of four largest follicles normalized to the beginning of expected deviation (Day 0; F1 ≥ 20.0 mm; n = 59 mares) and diameters for four individual mares. The four follicles were ranked F1, F2, F3, and F4 according to descending diameter on Day 0. The constant separation between F1 and F2 shown for the means occurred in 66% of individual mares as shown for Mares A and B. In the remaining mares, F1 was larger for at least Day –1 and –2 (17%; Mare C) or only for Day 0 (17%; Mare D). Adapted from Gastal *et al*., 2004.

B3. The predeviation follicle

In the most recent study in our laboratory (Ginther *et al*., 2004d), a predeviation follicle was found in 37% of 27 mares. A predeviation follicle was defined as a follicle that met the definition of being part of the ovulatory wave and reached a diameter at the beginning of deviation within the range for the future dominant follicle (19–27 mm) but began to regress before the beginning of deviation (Fig. 6). The predeviation follicles had the following average features: 1) earlier emergence than for the future dominant follicle, 2) a growth rate similar to the rate for the future dominant follicle during the common-growth phase, 3) maximum diameter similar to the diameter of the future dominant follicle at the beginning of deviation, and 4) maximum diameter occurring 1 day before the beginning of deviation. The day of observed beginning of deviation was obscured in these individuals until the predeviation follicle was recognized as a separate follicle type. Confirmation that the predeviation follicle is a specific follicle type will require elucidation of a mechanism that accounts for its regression before the beginning of deviation. Speculatively, the predeviation follicle may represent early cessation of growth before a deviation mechanism is in place or before LH has reached adequate concentrations (Section B7). A delay in development of the deviation mechanism may also account for the transient 3- to 5-day plateaus in growth of the future dominant and subordinate follicles (Fig. 7, Mare E) before deviation that has been reported for 13% of mares (Ginther *et al*., 2004d).

B4. Variation in follicle profiles

Classifying follicles as dominant, subordinate, or predeviation and centralizing the follicles to specific events results in an average profile that facilitates interpretation (Fig. 6). The follicles of the ovulatory wave may be obscured by follicles from a previous wave (Fig. 8). The intruding follicles can be from the previous ovulatory wave or can be from overlapping major or minor anovulatory waves. This intermingling of follicles can be avoided by ablating all follicles (e.g., ≥ 6 mm) and monitoring the follicles of the new wave beginning with emergence of each follicle. Examples from this approach are shown (Fig. 7).

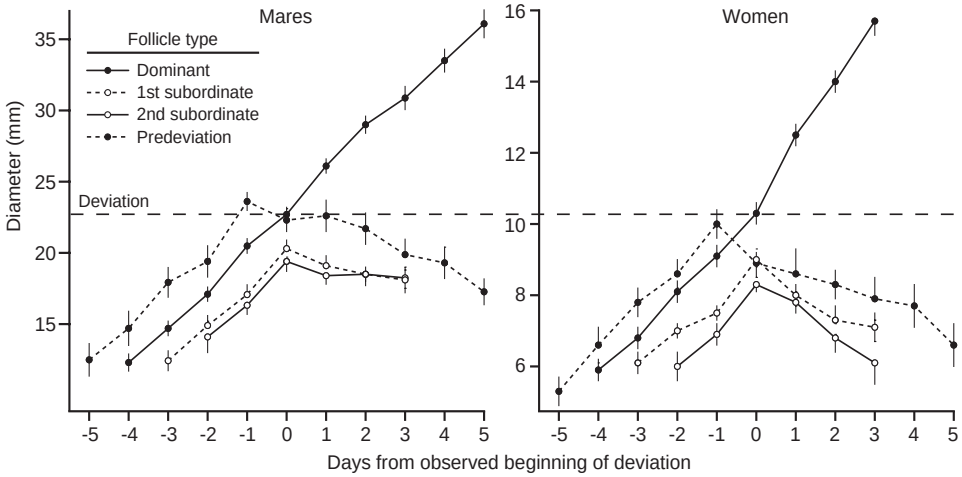

Figure 6. Mean ± SEM for diameters of four follicle types during ovulatory waves in 27 mares and 25 women. Numbers of waves for mares and women, respectively, with various follicle types were: dominant and first subordinate follicles (27 and 25), second subordinate follicles (14 and 9), and predeviation follicles (10 and 12). Dominant and subordinate follicles are centralized to the beginning of deviation (Day 0), and the predeviation follicle is centralized to the mean day at the beginning of its regression (Day -1). From Ginther *et al*., 2004d.

The profile of an individual ovulatory wave may not seem representative of the means. The number and therefore the complexity of the interrelationships among the growing follicles varies widely among waves. Unknown measuring and tracking errors would also contribute to the complexity, making interpretation difficult without averaging. The importance of guidelines for defining the follicle composition of a wave are apparent. In the set shown (Fig. 7), a follicle was not considered part of the wave if emergence was more than 2 days from emergence of the preceding follicle. Excluding defined predeviation follicles (Fig. 7, Mares C, E) and closely aligned companion follicles (Mare E) from the group of subordinate follicles has also been useful in interpreting individual waves (Ginther *et al*., 2004d).

B5. Capacity for dominance

The capacity for dominance is similar among the four largest follicles at the beginning of deviation, but dominance by a smaller follicle is blocked when a larger follicle is present. This conclusion is based on a study (Gastal *et al*., 2004) in which no follicles, the largest follicle, two largest follicles, or three largest follicles were ablated at the expected beginning of deviation. The largest remaining follicle became dominant in 76% of 34 mares with no differences among groups. The conclusion is compatible with the results of the studies that used the two-follicle model. The larger follicle at the beginning of deviation became dominant in 14 of 14 mares (Gastal *et al*., 1997) and in 8 of 8 mares (Gastal *et al*., 1999a). When the larger follicle was ablated at the expected beginning of deviation, the smaller follicle became dominant in 6 of 9 mares (Gastal *et al*., 1999a). The difference in diameter between the two follicles apparently determined whether the smaller follicle would become dominant (mean difference between follicles, 2.2 mm) or regress (difference, 5.9 mm). The recent study (Gastal *et al*., 2004) also found that the second-largest follicle retains the capacity for dominance in most mares for at least 2 days after the beginning of deviation. This was shown in 67% of 15 mares in which the largest follicle was ablated 1 or 2 days after the expected beginning of deiation. In this regard, the FSH concentrations of the wave-stimulating surge do not reach a nadir until a few days after deviation (Section B7), which may account for the prolonged postdeviation viability of the subordinate follicles.

B6. Similarities between mares and women

Follicle diameters during an interovulatory interval and the ovulatory wave were compared between 30 mares and 30 women, using similar methods for collecting and analyzing data (Ginther *et al*., 2004d). Follicles were tracked and measured daily by ultrasonography. Diameter at follicle emergence (mares, 13 mm; women, 6 mm) and the required minimal attained diameter for assessment of follicles (mares, 17 mm; women, 8 mm) were chosen to simulate the reported ratio between the two species in mean diameter of the largest follicle at the beginning of deviation (mares, 22.5 mm; women 10.5 mm). The differences between mares and women in dynamics of follicle development during an interovulatory interval and during the ovulatory follicular wave included: 1) a more complex ovulatory follicular wave in mares than

in women, owing to more follicles per wave and more intermingling of follicles from previous waves (Fig. 8), 2) greater growth rate of the dominant follicle after deviation than before deviation in women but not in mares, and 3) greater regression rate for nondominant follicles in women (Fig. 6). The similarities in the ovulatory wave between mares and women included: 1) emergence of the future dominant follicle before the future largest subordinate follicle, 2) similar length of intervals between sequential emergence of follicles, 3) similar percentage growth of follicles during the common-growth phase, 4) maintenance of a 2:1 ratio (mare:women) in diameter of the dominant follicle from the beginning of deviation to ovulation, and 5) similar incidence of predeviation follicles during ovulatory waves. The two species were also similar in the incidence of major anovulatory waves during the interovulatory interval.

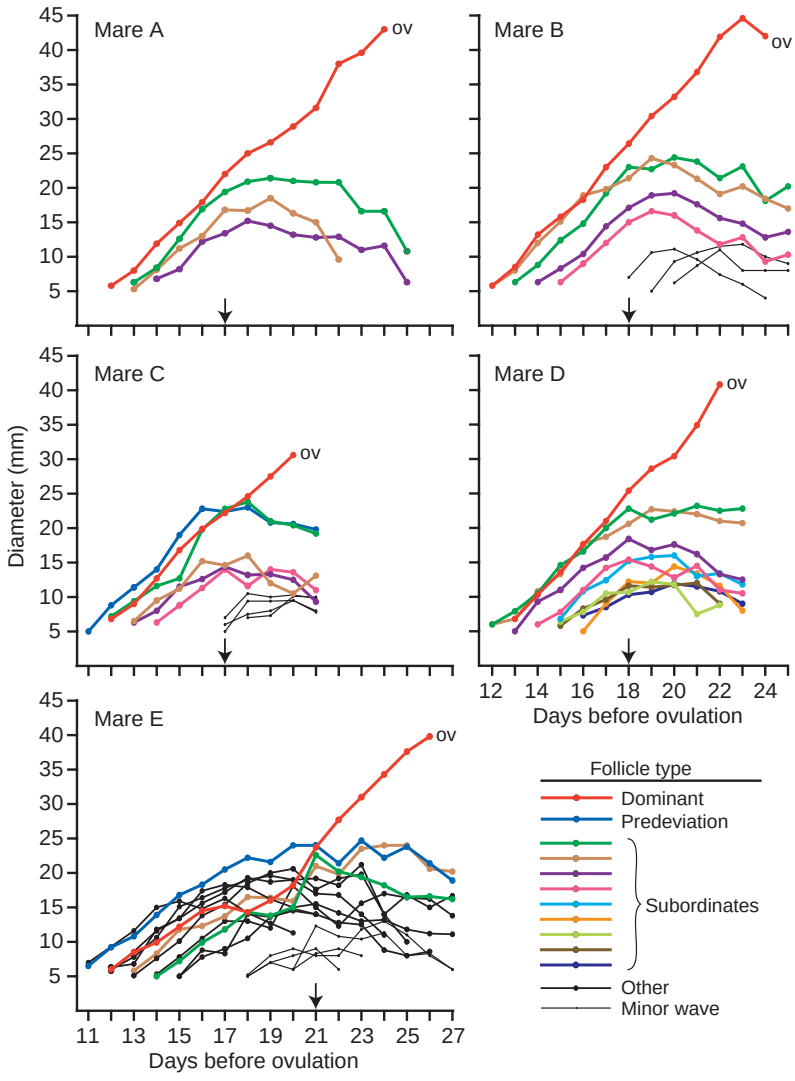

Figure 7. Ovulatory follicular waves in individual mares following ablation of all follicles ≥ 6 mm. Profiles are arranged according to ascending order of complexity. Note the following: 1) predeviation follicle in Mares C and E; 2) minor waves in Mares B, C, and E, as defined by emergence more than 2 days after emergence of the last follicle of the ovulatory wave, and 3) variation in number of follicles per wave, ranging from four (Mare A) to 12 (Mare E). In Mare E, the follicles designated "other' (black) appear to be more associated with the predeviation follicle than with the follicles designated "subordinates." These follicles and the predeviation follicle appear to cease growing before the designated day of the beginning of deviation. Similarly, growth of the dominant and two subordinate follicles (colored) appears to enter a transient, approximate 3-day plateau during the days that the "other" follicles were beginning to regress.

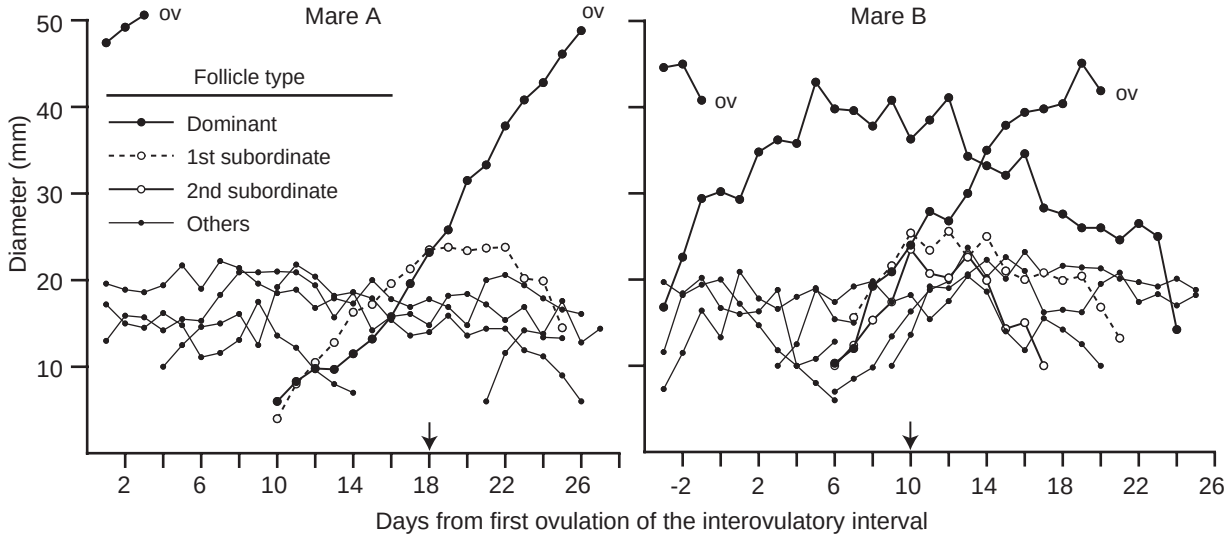

Figure 8. Ovulatory follicular waves in two mares, showing extreme examples of intermingling of follicles from a previous wave with the follicles of the ovulatory wave. The ovulatory wave is compromised by lingering follicles of a previous unknown wave (Mare A) or by the follicles of a preceding major secondary anovulatory wave (Mare B). This complexity can be managed for research purposes by ablating all follicles (example, on Day 10) and studying the new post-ablation wave. Compare with Figure 7. From Ginther *et al*., 2004d.

The striking similarities between mares and women in the dynamics of follicles during the interovulatory interval and during the ovulatory follicular wave (Fig. 6) encourage the potential use of the mare as a relevant experimental model for study of folliculogenesis in women. Although mares have added complexity from the intermingling of follicles from previous waves with follicles of the ovulatory wave, this aspect can be experimentally managed by ablating all follicles and studying the new follicular wave. Furthermore, the number of follicles in the new wave can be modified by selective ablations to simplify follicle tracking from examination to examination. The equine model allows hypothesis testing using invasive technologies that may provide additional information for consideration in human clinical medicine. The greater diameter of mare follicles before deviation begins allows superb access for *in vivo* experimental techniques, such as intrafollicular treatment, sequential sampling of follicular fluid, and assessing vascular changes by color Doppler ultrasonography. Comparative study between the two species on the similarities and differences in the systemic and local physiologic changes associated with morphologic follicle dynamics are needed.

B7. Circulating hormones

It has been established that the ovulatory wave in mares as well as major anovulatory waves and minor waves (Section A2) originate from the stimulation of an FSH surge (Fig. 4; Bergfelt and Ginther, 1992; 1993a; Ginther and Bergfelt, 1992; 1993; Gastal *et al*., 1997; 2000). The FSH surge reaches peak concentrations, on average, when the largest follicle is about 13 mm (Fig. 4). The changing concentrations of FSH and LH initially remain closely associated in mares, but dissociate beginning at the peak of the FSH surge—FSH decreases and LH continues to increase (Fig. 4; Bergfelt and Ginther, 1993b; Gastal *et al*., 1997; 2000). After the peak, the mean concentrations of FSH then decrease, with about a 3- or 4-day interval between peak concentrations and the beginning of deviation. The FSH decline is necessary for the establishment of deviation, as indicated by the development of several dominant follicles after administering FSH (Squires *et al*., 1986). Between the peak of the FSH surge and the beginning of deviation, all follicles of the wave contribute to the FSH decrease, as demonstrated by manipulating follicle numbers (Fig. 9; Donadeu and Ginther, 2001). In association with the end of the common-growth phase or the beginning of deviation, the FSH/follicle relationship changes from involvement of several follicles to involvement of only the developing dominant follicle (reviewed in Ginther *et al*., 2003).

Both estradiol and a proteinaceous fraction of follicular fluid either alone or synergistically suppress circulating concentrations of FSH (Miller *et al*., 1979; 1981; Bergfelt and Ginther, 1986). Circulating estradiol begins to increase about a day before deviation (Fig. 10; Gastal *et al*., 1999a; 1999c; Bergfelt *et al*.,

2001). Ablation of the larger follicle at the expected beginning of deviation prevents a further increase in circulating estradiol and results in an associated FSH increase; the FSH increase did not occur when only the smaller follicle of the two-follicle model was ablated (Gastal *et al*., 1999a).

Total inhibin concentrations began to increase before the beginning of the declining portion of the wave-stimulating FSH surge (Figs. 9, 10; Bergfelt *et al*., 1991; Irvine *et al*., 2000; Bergfelt *et al*., 2001; Donadeu and Ginther, 2001). Equivalent results were obtained for inhibin-A (Watson *et al*., 2002), indicating that inhibin-A is at least one of the active forms that depresses FSH before and during deviation. After the expected day of deviation, inhibin remains elevated (Figs. 9, 10), which is attributable to the dominant follicle. It has been concluded (Donadeu and Ginther, 2001) that the first 2 days of the decline in the FSH surge was caused by inhibin, based on a positive relationship between the number of experimentally retained follicles and the extent of the increase in circulating total inhibin concentrations and the corresponding extent of the decrease in FSH (Fig. 9). As noted above, increases in estradiol were not detected at this time, but a role for the basal levels of estradiol cannot be discounted.

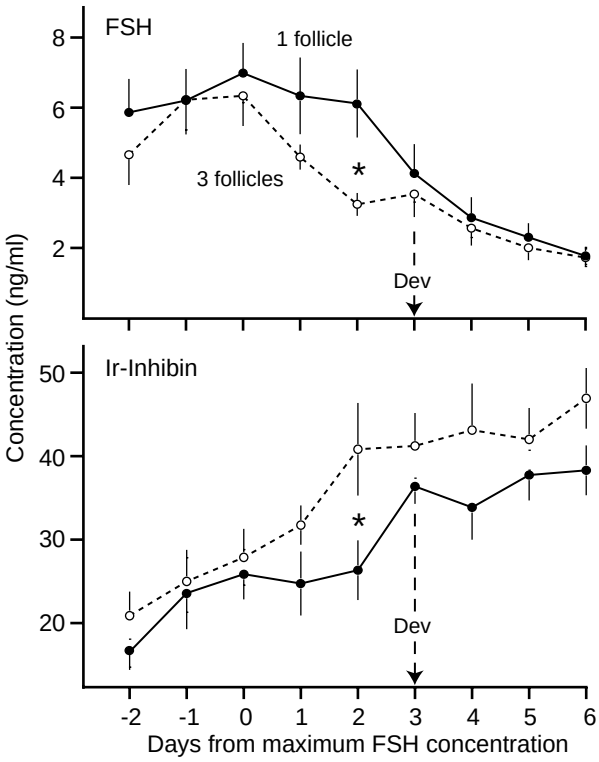

Figure 9. Mean ± SEM concentrations of FSH and inhibin in mares with one or three retained follicles per wave (n = 10 per group); all other follicles were ablated. The mean beginning of deviation (Dev) is shown. The asterisk indicates a significant difference between the one- and three-follicle groups on the day before the beginning of deviation. From Donadeu and Ginther, 2001.

A plateau or transient elevation in LH occurs during deviation as part of the ovulatory LH surge (Figs. 4, 10; Gastal *et al*., 1997; 1999a; 2000; Bergfelt *et al*., 2001). In this species, the LH surge encompasses about 6 or 7 days and reaches maximum 1 day after ovulation (Whitmore *et al*., 1973). Experimental reduction of LH encompassing deviation resulted in smaller postdeviation diameter of the largest follicle and lower circulating concentrations of estradiol and total inhibin compared to controls but did not delay deviation, based on unaltered diameters of the second-largest follicle (Gastal *et al*., 1999d; Bergfelt *et al*., 2001). These studies indicated that regulation of the production of inhibin, as well as estradiol, depends at least partly on LH in mares.

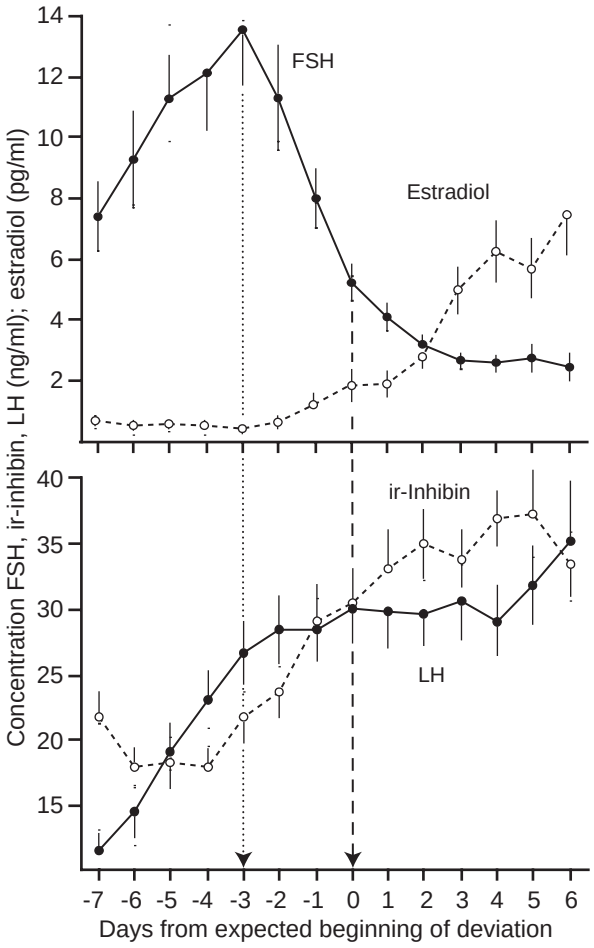

Figure 10.Mean ± SEM concentrations of four circulating hormones in 15 mares. Data are normalized to the expected beginning of deviation (largest follicle, ≥ 20 mm). The dotted and broken vertical lines indicate the mean peak of the FSH surge and the beginning of deviation, respectively. FSH continued to decrease after the beginning of deviation, and estradiol began to increase before deviation. Inhibin began to increase on the day before FSH began to decrease, and LH increased and formed a mean plateau encompassing deviation. From Bergfelt *et al*., 2001.

C. Hormonal and Molecular Aspects of the Follicle Selection Mechanism

A cascade of intrafollicular biochemical events precedes the beginning of diameter deviation. The mechanism which switches on this functional cascade involves reduced circulating FSH concentrationsand the attainment of a critical developmental stage by the future dominant follicle, including acquisition of granulosa cell LH receptors and enhanced responsiveness to gonadotropins. Based on a study in heifers, the stage reached by the most developed follicle may be more crucial than a specific low concentration of FSH (Haughian *et al*., 2004). Apparently, the future subordinate follicles have not reached a similar developmental stage and are not as responsive to low concentrations of gonadotropins, and as a result their growth rate decreases. The follicular-fluid factors that are candidates for the enablers of enhanced gonadotropin responsiveness of the future dominant follicle during deviation in mares include estradiol, insulin-like growth factor-1 (IGF-1), inhibin-A, and activin-A.

C1. Gonadotropins

The role of FSH after the peak of the surge involves the continued growth and development of all follicles before deviation and the developing dominant follicle after deviation. An equine tissue-culture study has indicated that FSH stimulates the production of IGF binding proteins (Davidson *et al*., 2002). Based on *in vitro* studies with granulosa cells in nonequine species, FSH stimulates the production of estradiol (cattle; Glister *et al*., 2001), IGF-1 (sheep; Khalid *et al*., 2000), activin-A (cattle; Glister *et al*., 2001), and inhibin-A (sheep; Campbell and Baird, 2001; cattle; Glister *et al*., 2001). That is, all of the proposed enablers for enhanced FSH responsiveness in mares have been implicated for that function by *in vitro* studies in nonequine species. There is limited comparable information from *in vitro* studies on the intrafollicular role of LH. However, the reduction in circulating estradiol and inhibin concentrations when LH is experimentally reduced in mares (Section B7) implies that LH stimulates the intrafollicular production of both estradiol and inhibin. In this regard, an early seasonal LH deficiency during the development of a dominant-sized anovulatory follicle was associated with reduced follicular-fluid concentrations of estradiol, inhibin-A, free IGF-1, and vascular endothelial growth factor (VEGF), but progesterone, androstenedione and activin-A were not altered significantly (Acosta *et al*., 2004a).

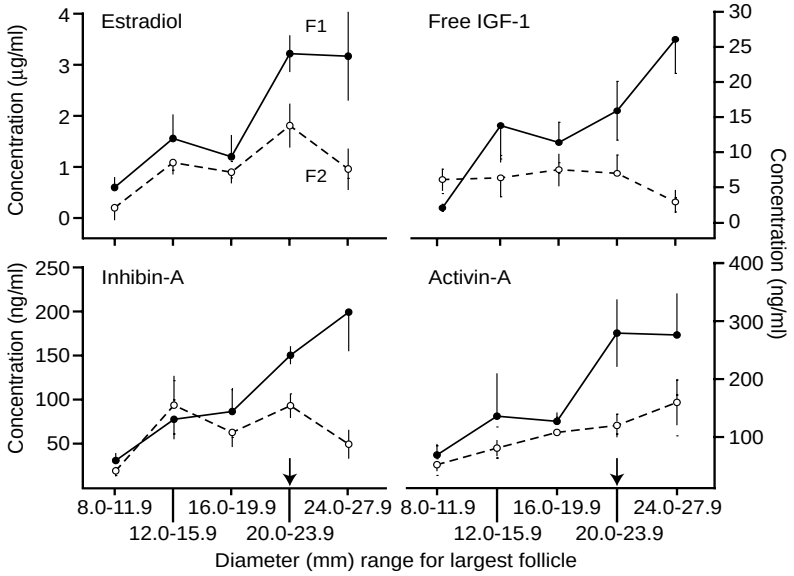

Figure 11.Mean ± SEM follicle diameters and concentrations of follicular-fluid factors for the two largest follicles (F1, F2). The beginning of diameter deviation (not shown) was in the 20.0–23.9 mm range (arrows). Differential changes between follicles in estradiol, IGF-1, inhibin-A, and activin-A began one diameter group before the beginning of diameter deviation. From Donadeu and Ginther, 2002.

C2. Estradiol

About a day before the beginning of deviation, when the largest follicle is approximately 18 mm, the follicular-fluid estradiol concentrations began to increase differentially in the future dominant follicle (Fig.

11; Gastal *et al*., 1999c; Donadeu and Ginther, 2002). This differential increase accounts for the increase in systemic estradiol concentrations and the systemic decrease after ablation of the largest follicle at the expected beginning of deviation (Section B7). The increase in estradiol concentrations was reduced in the future subordinate follicles as deviation approached. This is consistent with reduced utilization of the androgen substrate as indicated by the increasing androstenedione concentrations in the subordinate follicles (Donadeu and Ginther, 2002).

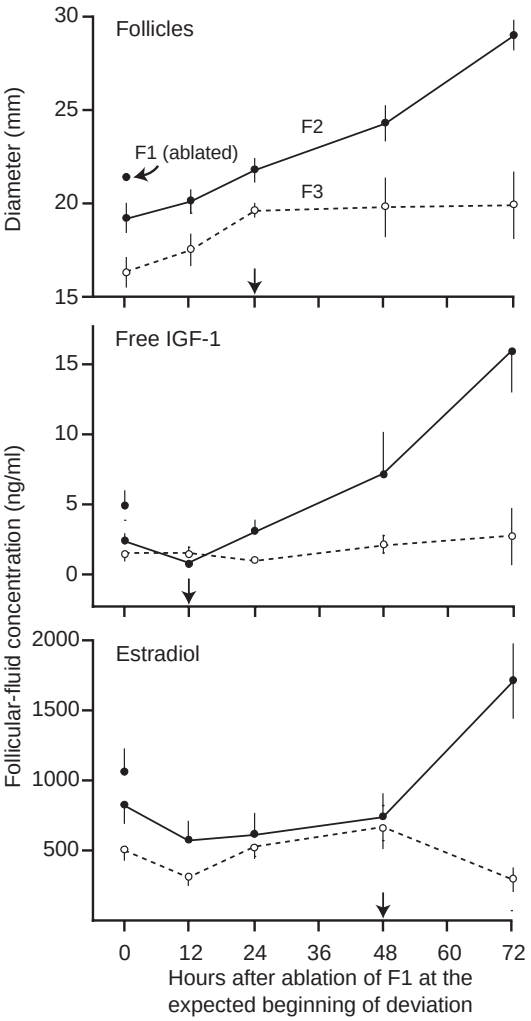

Figure 12.Mean ± SEM diameters and follicular-fluid concentrations of free IGF-1 and estradiol in F1, F2, and F3, as ranked in descending diameter at the expected beginning of deviation (Hour 0). F1 was ablated at Hour 0 and the experimental conversion of F2 and F3 to a dominant follicle and largest subordinate follicle, respectively, was studied. Experimental diameter deviation began at Hour 24 (arrow), whereas IGF-1 and estradiol began to deviate at Hours 12 and 48, respectively. Adapted from Ginther *et al*., 2002.

The relationship of estradiol to the initiation of deviation was studied in mares by ablating the largest follicle at the expected beginning of deviation and monitoring the conversion of the second-largest follicle to dominant status. In the two-follicle model, the retained smaller follicle continued to grow, indicating rapid conversion to dominant status (Gastal *et al*., 1999a). However, systemic estradiol did not begin to increase until 2 days later. Subsequently, changes in concentrations of follicular-fluid factors in the second-largest follicle after ablation of the largest follicle were considered in an all-follicle model (Ginther *et al*., 2002). Estradiol concentrations did not increase until 24 hours after the beginning of experimental deviation between the second- and third-largest follicles and 36 hours after an increase in IGF-1 (Fig. 12). Results of these studies indicated that an increase in estradiol, either systemically or locally, was not prerequisite to the initiation of diameter deviation in mares, even though estradiol increases differentially in the future dominant follicle before the beginning of natural deviation.

An intrafollicular role for estradiol is consistent with an increase in granulosa-cell content of steroidogenic acute regulatory protein and the enzymes P450 scc, 3ßHSD, and aromatase as the follicle grows from 20–24 mm (equivalent to the beginning of deviation) to ≥ 30 mm (dominant size; Belin *et al*., 2000). Estradiol has the capacity *in vitro* to augment its own synthesis by upregulating the thecal androgens (rats; Wrathal and Knight, 1995). Estradiol has not been shown to have direct effects on follicle growth in mares, but in other species it promotes development of preantral follicles and stimulates steroidogenesis in granulosa and theca cells *in vitro* (cattle), stimulates follicle growth and development *in vivo* and *in vitro* and inhibits granulosa cell apoptosis (mice and rats), increases the sensitivity of the granulosa cells to FSH and LH by promoting the expression of their receptors and regulating formation of gap junctions among granulosa cells *in vivo* (rats; reviewed in Rosenfeld *et al*., 2001). In pigs and sheep, estradiol promotes the synthesis of IGF-1 (Spicer and Chamberlain, 2000). Systemic treatment with anti serum against estradiol decreased the growth rate of the two largest follicles in heifers and delayed the beginning of deviation despite an increase in FSH (Beg *et al*., 2003).

C3. Androgens and progestins

These steroids are substrates for estradiol synthesis. Androgens are produced in the theca layer and are then aromatized in the granulosa layer in mares, as in other species (Fortune and Quirk, 1988). The synthesis of androgen in the mare, but not in cattle, involves progesterone as an intermediary steroid. The androgen concentrations in the subordinate follicles were higher than in the dominant follicle in mares, and

progesterone concentrations increased in the future dominant follicle at the beginning of deviation (Donadeu and Ginther, 2002). In heifers, androgen concentrations increased in the developing dominant follicle (Beg *et al*., 2002), contrasting with the increase in the subordinate follicles in mares. The higher androgen concentrations in the subordinate follicles in mares apparently results from the lack of aromatogenic activity in these follicles. The comparative (horses versus cattle) relationships of androgens and progesterone to the beginning of deviation and the high androgen concentration in subordinate follicles in horses versus dominant follicles in cattle should be productive research areas.

C4. IGF system

The IGF system includes IGF-1 and -2, IGF binding proteins (BPs) and IGFBP proteases (reviewed in Spicer, 2004). In mares the concentrations of free IGF-1 differentially increased in the future dominant follicle before the beginning of diameter deviation concomitant with the increase in estradiol (Fig. 11; Donadeu and Ginther, 2002). In addition, when the largest follicle was ablated at the beginning of deviation, free IGF-1 began to increase in the second-largest follicle 12 hours before the beginning of experimental diameter deviation between the two largest retained follicles (Ginther *et al*., 2002) and was the first noted change among the potential intrafollicular gonadotropin-enabling factors (Fig. 12). Concentrations of estradiol, inhibin-A and activin-A also increased differentially in the largest retained follicle, but only after experimental deviation had begun, indicating a key role for the IGF-1 system in the initiation of the selection mechanism in mares.

Recently the role of IGF-1 was studied by injecting pharmacologic doses (250 and 500 µg) of recombinant human (rh) IGF-1 into the second-largest follicle at the expected beginning of deviation (Ginther *et al*., 2004c). More IGF-1 injected second-largest follicles continued to grow, became dominant, and ovulated than in saline injected controls. A low physiologic dose (2.5 µg) was injected into the second-largest follicle at the expected beginning of deviation and the two largest follicles were sampled 24 hours later. The follicular-fluid concentrations of free IGF-1 in the treated second-largest follicle were higher than in saline controls and were similar to the natural concentrations in the largest follicle (Fig. 13). The physiologic dose of IGF-1 stimulated an increase in inhibin-A, activin-A, and VEGF, but did not increase the estradiol concentration. In contrast, in cattle rh-IGF-1 injection into the second-largest follicle at the beginning of deviation (Ginther *et al*., 2004b) or into the ovarian stroma (Spicer *et al*., 2000) increased the follicular fluid estradiol concentrations.

In nonequine species, *in vitro* studies have shown that IGF-1 increased the proliferation of bovine follicle cells, enhanced their sensitivity to FSH, and increased the secretion of estradiol., inhibin-A, and follistatin from granulosa cells and also enhanced LH-stimulated androgen secretion from theca cells (reviewed in Webb *et al*., 1999; Glister *et al*., 2003). Similar tissue-culture studies have not been done in mares.

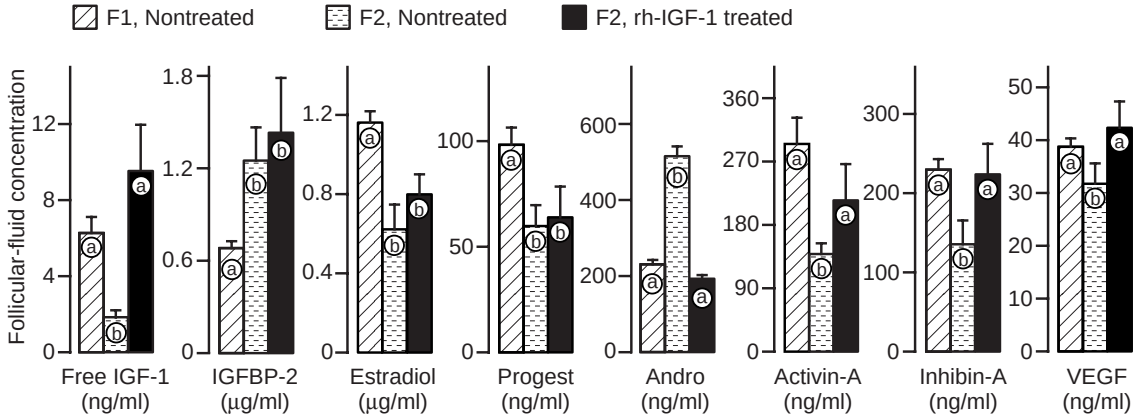

Figure 13. Mean ± SEM for follicular-fluid concentrations of hormones 24 hours after injection of F2 (second-largest follicle) with a physiologic dose of rh-IGF-1 at the expected beginning of deviation (F1, ≥ 20 mm). Controls were not treated. Significant differences within each factor are indicated by the lower-case letters. Progest = progesterone. Andro = androstenedione. Adapted from Ginther *et al*., 2004c.

C5. IGF binding proteins and proteases

At least 4 to 5 different IGFBPs (BP-2, -3, -4, -5 and a high molecular weight complex of 90-135 kD) have been reported in follicular fluid of mares (Gerard and Monget 1998; Bridges *et al*., 2002). IGFBP-2 is the only BP that has been studied in relation to deviation in mares (Donadeu and Ginther., 2002); concentrations began to increase in the future subordinate follicles in temporal association with the beginning of de viation but did not change in the future dominant follicle. Other studies have shown higher levels of low molecular weight IGFBPs (BP-2, -4 and -5) in subordinate follicles and lower levels in dominant follicles of mares (Gerard and Monget, 1998; Bridges *et al*., 2002; Monget *et al*., 2002). Also, BP-2 and BP-5 have been shown to be produced by equine granulosa cells in culture when stimulated with FSH (Davidson *et al*., 2002).

The functional relationships of IGFs and IGFBPs to the deviation mechanism are further complicated by the presence of specific BP proteases in the follicular fluid (reviewed in Spicer, 2004). The proteases degrade the binding proteins and thus increase the bioavailability of IGF-1 in the follicles. Proteolytic activity for BP-2 (Mazerbourg *et al*., 2003), BP-4 (Mazerbourg *et al*., 2000), and BP-5 (Bridges *et al*., 2002) has been reported in dominant follicles during the follicular phase in mares. Recently, the role of the IGF system in follicle selection was studied in mares by injecting rh-IGFBP-3 into the largest follicle at the expected beginning of deviation, and sampling follicular fluid 24 hours later (Ginther *et al*., 2004a). It was expected that the BP-3 would bind the IGFs and thus interfere with the follicle selection mechanism. More follicles injected with BP-3 stopped growing and regressed than for saline injected follicles, and the second-largest follicle became the dominant follicle in the BP-3 injected group. In addition, injection of BP-3 into the largest follicle decreased the follicular fluid concentrations of estradiol, free IGF-1, activin-A, and inhibin-A and increased androstenedione concentrations within 24 hours (Fig. 14). It was concluded that the IGF-1 system plays a critical initiating role in follicle selection and the development of follicle dominance in mares.

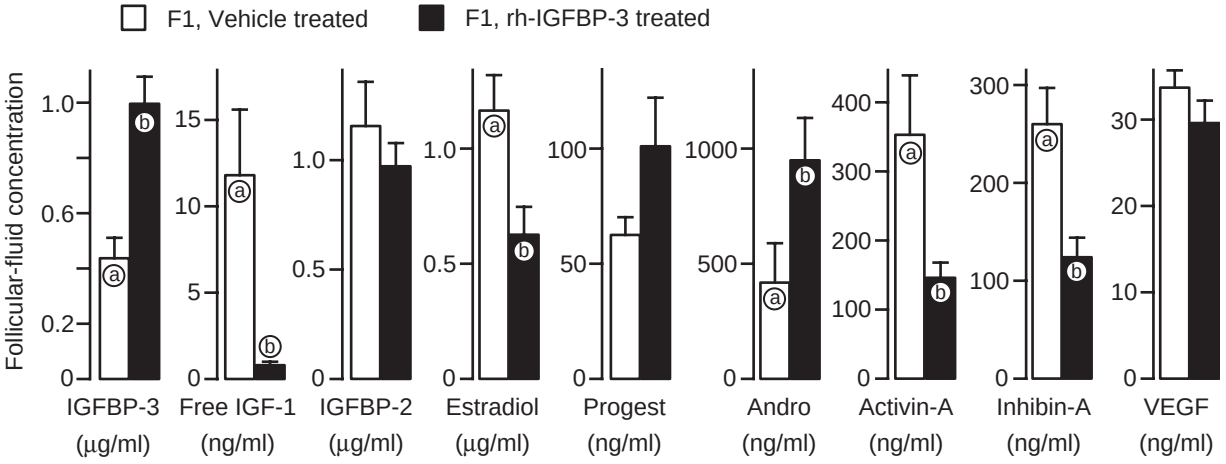

Figure 14. Mean ± SEM for follicular-fluid concentrations of hormones 24 hours after injection of F1 (largest follicle) with vehicle or rh-IGFBP-3 at the expected beginning of deviation (F1, ≥ 20 mm). Significant differences within each factor are indicated by the lower-case letters. Progest = progesterone. Andro = androstenedione. Adapted from Ginther *et al*., 2004a.

C6. Inhibin-A

Inhibin-A began to increase in the future dominant follicle differentially between follicles before the beginning of diameter deviation at the same diameters as for estradiol, free IGF-1, and activin-A (Fig. 11; Donadeu and Ginther, 2002). Inhibin-B concentrations did not change in the future dominant follicle but a decrease in the future subordinate follicle began at the beginning of deviation. When the largest follicle was ablated at the beginning of deviation, inhibin-A concentrations, like estradiol concentrations (Fig. 12), increased differentially in the second-largest follicle but not until after an increase in the free IGF-1 and after the beginning of experimental deviation (Ginther *et al*., 2002). In addition, the follicular-fluid inhibin-A concentrations increased in the second-largest follicle within 24 h after an injection of a physiologic dose of rh-IGF-1 (Fig. 13; Ginther *et al*., 2004c). Injection of rh-IGFBP-3 into the largest follicle decreased the inhibin-A concentrations (Fig. 14; Ginther *et al*., 2004a).

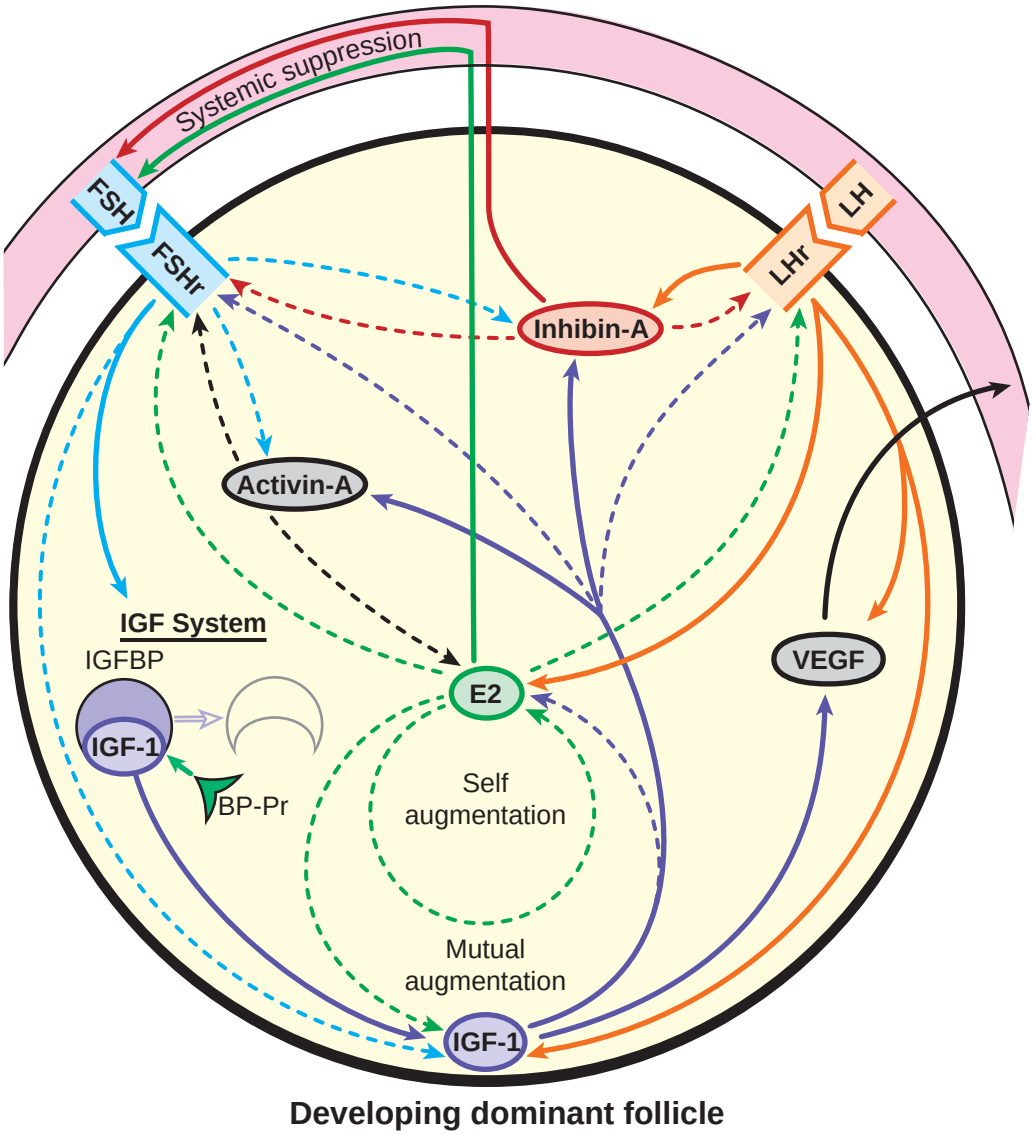

Figure 15. The postulated interactions of systemic and local aspects of follicle deviation in mares. The follicle is shown as a whole without regard to its cellular layers. When the wave-stimulating FSH surge is declining and the future dominant follicle is near the beginning of diameter deviation, it develops the indicated biochemical relationships. The critical intrafollicular initiator of the diagrammed effects is the IGF system. IGFBPs (binding proteins) are degraded by binding-protein proteases (BP-Pr) and free IGF-1 is released. The free IGF-1 stimulates the production of inhibin-A, activin-A, and VEGF but does not have an immediate effect on estradiol (E2) in mares. The overall effect of these concentration changes is greater LH and FSH responsiveness of the developing dominant follicle so that it is the only follicle of the wave that is able to continue growing in the presence of low concentrations of circulating FSH. The solid arrows represent the direction of positive effects between factors or between factors and the gonadotropins that have been shown *in vivo* in mares or *in vitro* with equine granulosa cells. The dashed lines are based on studies *in vivo* or *in vitro* in cattle and other species and have not been studied in mares. The literature sources for the arrows are given in the text.

In vitro, inhibin-A increased the LH induced androgen production from theca cells (rats; Wrathal and Knight, 1995) and increased FSH-induced estradiol secretion from ovine granulosa cells (Campbell and Baird, 2001). Conversely, a recent report has also indicated a negative paracrine role for inhibin on estra-

diol synthesis in bovine granulosa cells (Jimenez-Krassel *et al*., 2003). Except for the latter study, the *in vitro* results in nonequine species are compatible with the equine *in vivo* results, thereby supporting the concept that inhibin-A is an additional enabling factor for enhancing the responsiveness of the future dominant follicle in mares.

C7. Activin/Follistatin

Activins are dimeric glycoproteins and follistatin is a monomeric glycoprotein and both are present in the follicular fluid (Austin *et al*., 2001). Follistatin acts as a binding protein for activin and inhibin. Reported studies (reviewed in Knight and Glister, 2001) have indicated that activin induced proliferation and expression of FSH receptor in rat granulosa cells and increased the granulosa-cell steroidogenesis and aromatase activity in rats and cattle. Temporally, in mares, activin-A concentrations differentially increased in the largest follicle concomitant with an increase in estradiol, free IGF-1 and inhibin-A before the beginning of deviation (Fig. 11; Donadeu and Ginther, 2002). Furthermore, the response of activin-A was similar to the response of inhibin-A (Section C6) to experimentally induced deviation or to the intrafollicular injection of rh-IGF- (Fig. 13) or rh-IGFBP-3 (Fig. 14). In conclusion, activin-A may also be an enabling factor for increasing the gonadotropin responsiveness of the developing dominant follicle in mares.

C8. VEGF and vascularity

Follicular-fluid concentrations of VEGF, an angiogenic factor, are higher in the dominant follicle than in the subordinate follicle of mares 1 day after the expected beginning of diameter deviation (Fig. 13; Ginther *et al*., 2004c); the earlier temporal relationships before deviation are not known. Thus, follicle-produced VEGF is a candidate for a role in development of the vascularity of the follicles during diameter deviation. In this regard, VEGF has been shown to stimulate mitosis of endothelial cells and to increase vascular permeability and angiogenesis in other species (reviewed in Redmer and Reynolds, 1996; Martiez-Chequer *et al*., 2003). Furthermore, VEGF production increased in cultures of granulosa cells in cattle (Schams *et al*., 2001) and monkeys (Martinez-Chequer *et al*., 2003) when exposed to IGF-1. In mares, VEGF increased in the second-largest follicle within 24 hours after injection of rh-IGF-1 at the beginning of expected deviation (Ginther *et al*., 2004c). An expanded anechoic layer within the wall of dominant follicle becomes apparent near the beginning of deviation in mares and has been attributed to increased vascularization (Gastal *et al*., 1999b).

Recently the relationship of follicle vascularity to the beginning of deviation was studied directly by Doppler ultrasonography in mares (Acosta *et al*., 2004b). The blood flow velocities and the blood flow area in the follicle wall began to decrease in the future subordinate follicles but not in the future dominant follicle 1 or 2 days before the beginning of deviation. The cause and effect relationships of follicle vascularity and VEGF and the beginning of deviation are not known. However, a continued increase in vascularity of the future dominant follicle is a likely advantage for the follicle in receiving preferential supply of growth factors, gonadotropins, steroid precursors, and other nutrients required for its continued preferential development. A diagrammatic summary of the postulated systemic and local aspects of follicle deviation in mares is shown (Fig. 15).

C9. Genomics of the selection mechanism

The availability of highly specialized techniques including suppressive subtraction hybridization and gene array techniques has made it possible to screen and identify genes that are differentially expressed in dominant follicles versus subordinate follicles. Gene profiling will aid in identifying the mechanisms at the molecular level that determine the destiny of future dominant and subordinate follicles. Gene profiling during follicle selection has been reported in cattle (Sisco *et al*., 2003; Fayad *et al*., 2004; Evans *et al*., 2004). Gene profiling during follicle selection in mares has not been reported. In this regard, a limited number of equine specific genes have been sequenced, and equine specific micro arrays are not commercially available. These needs presumably will be resolved as the mare becomes an increasingly important research model (Section B6).

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