

**Reproduction and Endocrine Aspects of Early and Mid Lactation Holstein
Cows**

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(ABSTRACT)

This study was designed to determine the effects of stage of lactation and subsequent energy status on metabolic and endocrine measures, follicular development, and the quality of oocytes obtained from Holstein cows. Holstein cows were selected prior to calving and assigned to the early lactation (EL) group (n=8) while, cows at d 90 postpartum were selected for the mid-lactation (ML) group (n=7). Blood samples were taken twice weekly from 4 wk prior to the start of follicular aspirations and then on through the aspiration periods for metabolite and hormone determination. Ultrasound-guided transvaginal follicular aspiration (TVFA) was conducted twice weekly for a 10-wk period on all cows. Follicular fluid samples were obtained from the largest follicle, > 10 mm in diameter, for hormone determination. All data were analyzed by ANOVA, using the general linear model procedures. Mean energy balance was positive for (2.43 ± 0.32 Mcal/kg) for ML cows and negative (-1.55 ± 0.33 Mcal/kg) for EL cows. In ML cows serum progesterone (P_4) decreased rapidly from 2.7 ± 0.1 ng/ml at the first aspiration session to a nadir of 0.33 ± 0.1 ng/ml at wk 8, while follicular fluid P_4 increased from 0.9 ± 0.5 to 5.6 ± 0.5 ng/ml. In the EL cows serum and follicular fluid P_4 remained relatively constant over the course of aspirations. There was a linear increase in follicular fluid insulin-like growth factor I (IGF-I) for EL and ML cows, however the increase was more rapid for ML cows (159 ± 36 to 200 ± 36 ng/ml) than for EL cows (145 ± 36 to 164 ± 36 ng/ml). Over the aspiration period nonesterified fatty acids (NEFA) declined rapidly for the EL cows (0.32 ± 0.2 to 0.22 ± 0.2 mEq/L), while serum NEFA for the ML cows were relatively stable (0.19 ± 0.2 to 0.22 ± 0.2 mEq/L). The number of

follicles observed during the aspiration sessions increased linearly for both EL and ML cows ($P < 0.05$) over the 10-wk period. However, the increase was larger for the ML cows than for the EL cows, going from 14.2 ± 0.5 to 18.1 ± 0.5 and 14.9 ± 0.3 to 15.7 ± 0.5 , respectively. These results show that cows in early lactation are physiologically under more production stress than cows in mid lactation. Furthermore, increasing levels of serum and follicular fluid IGF-I in mid lactation may reflect differences in follicle and oocyte measures.

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INTRODUCTION

As uses for reproductive technologies increase, our understanding of factors surrounding these technologies must increase as well. One such technology is the use of transvaginal follicular aspiration (TVFA) as a means of collecting follicular contents. This technique was adapted for bovine use in the Netherlands by Pieterse et al.(1987) and now provides a means of collecting potentially large numbers of unfertilized ova from domestic livestock. When TVFA is used on livestock the collected ova can be used by breeders, the pharmaceutical industry, or for research. However the potential uses for this technology could be affected by the primary role of livestock, to produce food. For instance, could the level of milk production in a dairy cow affect the efficiency of TVFA recoveries in that cow? And, if so, at what stage of lactation is collection of ovum most efficient and beneficial to the user of this technology?

The most obvious function of a dairy cow is to produce milk or to lactate. As the cow progresses through lactation her body undergoes many changes. These changes can affect everything from her body condition to her lactation performance. Within these constraints lies reproduction. Reproduction is linked to lactation by energy constraints, changes in hormone secretion, and hormone availability. All of these factors change as the cow advances through lactation. Therefore, since hormones control the development of the ovum, the stage of lactation may play a role in the quality and quantity of oocytes produced.

To enhance the understanding of these issues, this study was designed to address three objectives. Each objective investigated the effect of stage of lactation on reproductive characteristics. The first objective was to determine the effect of stage of lactation on a biweekly assessment of follicular development. The second objective was to look at the effect of stage of lactation on the quality of recovered oocytes. The third and final objective was to determine relative differences between early and mid lactation dairy cattle in the concentration of serum progesterone, follicle stimulating hormone, luteinizing hormone, insulin-like growth factor-I, growth hormone, nonesterified fatty acids, β -hydroxybutyrate, and follicular fluid progesterone, estradiol, and insulin like

growth factor I. By integrating the results of each objective, a more defined picture of the role of lactation can be made on reproduction.

Review of Literature

The advances made in reproductive technologies through the mid-eighties allowed the dairy industry (DI) a variety of means to maximize genetic improvement and profitability from superior genetics. Procedures such as artificial insemination, superovulation, embryo flushing, and embryo transfer allowed the DI to utilize genetically superior cows as the building blocks for the next generation and as marketable commodities. However, not all cows respond to these treatments equally. Likewise, there are many limitations to these standard procedures, such as reproductive status, time constraints, and costly drug protocols. Therefore, there exists room for still more increases in the options available.

Collection of oocytes from the cow, followed by in vitro fertilization could provide an alternative. However, oocyte collections are not without limitations. Early attempts at oocyte collection involved surgical procedures, which were costly, potentially dangerous for the cow, and only yielded marginal results. For these reasons, researchers attempted to modify a technique used in humans that was both non-surgical and more cost effective. This procedure involved the use of ultrasound imaging to visualize follicles and then guide a needle to them for aspiration of their contents. Modifications of this human medical procedure were first seen in animals as early as 1987. Since this time many other modifications have developed such as, changes in the frequency of collection, the media used, the type of tubing, the culture system, and time of collection. All of these changes have made TVFA a more efficient procedure as well as allowing the practitioner to adapt the system to his clients needs.

Lactation

At the onset of lactation a cow's body undergoes many dramatic changes. Most of the cow's energy intake is utilized for milk production. In over 80% of all dairy cattle milk production causes the cow to go into a negative energy balance (NEB) shortly after parturition. The NEB reaches nadir in most cows between day 10 and 15 of lactation, which may be several weeks before peak milk production. As a result of the NEB serum

insulin-like growth factor I (IGF-I) is lower in early lactation than in late lactation (Stanisiewski et al., 1992). Similarly, Sharma et al. (1994) discussed the possible effects of different serum profiles of insulin-like growth factor binding proteins (IGFBP) in early lactation and late lactation, suggesting that these different profiles of IGFBP may cause different rates of clearance of IGF-I.

As dairy cattle progress into late lactation they regain a positive energy balance (EB) and begin to gain body conditioning. Rose et al. (1996) stated that the transition from using body fat reserves to depositing them is believed to be due to changes in the body tissue's ability to respond to insulin and other hormones such as GH, glucagon, IGF-I. This change in response is likely due to an increase in availability of nutrients. More nutrients may be available for numerous reasons: an increase in feed intake, change in ration density and feed availability, milk production, and changes in the body's response to growth hormone. The changes in late lactation result in stabilization and in potential increases in body condition score (BCS).

Energy Balance

Because changes in EB are one of the major differences between early and late lactation, it is worthwhile to go into more detail on how EB affects reproduction. For example, EB has an effect on the resumption of the estrous cycle. Beam and Butler (1997) reported that the number of days to first ovulation is positively correlated with days to EB nadir. In other words, a shorter time to nadir results in fewer days to first postpartum ovulation, and potentially a shorter calving interval. They also reported that higher energy, similar to that of late lactation, results in more large follicles (>15 mm).

Yung et al. (1996) studied the effects of NEB on the corpus luteum (CL). They found that not only did NEB cause a decrease in CL size, but it resulted in a decrease in progesterone derived from the CL as compared to cows on a higher plane of nutrition. Additionally, they noted lower levels of serum IGF-I in NEB cows when compared to cows in positive EB.

The duration of NEB affects the response of the cow to the NEB. Looper et al. (1996) showed that a short interval of NEB would result in increased follicle stimulating hormone (FSH) and a decrease in luteinizing hormone (LH). However, a long period of NEB would decrease both FSH and LH. These hormonal changes were related to

changes in body condition. Moderately conditioned cows had greater serum LH and FSH concentrations than thin condition cows.

Purvis et al. (1996) found body condition related to reproduction. They demonstrated that the body condition score (BCS) at calving was negatively correlated with days to first estrus. Furthermore, Domecq et al. (1997) reported that a decrease in BCS during the first month of lactation was associated with a decreased likelihood of conception at the first AI service.

Reproductive Hormones

Many hormones are involved in the control and regulation of the estrous cycle. These hormones control follicular growth and CL development and maintenance. Follicle stimulating hormone (FSH), LH, estradiol (E2), and progesterone (P4) are the hormones most commonly mentioned when discussing the estrous cycle. However, there are other hormones that play roles in controlling the estrous cycle in either an autocrine or paracrine fashion within the ovary. Inhibin, oxytocin, activin, IGF, IGFBP, follistatin, ubiquitin, testosterone, transforming growth factor α (TGF α), and TGF β are others that regulate cyclicity of estrus (Borromeo et al. 1996). For instance, it was shown that immunization against inhibin resulted in an increased number of all follicle sizes (Konishi et al., 1996). Similarly, Kaneko et al. (1995) showed that not only was inhibin important in regulating FSH, but it may also have a synergistic effect on FSH when combined with E2. Meidan et al. (1993) reported that granulosa cells secrete oxytocin into the follicular fluid, suggesting a role in ovarian function.

The presence of these hormones are important, but the ratios of one to another become important as well. Healthy follicles have a high ratio of E2 : P4 (Meidan et al., 1993). However, this may be deceiving, as some atretic follicles will have the same type of ratio (Skinner and Osteen, 1988). Within the follicle it may be the ratio of IGF-I to various IGFBP that determines if a follicle is to ovulate or become atretic. Funston et al. (1996) showed that only IGFBP-3 was present in significant levels in ovulatory follicles. Whereas, in smaller follicles and atretic follicles levels of IGFBP 2, 3, 4, and 5 could be detected as well. They also demonstrated that IGFBP- 4 and 5 were inversely associated with androstenedione concentrations. Serum IGFBP-2 decreased by as much as 50%

after estrus. Another study noticed that the ratio of IGF-I to IGFBP decreased with the atresia of the dominant follicle (de la Sota et al., 1996).

Other hormones have not been studied as extensively as IGF-I and IGFBP. One of these hormones is ubiquitin. Ubiquitin can serve as an indicator of cellular replicative ability (Einspanier et al., 1993). Therefore, the presence of ubiquitin in follicles is not surprising. However, in the preovulatory follicle ubiquitin concentrations increase from around 1.6 µg/ml to 2.3 µg/ml.

Other pituitary hormones play a role in follicular growth and development. Two such hormones that have been found at high levels within follicles are growth hormone and prolactin (Borromeo et al., 1996). Although their roles are not completely understood, it is known that these hormones are found within healthy, atretic, and cystic follicles.

Follicular Development

In order for the estrous cycle to occur, a pool of follicles must develop, typically one will become the dominant follicle, mature, ovulate, and finally luteinize to form a CL. An interruption at any of these steps will hinder follicular dynamics and hence reproductive efficiency. Many factors can impair these steps. Climate, nutrition, suckling, and management have been shown too affect follicular growth and development (Dominguez, 1995).

The gonadotropins are generally given credit for controlling the amount and rate of follicular development. One function of gonadotropins is to regulate androgen and estrogen production and secretion by theca and granulosa cells by inducing several key biosynthetic enzymes (Meidan et al., 1993). Similarly, IGF-I has been found to be a potent mediator of steroidogenesis and proliferation of granulosa cells by acting through an autocrine and/or paracrine mechanism (Gutierrez et al., 1996). Complementing this work, Spicer et al. (1996) found that IGFBP-3 plays a role in steroidogenesis within the developing bovine follicles by inhibiting IGF-I action. Beam and Butler (1997) found that higher plasma IGF-I leads to fewer days to first ovulation after parturition.

Gonadotropins have also been implicated in the selection of the dominant follicle. Ginther et al. (1996) reported that FSH peaks at the emergence of a cohort of follicles and is lowest at the day the dominant follicles rate of development deviates from others in the

cohort. Therefore, FSH can not be the factor that determines which follicles will become dominant and which will be subordinate, suggesting that dominance depends on LH.

Ginther et al. (1996) suggested changes in E2 concentrations determine the dominant follicle. However, Ginther et al. (1997) demonstrated that changes in E2 are not detectable until after emergence of the dominant follicle. Also, size can not be used to determine dominance until after the growth rate between dominant and subordinate follicles deviate. In sheep Dodson et al. (1997) showed that atresia was determined within the follicle. They demonstrated that gonadotropins did not determine which follicles would become atretic and which would ovulate.

The end point of follicular development is ovulation and the conversion of the follicle into a CL. Luteinizing hormone is responsible for this transformation. The CL is highly vascularized and is responsible for the production of P4 and maintenance of pregnancy. However, oocytes collected in the presence of a CL were few of high quality, even though there were greater numbers of follicles (Dominquez, 1995).

Ultrasound Guided Transvaginal Follicular Aspiration

Ultrasound guided transvaginal follicular aspiration is a process in which oocytes may be collected from live donors for use in vitro embryo production. Advantages to TVFA over oocytes obtained from an abattoir or superovulation are: oocytes may be collected regularly from the same donor, oocytes are coming from a live donor and thus having higher potential for known genetic superiority, oocytes can be collected regardless of stage of estrous cycle or condition of reproductive tract, and oocytes can be collected from donors that do not respond to superovulation treatments (Boni et al., 1996).

The process of TVFA involves inserting a vaginal ultrasound probe that is equipped with a needle guide into the vagina of a cow. The ovaries are located via palpation and are brought into contact with the probe. A 17 gauge needle is inserted through the guide and can puncture the follicle. While the needle remains in the cavity of the follicle a vacuum is applied and the oocyte and surrounding fluid are removed.

Other applications of TVFA have been devised for specific purposes. Ginther et al. (1997) developed a technique for sampling 20 μ l of follicular fluid without damaging or disturbing follicular development. Becker et al. (1996) made a direct comparison between endoscopic follicular aspiration and ultrasound guided TVFA. They found that

endoscopic technique causes less trauma to the ovary, but more damage to the oocyte and organs surrounding the ovary.

Frequency of oocyte pickup plays a major role in TVFA. Since it is possible to remove all large follicles, it is also possible to eliminate the presence of a dominant follicle. Likewise, when performing twice weekly retrieval rather than once weekly retrieval of oocytes, the number of potential follicular waves doubles. This not only increases the number of oocytes recovered, but it also reduces losses to atresia. Moreover, the interval that follicles are aspirated may also play a role in oocyte quality. Boni et al. (1996) found higher quality oocytes and greater development from follicles that were aspirated on shorter intervals (3 d vs. 4 d) in the buffalo.

In an attempt to increase the number of recovered oocytes Konishi et al. (1996) combined a hormonal treatments with TVFA. Cows were immunized against inhibin to reduce negative feedback of FSH on follicular development. In comparison with controls, the immunization increased both the number and size of follicles aspirated.

Individual technique influences oocyte recovery rates. Pieterse et al. (1991) recovered oocytes from 55% of all follicles aspirated, while earlier they only recovered oocytes from 27.4% of all follicles punctured (Pieterse et al., 1988). Recently, Boni et al. (1996) had an oocyte recovery rate of 49.4%. The differences in recovery rates could be attributed to many factors such as: tubing, filter system, vacuum pressure, aspirator experience, needle size, needle sharpness, and variations within animals.

The size of follicles aspirated had an effect on the ability of the oocyte to endure in vitro development (Machatkova, 1996). More specifically, follicles in the range of 2 to 7 mm produced oocytes that were more competent due to greater nuclear maturation. Thus, these oocytes had a higher cleavage rate and greater numbers of cells within each blastocyst than oocytes from follicles that were either larger or smaller than 2 to 7 mm.

Bovine In-Vitro Maturation, Fertilization, and Culture

After the collection of oocytes via TVFA, in vitro maturation, fertilization, and culture (IVM, IVF, IVC) provide a convenient means of developing oocytes in the laboratory and determining oocyte competency. Oocytes can be cultured until they have reached the blastocyst stage and then hatch through the zona pellucida. There are many

factors which may affect in vitro oocyte development, such as the quality of the oocyte, temperature, environment, and media used.

The temperature at which oocytes are collected and cultured is the first problem encountered in IVM. Pollard et al. (1996) found that the quantity and quality of oocytes that developed was significantly decreased when the temperature fell below 35°C for even brief intervals of time. The most suitable range for oocyte development was between 37°C and 39°C, a temperature corresponding to the physiological temperature of the donor.

The next most important element for successful in vitro development is the quality of the starting product. Oocytes can be graded by cytoplasmic appearance and the density of the cumulus surrounding the oocyte. This combination of cumulus and oocyte is called the cumulus oocyte complex (COC). These COC can be placed into one of four categories dependent upon the number of layers of cumulus surrounding the oocyte and its cytoplasmic consistency. Oocytes classified as a 1 would have a compact multilayered cumulus of greater than three layers and a homogeneous ooplasm. Oocytes classified as a 2 would have one or two compact layers of cumulus with a homogeneous ooplasm. Oocytes classified as a 3 would have a less compact irregular cumulus and the ooplasm contains dark clusters. The final classification is a 4. These oocytes are either nude or have an expanded cumulus mass with an irregular ooplasm (DeLoos et al., 1989). Laurincik et al. (1996) reported differences in development between the four classes of COC. Class 1 had the highest rate of development, with class 2 developing slightly less. Class 3 oocytes had some cleavage, but the cleavage rate dropped significantly, and class 4 oocytes had very little cleavage. Even though this method of predicting oocyte competence is not 100% reliable, it does serve as a good means of categorizing oocytes in terms of their developmental and fertilization potential.

The first culture stage is IVM. This allows the oocyte time to mature, more specifically it is the time for the nuclear components of the oocyte to mature. Work in the porcine species has shown that a preincubation before the addition of gonadotropins helps facilitate the number of oocytes reaching the blastocyst stage (Funahashi et al., 1997).

After IVM the COC undergo IVF. There are many different procedures and timing intervals used for IVF. For instance, in semen preparation a migration/sedimentation technique may be used to obtain spermatozoa that are capable of fertilization (Risopation et al., 1996). Likewise, either synthetic oviduct fluid (SOF) or Tyrode's fertilization medium can be used for IVF with similar results (Choi et al., 1991). Regardless of the methods used, there are still fundamental differences between IVF and in vivo fertilization. The most notable is the presence of the cumulus complex surrounding the oocyte. Sun et al. (1994) reported that the cumulus layer not only matures the oocyte, but provides a delay of syngamy and a block to polyspermy by engulfing the spermatozoa. The cumulus layer also facilitates the acrosomal reaction of the sperm once in contact with the zona pellucida.

The final step of in vitro development is IVC. There are many factors that surround the success of IVC, but the most important is providing the adequate nutrients for the developing embryo. Synthetic oviduct fluid media supplemented with alanine and glycine has been shown to have a significant effect on increasing embryo development (Lee and Fukui, 1996). Likewise supplementation with fetal calf serum before the developmental block stage was reported to increase the speed of development after the block stage is passed (Langendonck et al., 1997). Other factor also effect development, such as media renewal. Fukui et al. (1996) showed that media renewal hindered the development of embryos. Even with all the pitfalls of the IVM, IVF, and IVC systems, they still provide an alternative means to culturing embryos without having to rely on an animal host.

MATERIALS AND METHODS

Treatment Groups

A total of fifteen multiparous Holstein cows were randomly selected for one of two treatment groups. The first group consisted of multiparous cow between parity number 3 and 6 that had previously demonstrated moderate to high milk yields. All cows in this group (n = 8) calved between January 12, 1997 and February 20, 1997, had no dystocia, no illness (other than mastitis), and maintained moderate to high milk production throughout the study. This group was designated as Early Lactation (EL) and began aspiration on d 28 postpartum. Likewise, the cows that composed group two (n = 7) were all between their 3rd and 6th lactation. However, these cows calved between October 5, 1996 and October 20, 1996. Just as in group one, all of these cows had no dystocia, no illness (other than mastitis), and maintained moderate to high milk production throughout the study. This group was designated as the Mid Lactation (ML) cows and aspiration began on d 117 postpartum. Cows in both treatment groups were fed identical rations (Appendix Table 1), maintained non-pregnant, and lactating until the conclusion of all aspiration sessions.

Body Condition Scores and Body Weight

On each Tuesday throughout the study all cows were evaluated for their body conditioning status. Body condition scores (BCS) were assigned using the guidelines established by Braun et al. (1986). This method grades the cow's conditioning status on a scale of 1 to 5, with 1 being the least amount of body conditioning, and 5 being the highest. The cows were evaluated immediately after milking, before being given access to feed, and before oocyte retrieval. Immediately after BCS were assigned, each cow was weighed individually using a digital scale. After both measurements had been recorded the cow was then considered ready for oocyte retrieval.

The time frame in which both BCS and weights were recorded started at 14 d prepartum through 98 dpp for EL cows and 75 through 187 dpp for the ML cows. During the first 6 wk all measurements were taken on Tuesdays. However, for EL cows, there was a two-week time span before calving, in which feed intake could not be monitored or controlled. During this time all measurements were taken at the same time as the other cows in the study. No adjustments for body fill, or water intakes were made.

Daily Feed Intake & Energy Balance

Average daily feed intake was estimated for each animal by using data obtained from the Pinpointer system (4000B, AIS Corp., Cockeville, TN). The Pinpointer system was designed to measure the individual feed intake of a cow. Six cows are given access to one feed source that was located on an electronic scale. The pen was designed so only one cow could eat at a time. When the cow entered the feeding area, it triggered a sensor, which alerted the data recorder to the presence of a specific cow. The weight of the total mixed ration (TMR) within the feed bin was recorded. The system activated a sensor, which identified the cow by a transponder located on her neck chain. When the cow left the stall the recorder weighed the bin's contents again. The difference between the initial and final weight was added to the daily intake of the cow. Each cow's intake was printed daily. The system was checked daily, to determine if all parts were operating correctly. Feed intakes were checked for each cow to verify that they were all eating properly and adapting to the system. Residual feed was cleaned from the feed bin and weighed in order to check the accuracy of the printouts. Cows were rotated through the system in intervals of 12 d (data obtained on d 1 and d 2 were discarded thus giving cows time to adjust to the system). A group of six cows, three from each treatment group, were in each rotation. Entry and exit to the rotation was staggered, so that only two cows entered and left the system at any one time to avoid hierarchy problems between cows. Each cow went through this rotation at least three times (Appendix Figure 15). The average daily feed intake between treatment groups is reported in Appendix (Figure 16). We used milk yield, milk fat content, feed intake, BW, and estimated energy from the feed in conjunction with NRC factors to estimate net energy balance (Beam and Butler, 1997).

Blood Sampling

Jugular blood samples were collected twice weekly. Sampling started on the first Tuesday or Friday after calving for EL cows. Samples were collected on every Tuesday and Friday starting at 90 d postpartum for ML cows. Samples taken on Tuesday were taken after the PM milking and samples taken on Friday were taken after the AM milking. After the start of oocyte collections, jugular blood samples were taken from both treatment groups before TVFA and before any drugs had been administered. Each sample was stored at 4°C for 24 h. After 24 h the samples were centrifuged at 2700 × g

for 30 min and the serum collected. The serum was divided into two aliquots, 1 ml was stored at -80 °C and the remaining 4 ml stored at -20 °C.

Milk Fat and Protein

Milk samples were collected weekly, starting at 7 dpp for EL cows and 96 dpp for the ML cows. Samples were taken during the Thursday PM and Friday AM milkings. Each milk sample was analyzed for fat and protein content by the VA Tech DHI lab. These measurements could then be used for calculating energy balance and to monitor the animals' production performance.

Ultrasound-Guided Transvaginal Follicular Aspiration

Ultrasound-guided transvaginal follicular aspiration was performed twice weekly starting at approximately 117 d postpartum for ML cows and at 28 d postpartum for EL cows (oocyte retrievals for each animal started at the next weekly session once the cow had reached the appropriate number of days postpartum; dpp). The TVFA procedure was performed for 10 wk, with aspiration sessions occurring on Tuesday afternoons and Friday mornings. The equipment used in TVFA consisted of an ultrasound transducer (5MHz), a vaginal probe equipped with a needle guide, a 55 cm 17 gage needle with an echogenic tip (RAM Consulting, Madison, WI), and a vacuum pump fitted with a filter system to recover oocytes.

Acepromazine maleate was injected intravenously (44 mg/100 kg BW; Aveco Co., Inc., Fort Dodge, IA) to sedate each cow. A 6 ml epidural consisting of 2% lidocaine hydrochloride (Phoenix Scientific, Inc., St. Joseph, MO) was given to block rectal motility. In the event of an incomplete block of rectal motility, the epidural was supplemented with up to .3 ml of Rompun[®] (20 mg/ml; Moby Corporation, Shawnee, KS).

The cleansing of the anal area consisted of disinfecting the vulva and the anus using a diluted Nolvasan[®] solution (Fort Dodge Laboratories, Fort Dodge, IA). The vagina was also infused with two 60 ml saline solutions. The first was a 1:10 dilution of lidocaine in 0.9% sterile saline solution and the second was a 1:10 dilution of betadine in 0.9% sterile saline solution.

Follicles were observed via an Aloka 500V ultrasound monitor (Corimetrics, Wallingford, CT). The number and size of follicles on each ovary were recorded

immediately before the start of the oocyte retrieval. Follicular fluid was withdrawn from the largest follicle for hormonal analysis, when this follicle was larger than 10 mm in diameter.

Dulbecco's phosphate buffered saline (DPBS; Gibco, Long Island, NY) was used as a carrier for the oocytes. The DPBS was enriched with 10% vol/vol Newborn Calf Serum (NCS; Gibco), 1% vol/vol Penicillin/Streptomycin (PS) and 25 ug/ml Heparin (Sigma Chemical, St. Louis, MO). Oocytes were transferred through 1.25 m of Chemfluor Teflon tubing with an inner diameter of 1.59 mm (Atlantic Plastics, Roanoke, VA) under an approximate vacuum of 50 mmHg. The oocytes were then collected in an Em-con filter (Professional Embryo Transfer Supply, Canton, TX), and rinsed with DPBS. Oocytes were washed onto a search plate (Falcon 1012; Becton Dickinson and Co, Lincoln Park, NJ), located, and removed using a 5 to 10 μ l Drummond microdispenser pipette (Drummond Scientific Co., Broomall, PA). Oocytes were washed in TL HEPES supplemented with BSA (3 g/L; Sigma Chemical), classified, and then placed into maturation media containing TCM199 (Gibco), 10% vol/vol Fetal Calf Serum (FCS; Hyclone, Logan, UT), bFSH, bLH (0.01 U/ml each; NOBL Labs, Sioux Center, IA) and 1% vol/vol PS (Gibco; Gibbons et al., 1994). Treatment groups were kept in separate vials which contained 0.5 ml of maturation media and were incubated for 22 to 24 h at 39 °C and 5% CO₂.

Oocyte Evaluation

Oocytes were classified into one of four classes based on a system proposed by DeLoos et al. (1989). Classes were determined as follows: 4- compact multilayered cumulus with greater than three layers and a homogeneous ooplasm; 3- compact cumulus with only one to two layers with a homogeneous ooplasm which may appear coarse and had a darker shaded zona pellucida; 2- oocytes had less compact cumulus with irregular ooplasm that contained dark clusters; and 1- nude oocytes or oocytes that had an expanded cumulus that consists of a jelly-like matrix and irregular ooplasm (Figure 1).

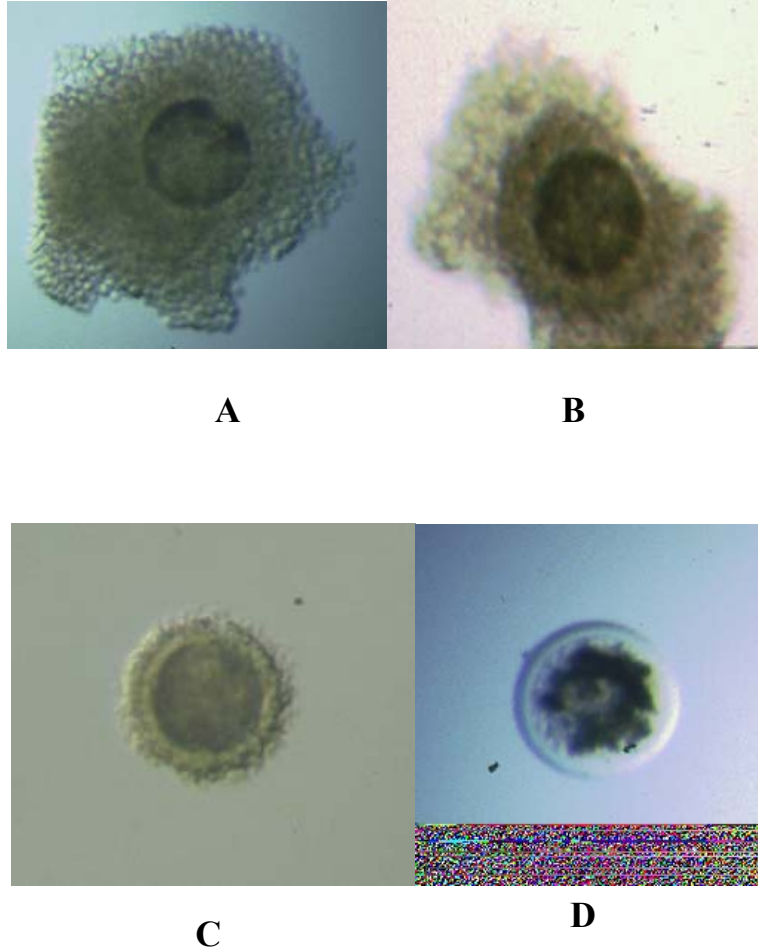


Figure 1. Example of the quality grades of oocytes obtained through transvaginal follicular aspiration.

- A. Good + (Score 4, G+): compact multi-layered cumulus with greater than three layers and a homogeneous ooplasm.
- B. Good (Score 3, G): compact cumulus of one to two layers with homogeneous ooplasm having a coarse appearance and a darker zona pellucida.
- C. Good - (Score 2, G-): less compact cumulus with irregular ooplasm containing dark clusters.
- D. Poor (Score 1, P): nude oocyte or expanded cumulus, irregular ooplasm with jelly-like matrix.

Assay Procedures

Double antibody radioimmunoassay procedures were used to determine the concentrations of GH, FSH, LH, and IGF-I in serum (Bolt and Caldwell, 1992). The same assay procedures were used to determine concentration of IGF-I in follicular fluid.

IGF-I concentrations: Frozen follicular fluid or serum samples were thawed and mixed. Eight hundred microliters of an extraction mixture made up of 87.5% ethanol (100%) and 12.5% 2N HCl was added to a 200 μ l aliquot of a follicular fluid sample in a microfuge tube, vortexed and then incubated for 1 h. The samples were placed in a Hermle Microfuge Z231 and spun for 10 min at 9982 x g. Five hundred microliters of the supernatant was added to 200 μ l of 0.855 M/L Tris Base to neutralize the sample. The samples were incubated at -20° C for 1 h. The samples were removed from the freezer and placed in a Beckman J6-B clinical centrifuge for 30 min at 3935 g. The supernatant was poured off into a 12 x 75 mm plastic tube (VWR, Bridgeport, NJ) and stored at -20° C until assayed.

On d 1 of the assay, 100 μ l of the tracer plus 100 μ l of the first antibody (mouse anti-IGF-I) were added to the 40 μ l of the thawed supernatant in a PBS EDTA buffer and stored 4° C for 24 h. One hundred microliters of the second antibody (goat anti-mouse) was added on d 2 to each sample followed by an additional 72 h incubation at 4° C. After incubation 1 ml of cold double distilled PBS was added to each sample and the samples were centrifuged at 2,200 g for 30 min. The samples were decanted and allowed to dry for 24 h. Samples were then counted for 1 min on a gamma counter (Weber et al., 1998).

Luteinizing hormone concentrations in bovine serum samples: The concentrations of LH in the serum samples were obtained using the methods of Bolt and Caldwell (1992). Serum samples and a standard curve formed from a purified form of LH (USDA-bLH-B-6) were placed in PBS assay buffer (500 μ l) and incubated with USDA-309-684p antibody (1:35,000) for 48 h. Then, 125 I radio-labeled LH was added to each sample and kept at room temperature for 48 h. The second antibody (anti-rabbit gamma globulin serum, [1:8 dilution]) was added to each sample, followed by an additional 48 h of incubation at 4° C. The samples were then centrifuged at 2200 g for 45 min and the supernatant was decanted. The tubes were allowed to dry for 24 h before being placed on a gamma counter where each tube was subjected to a 1 min count.

Follicle stimulating hormone concentrations in bovine serum samples: Serum FSH concentrations were obtained using a double antibody radioimmunoassay procedure described by Bolt and Caldwell (1992) with the following changes. The FSH assay utilized rabbit ovine-FSH antiserum (NIDDK-anti-oFSH-1, [1:80,000 dilution]) as the primary antibody and highly purified bovine FSH (USDA-bFSH-I-2) as both iodinated tracer and reference standard. Sheep anti-rabbit gamma globulin (1:8 dilution) was used as the second antibody. The ovine-FSH antiserum bound 15% of radiolabeled FSH in the absence of the unlabeled hormone with the sensitivity of the assay being < 0.1 ng/ml of the reference standard.

Growth hormone concentrations in bovine serum samples: The concentration of bovine growth hormone (bGH) in serum was obtained using recombinant bovine growth hormone (rbGH) as a control. A dilution of 1:500 rabbit-anti-BGH in 1% BSA was prepared for the first antibody of this double antibody assay. On the day of the assay, serum samples were thawed and mixed. Three hundred microliters of serum was added to 200 μ l of 1% BSA assay buffer. An additional 100 μ l of the first antibody was added to all tubes (excluding background controls and total count tubes). One hundred microliters of the 125 I tracer was added and the mixture was allowed to incubate for 48 h at room temperature. Following incubation, an additional 100 μ l of second antibody, sheep anti-rabbit, was added and the solution was allowed an additional 72 h of incubation at 4 $^{\circ}$ C. Following the final incubation, 1 ml of cold PBS was added to each tube. The tubes were then centrifuged at 2,200 g for 30 min. The samples were decanted and allowed to dry for 24 h. Samples were then counted for 1 min on a gamma counter.

Estradiol and Progesterone concentrations in bovine follicular fluid: Serial dilutions of 1:100, 1:200, and 1:400 were prepared using bovine follicular fluid and DPC zero calibrator, respectively (Diagnostic Products, Los Angeles, CA). One hundred microliters of the diluted sample was placed in polypropylene antibody coated tubes (Diagnostic Products, Los Angeles, CA), and mixed with 1 ml of 125 I-labeled progesterone/estradiol (Kendrick et al., 1999). The hormones were allowed 3 h to compete for estrogen/progesterone binding sites. After 3 h the supernatant was decanted to terminate the competition. Tubes were given 24 h to dry and then were counted using an 125 I gamma counter (1 min per sample).

Progesterone concentrations in bovine serum samples: Serum samples (0.1 ml) were analyzed without dilution using the same RIA kit (Diagnostic Products) and the same assay procedure as follicular fluid (Holt et al., 1989).

Non-esterified fatty acid and β -hydroxybutyrate concentrations in bovine serum. Light absorbancy was used to measure the concentration of β -hydroxybutyrate and non-esterified free fatty acids (NEFA) in serum. To measure NEFA, the NEFA C kit, was purchased and modified for expected concentrations (Wako Chemicals, Dallas, TX; Eisemann et al., 1988). Serum samples that had been stored at -80°C were thawed and mixed the morning of the assay. Color reagents and serum were mixed and incubated according to protocol, then transferred to micro-titer plates for absorbance readings.

To measure the relative differences in the concentration of β -hydroxybutyrate (BHB) between treatment groups, the β -hydroxybutyrate kit was purchased from Sigma Diagnostics (Sigma Diagnostics, St. Louis, MO). The assay procedure was done according to Sigma's protocol, however, all reagent and sample volumes were reduced by a factor of ten to accommodate the capacity of micro-titer plates. All samples were run in duplicate and read at 340 nm on a Titertek plate reader.

Post Aspiration Synchronization

As each cow completed the 10 wk of TVFA, her estrous cycle was synchronized. Synchronate B[®] ear implants (6 mg norgestomet) were used to accomplish this. Two doses of GnRH were given at 100 μg each. The first dose of GnRH was given at d 5 after the last TVFA session, and the second dose was given at d 18, 48 h after implant removal. Synchronate B[®] implants were administered on 7 d after the last TVFA session. On d 15, PGF_{2 α} was given to each cow, and on d 16 the Synchronate B[®] implants were removed. Throughout the post-aspiration treatment period follicular development was recorded using a transrectal ultrasound probe. Cows were scanned daily, growth of follicles on each ovary was monitored and recorded. Estrus was monitored using the Heatwatch[®] system (DDx Incorporated, Boulder, CO). All cows showing estrus were inseminated using AI. Cows that became cystic were monitored via a transrectal ultrasound probe. Cystic cows were given treatments of PGF_{2 α} or GnRH depending upon the type of the cyst.

Statistical Analysis

We analyzed data by using the general linear model (GLM) procedures in SAS. We used analyses of variance to evaluate effects of treatment; cow within treatment; and the linear, quadratic, and cubic effects of the interaction of treatment by week of aspiration on hormone and metabolite profiles in serum and follicular fluid; on follicle numbers; number of oocytes recovered; and oocyte quality (Appendix Tables 2 to 14).

RESULTS

The data revealed many significant differences between EL (28 to 98 dpp) and ML cows (117 to 187 dpp) when evaluated with respect to time. However, only energy balance was significantly ($P < 0.01$) different by treatment. The mean energy balance values for EL and ML cows were -1.55 ± 0.33 Mcal/kg and 2.43 ± 0.32 Mcal/kg, respectively. Energy balance was significantly affected ($P < 0.001$) by a cubic time by treatment interaction. The EL cows fell into a negative energy balance after parturition (Figure 2), going from 2.76 ± 0.33 Mcal/kg at parturition to -4.39 ± 0.323 Mcal/kg by 40 dpp (retrieval wk 1.5). The EL cows regained a positive energy balance by 85 dpp (retrieval wk 7.5; 0.22 ± 0.33 Mcal/kg). The ML cows were at a positive energy balance (3.36 ± 0.32 Mcal/kg) by the start of aspirations (117 dpp) and maintained this positive energy balance throughout the study.

Changes in energy balance were reflected in changes in BW and BCS. The treatment by time interaction was quadratic ($P < 0.0001$) for both BW and BCS (Figure 3). The EL cows decreased in BW until retrieval wk 4 (56 dpp), going from 621.2 ± 6.8 kg to 564.0 ± 6.8 kg, and then recovering to 589.8 ± 6.8 kg by the completion of aspirations (98 dpp). The ML cows had little variation in BW, increasing slightly from 574.3 ± 6.5 kg to 589.8 ± 6.5 kg over the course of the study. The BCS decreased for both treatment groups (Figure 3). The BCS in EL cows fell at a faster rate than BCS for ML cows, going from scores of 2.75 ± 0.07 (14 d prepartum) and 2.33 ± 0.06 (80 dpp) to scores of 2.1 ± 0.07 (98 dpp, retrieval wk 10) and 2.25 ± 0.06 (190 dpp, retrieval wk 10), respectively. The curvilinear changes in milk production of the EL cows were nearly the reciprocal of their energy balances (Figure 2). Milk production for the EL cows peaked at 45.0 ± 1.1 kg/d by 40 dpp (retrieval wk 1.5) and then fell to 38.1 ± 1.1 kg/d by 98 dpp (retrieval wk 10). Milk production in the ML cows only fell slightly, starting at 41.3 ± 1.1 kg/d (90 dpp) and declining to 35.5 ± 1.1 kg/d by the end of the study (190 dpp). However, milk components were in higher concentration for the ML cows than for EL cows. For instance, the percentage of milk fat fell quickly in the EL cows (4.10 ± 0.15 % to 3.30 ± 0.15 %) dropping below the ML cows (3.65 ± 0.14 %) by 14 dpp (Figure 4).

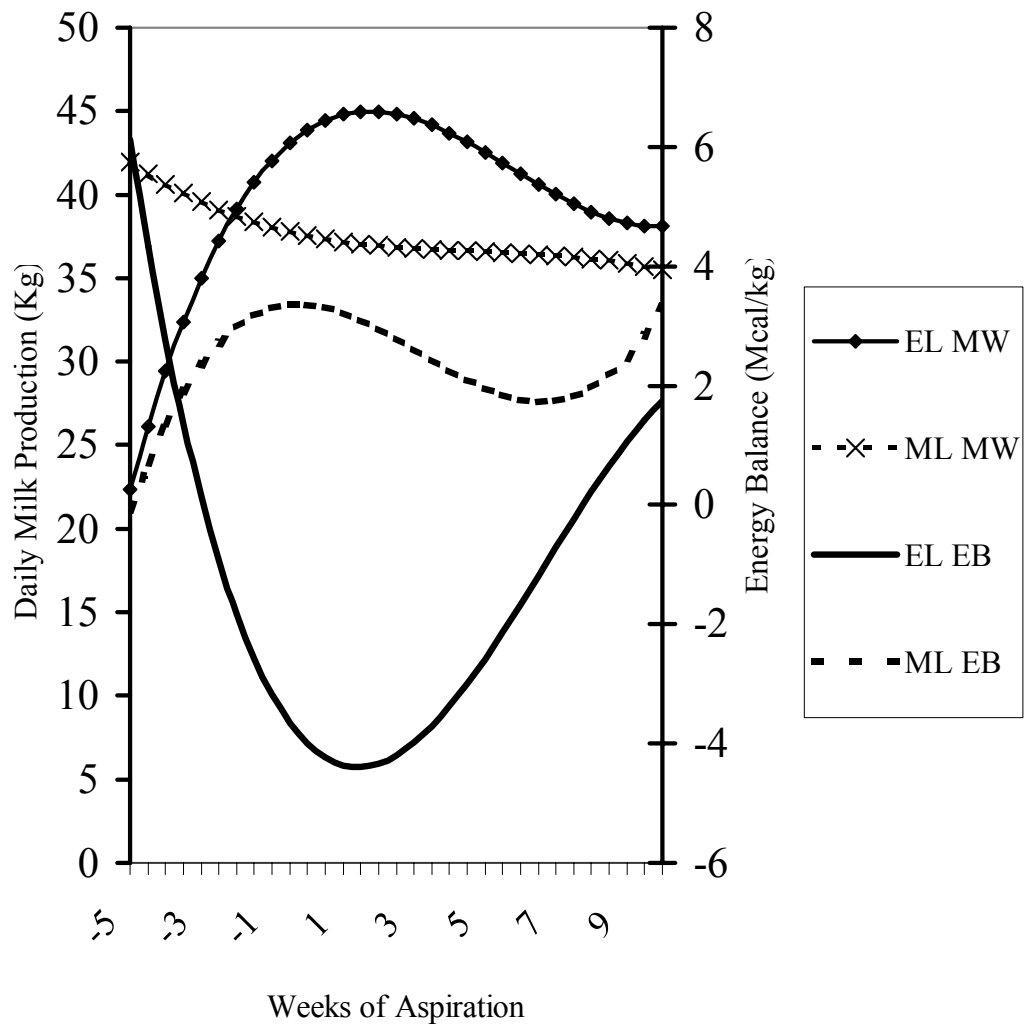


Figure 2. The daily milk production (MW) and estimated energy balance (EB) of early (EL) and mid lactation (ML) Holstein cows before and after the start of aspiration sessions

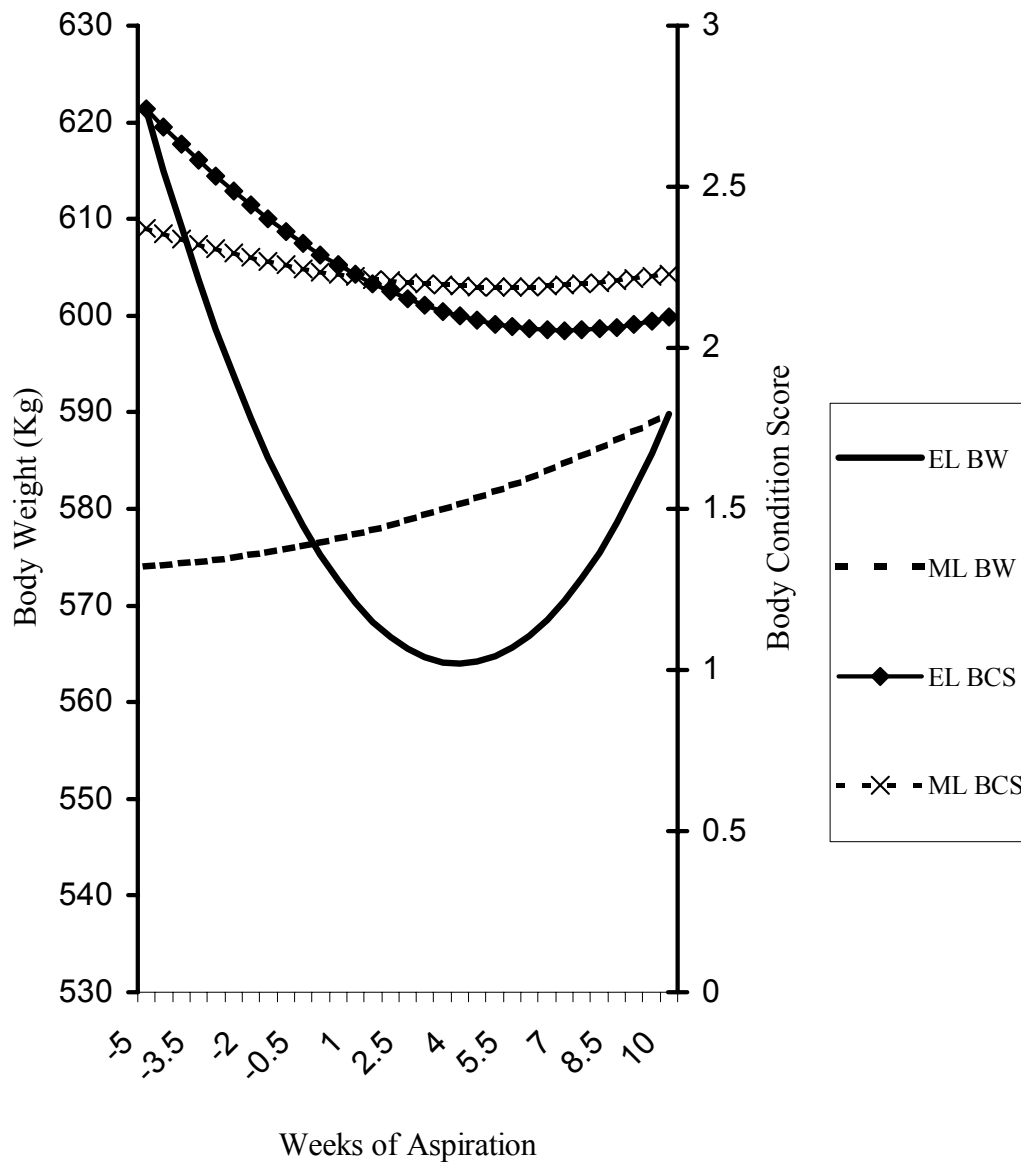


Figure 3. The body weight (BW) and body condition score (BCS) of early lactation (EL) and mid lactation (ML) Holstein cows prior to and during ten weeks of TVFA that was conducted twice weekly.

Milk protein change was similar to milk fat, going from 3.5 ± 0.05 % (1 dpp) to 2.7 ± 0.05 % (56 dpp, retrieval wk 4) and then recovering to 2.9 ± 0.05 % (98 dpp, retrieval wk 10) for the EL cows. The milk protein percentage in ML cows increased slightly throughout the study, going from 3.0 ± 0.05 % (90 dpp) to 3.15 ± 0.05 % (190 dpp, retrieval wk 10).

Likewise, the concentration of NEFA fell in the EL cows, going from 0.5 ± 0.012 mEq/l to 0.22 ± 0.012 mEq/l (Figure 5). However, the NEFA concentration for the ML group was stable at 0.18 ± 0.012 mEq/l throughout. There was a significant stage of lactation effect ($P < 0.05$) for BHB concentrations, with EL cows averaging 4.9 ± 0.2 mg/dl serum compared with 3.55 ± 0.21 mg/dl for the ML cows. The serum concentration of BHB in the EL cows increased from 3.83 ± 0.2 to 4.01 ± 0.2 mg/dl (Figure 5). However, the serum BHB concentrations of ML cows decreased from 3.27 ± 0.21 to 2.96 ± 0.21 mg/dl after 6.5 wk of oocyte retrievals (163 dpp), but then recovered to 3.05 ± 0.21 mg/dl by retrieval wk 10.

Aspiration Data

Over the course of oocyte retrievals a significant difference ($P < 0.01$) was revealed between aspirators and the number and proportion of different quality grades of oocytes they could retrieve. However, this difference was independent of treatment and thus does not bias the differences determined between treatment groups.

The data obtained following transvaginal follicular aspiration revealed many significant differences between treatment groups for ovarian and oocyte data. For instance, total number of follicles increased in both groups over the course of aspirations (Figure 6). However, the ML cows (13.6 to 17.9 ± 0.3) had a greater increase ($P < 0.01$) than the EL cows (14.3 to 15.0 ± 0.3) as aspirations progressed. Most of the increase in total follicle number was because of the increase in the number of small follicles (Figure 6). The EL cows started aspirations with an average of 9.4 ± 0.3 follicles per cow that increased to 10.8 ± 0.3 by retrieval wk 10. The number of small follicles in ML cows increased from 9.7 ± 0.3 to 13.0 ± 0.3 from the beginning to the end of oocyte retrieval sessions. There were no differences between treatment groups in very large (0.50 ± 0.06), large (0.6 ± 0.06), or medium size follicles (3.02 ± 0.19).

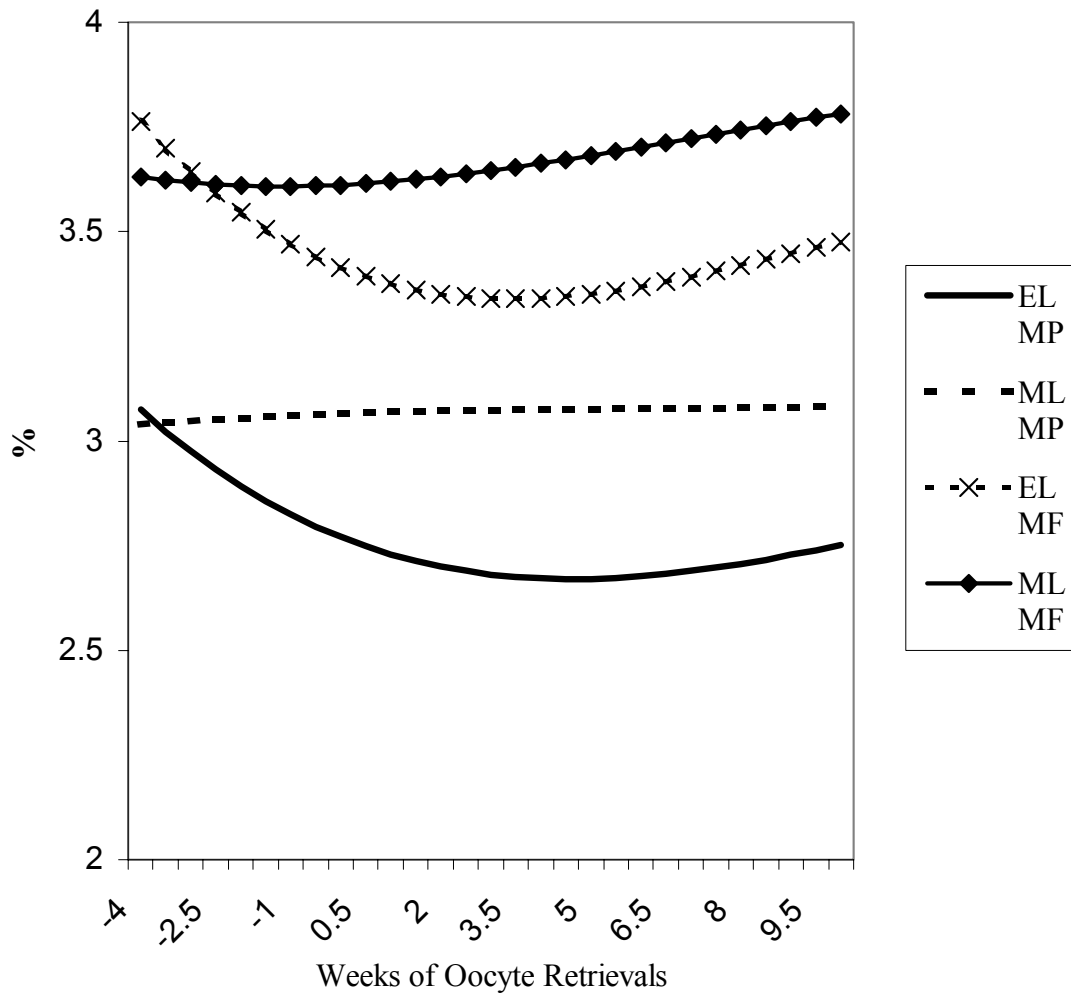


Figure 4. The average milk fat (MF) and milk protein (MP) percentage of early (EL) and mid lactation (ML) cows for the period prior to the start of oocyte retrievals and for the duration of the study.

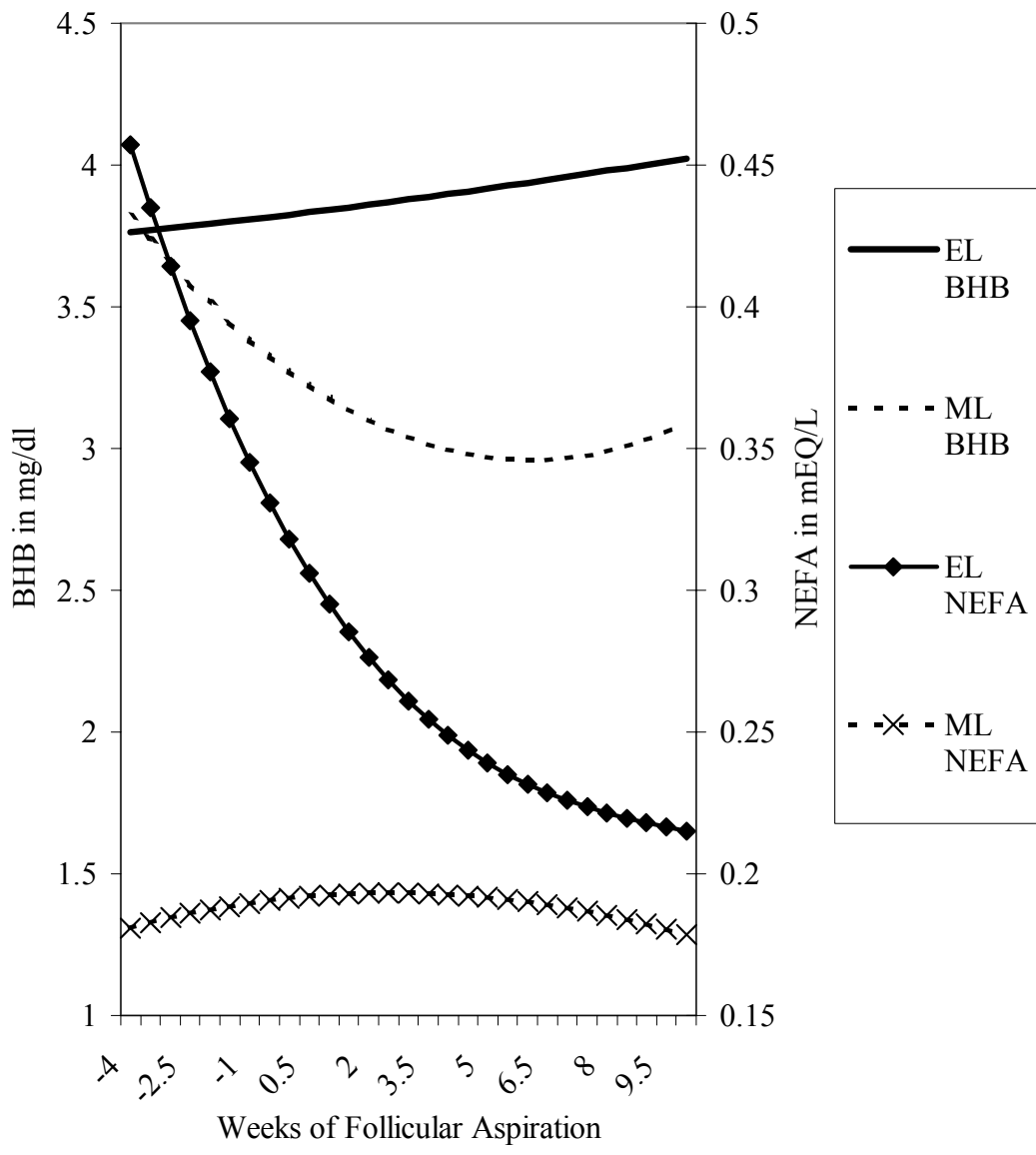


Figure 5. The serum concentrations of non-esterified fatty acids (NEFA) and Beta-hydroxybuterate (BHB) in early (EL) and mid lactation (ML) cows for the weeks preceding and during follicular aspiration.

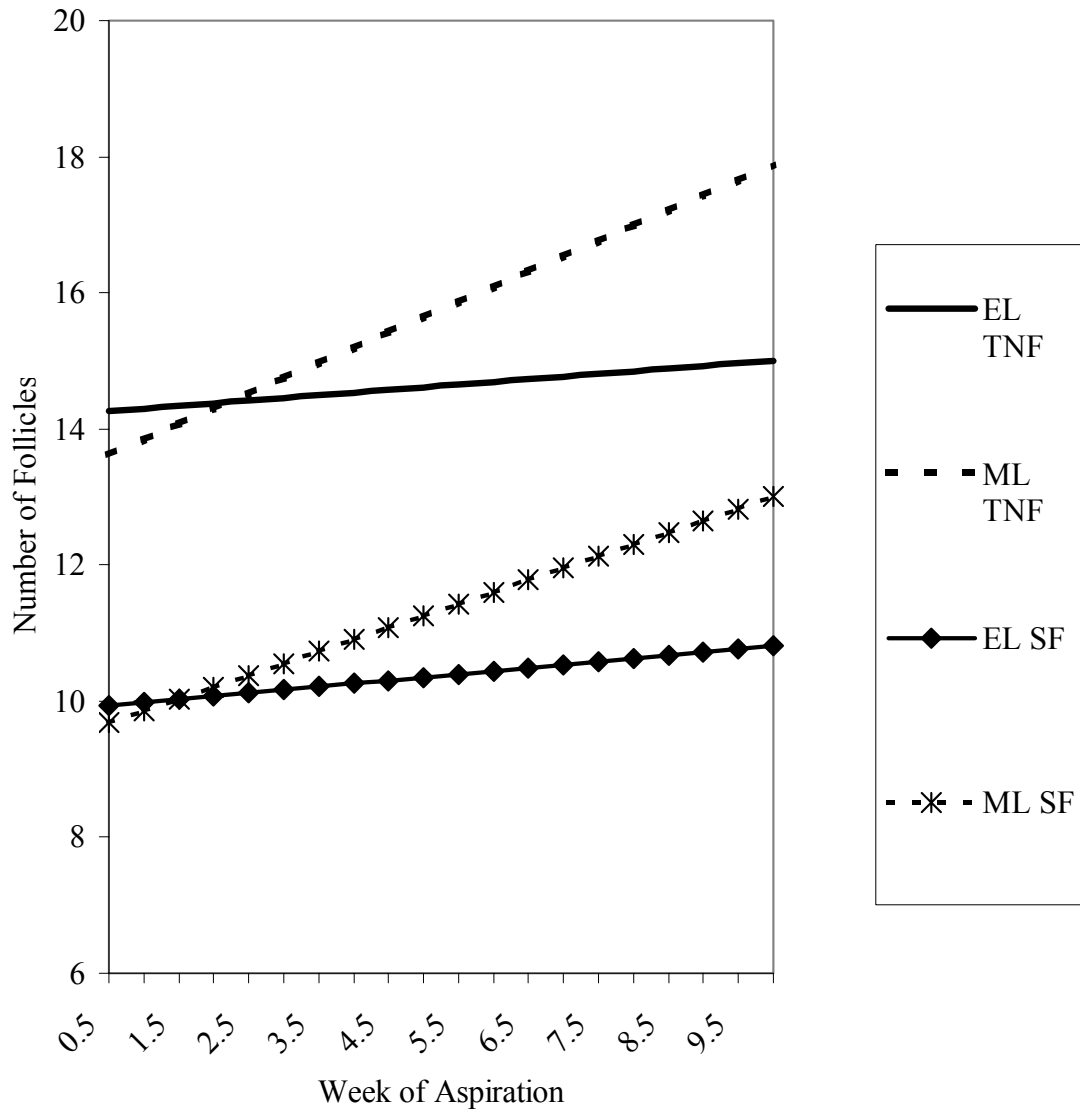


Figure 6. The number of small follicles (SF) and the total number of follicles (TNF) for early (EL) and mid lactation (ML) Hostein cows during ten weeks of TVFA conducted twice weekly.

There was no significant difference in the total number of oocytes recovered, only differences in the proportion of the oocyte quality grades. However, there was a noticeable trend ($P=0.054$) in the total number of oocytes recovered between treatment groups and their interaction with time. The ML showed a nearly linear decrease going from 9.13 ± 0.95 to 7.14 ± 0.95 oocytes recovered per cow over the course of aspirations. The EL cows exhibited a more rapid decrease going from 9.38 ± 0.99 to 5.54 ± 0.99 oocytes per cow by week 6.5 of retrievals (104 dpp), but then recovered to 5.96 ± 0.99 oocytes per cow by the end of retrieval sessions. The overall means of total oocytes recovered per cow per retrieval session was 6.08 ± 0.29 and 7.50 ± 0.30 for EL and ML cows, respectively.

The changes of oocyte quality did not follow the linear increases of follicle numbers, but instead all significant differences were curvilinear interactions with time. For instance, the number of poor oocytes collected in the EL cows started high at 5.9 ± 0.2 and then fell to an average of 3.8 ± 0.2 from retrieval wk 3 to 8 (49 dpp to 84 dpp) and then declined again to 2.1 ± 0.2 . The number of poor quality oocytes from ML cow was relatively constant with a mean of 4.9 ± 0.3 (Figure 7). There were no significant differences between treatment groups in the number of good plus (0.34 ± 0.76), good (0.71 ± 1.11), or good minus oocytes (1.61 ± 1.54) collected.

The percentage of good minus oocytes in the EL cows rapidly increased from $8.7 \pm 2.6\%$ (30 dpp) up to $33.0 \pm 2.6\%$ by retrieval wk 8 (84 dpp), where it plateaued for the remainder of the study (Figure 8). On the other hand, the good minus percentage in ML cows fell from $25.0 \pm 2.1\%$ to $17.8 \pm 2.1\%$ at the mid-point of retrieval sessions (152 dpp), and then increased again to $32.5 \pm 2.1\%$ by the end of oocyte retrieval. The percentage of good plus oocytes change resembled the trends in number of poor oocytes (Figure 7). The EL group started with only $1.6 \pm 0.9\%$ good plus oocytes, and then the percentage increased to $10.7 \pm 0.9\%$ by retrieval wk 2.5 (48 dpp), and then decreased to $0.2 \pm 0.9\%$ at retrieval wk 7.5 (81 dpp), but once again increased to $9.8 \pm 0.2\%$ by retrieval wk 10 (98dpp). Once again, the ML group was more stable with a starting

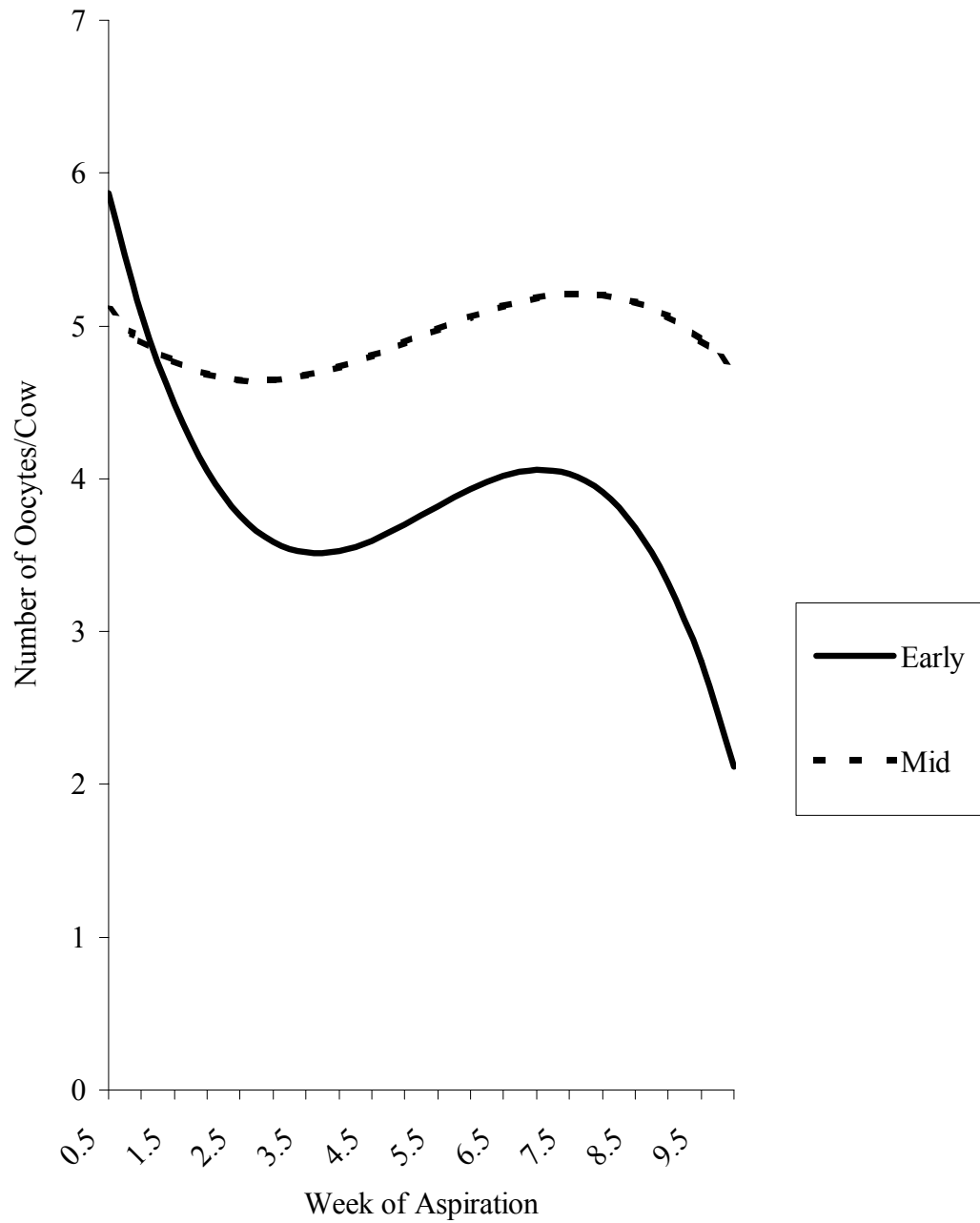


Figure 7. Number of poor oocytes collected for early and mid lactation Holstein cows during ten weeks of TVFA conducted twice weekly.

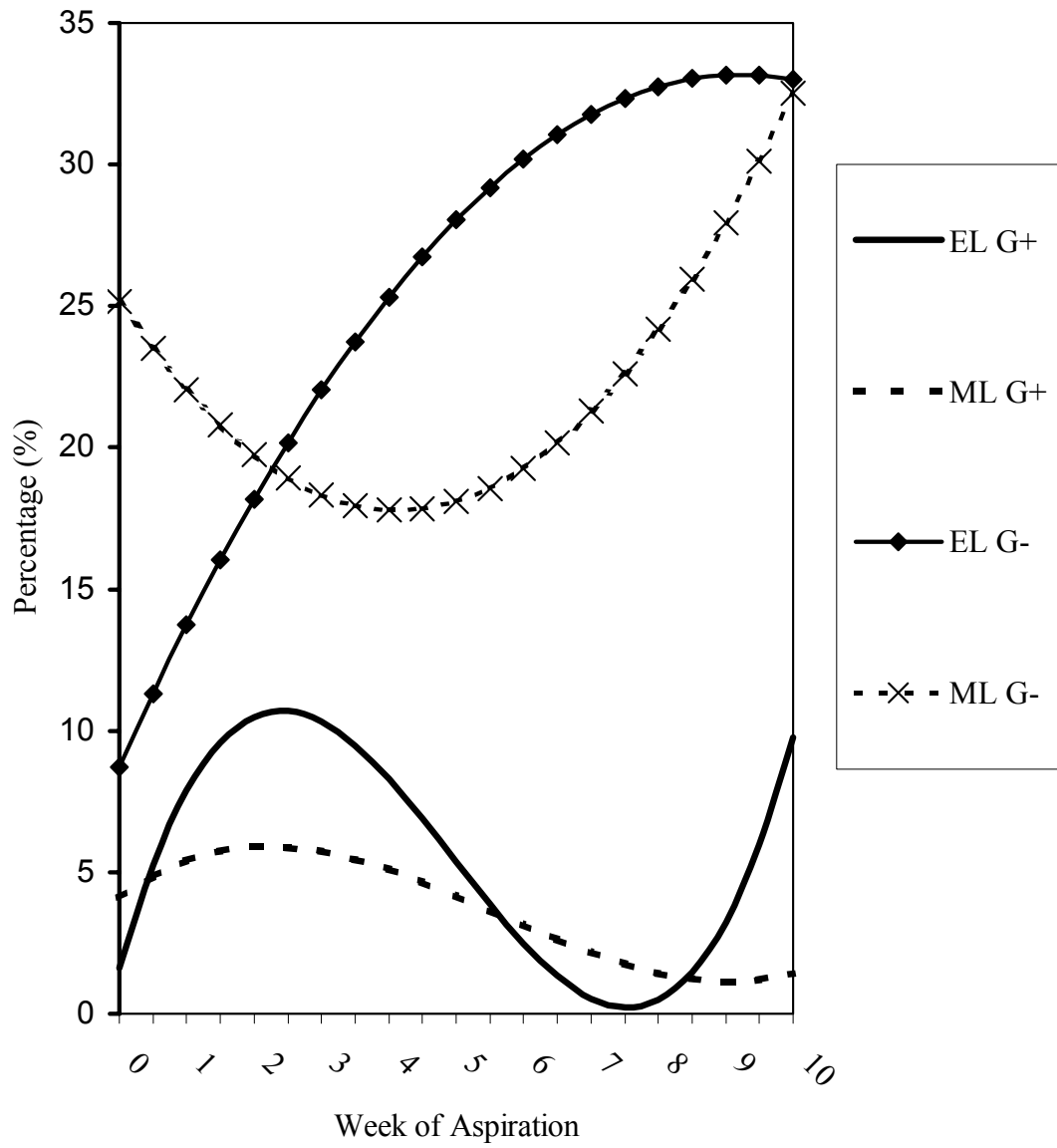


Figure 8. The percentage of good plus (G+) and good minus (G-) oocyte collected from early (EL) and mid lactation (ML) cows over ten weeks of TVFA conducted twice weekly.

percentage of $4.1 \pm 0.9\%$ good plus oocytes, which only increased to $5.9 \pm 0.9\%$ by retrieval wk 2.5 (135 dpp) and then slowly declined to $1.4 \pm 0.9\%$ by retrieval wk 10 (190 dpp; Figure 7). There was no significant difference in the number or percentage of good oocytes with an average of 1.7 ± 0.2 oocytes per session per cow, which made up an average of $9.9 \pm 1.2\%$ of the oocytes collected each session.

The presence of a CL was also noted during TVFA. A CL was detected in $64.4 \pm 3.1\%$ of the EL group (Figure 9). The percentage of cows having a CL decreased to $27.4 \pm 3.1\%$ by retrieval wk 6.5 (67 dpp), but then increased to $44.8 \pm 3.1\%$ by retrieval wk 10 (98 dpp). All ML cows started aspirations having a CL. The presence of a CL quickly decreased until retrieval wk 7.5 (170 dpp), where it reached its nadir at $12.8 \pm 3.3\%$ and then increased to $25.2 \pm 3.3\%$ by retrieval wk 10 (187 dpp).

Hormonal Profiles

Concentrations of serum gonadotropins were measured and analyzed over time. There was a significant treatment by time interaction ($P < 0.01$) for serum LH (Figure 10). Serum LH concentrations in ML cows were consistently higher than for the EL cows during aspiration session, even though the main effect of treatment was not different (EL 0.38 ± 0.03 ng/ml; ML 0.47 ± 0.03 ng/ml). The concentration of serum LH increased in the EL cows from 0.26 ± 0.03 ng/ml at retrieval session 1 (30 dpp) to 0.48 ± 0.03 ng/ml at wk 6 (70 dpp), and then decreased to 0.2 ± 0.03 ng/ml by the final session (98 dpp). The serum concentration of LH for the ML cows followed the same pattern going from 0.33 ± 0.03 ng/ml at retrieval session 1 (117 dpp) to 0.57 ± 0.03 ng/ml at wk 6 (159 dpp) and then falling to 0.34 ± 0.03 ng/ml by the final session (190 dpp). Unlike LH, serum FSH peaked at higher concentrations in EL cows rather than in ML cows. The quadratic pattern of FSH secretion ranged from 0.18 ± 0.026 ng/ml to 0.50 ± 0.026 ng/ml with a nadir at 48 dpp (retrieval wk 3) for the EL cows (Figure 10). Whereas, FSH in ML cows increased slightly, going from 0.20 ± 0.027 ng/ml to 0.25 ± 0.027 ng/ml over the course of the study.

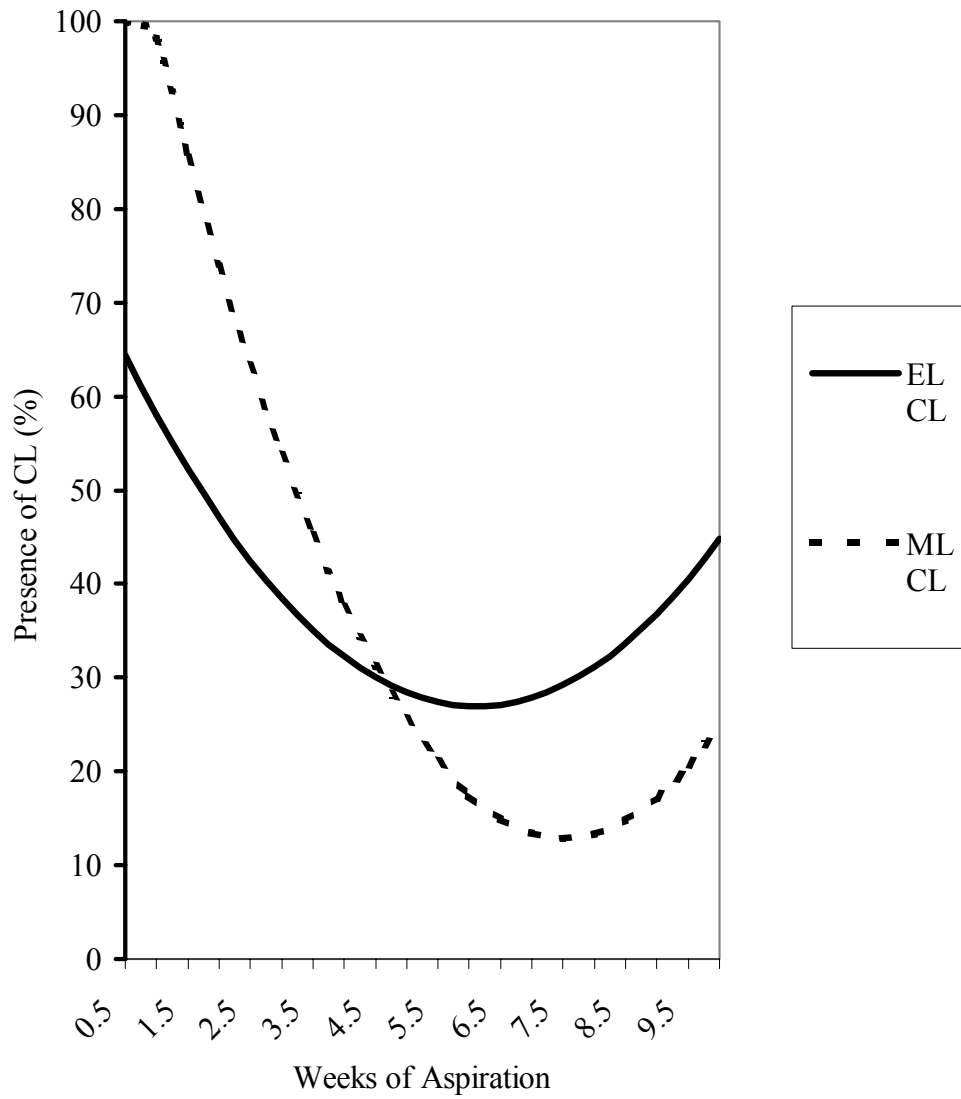


Figure 9. The percentage of Holstien cows in the early (EL) and mid lactation (ML) which had a Corpus Luteum present at the time of follicular aspiration.

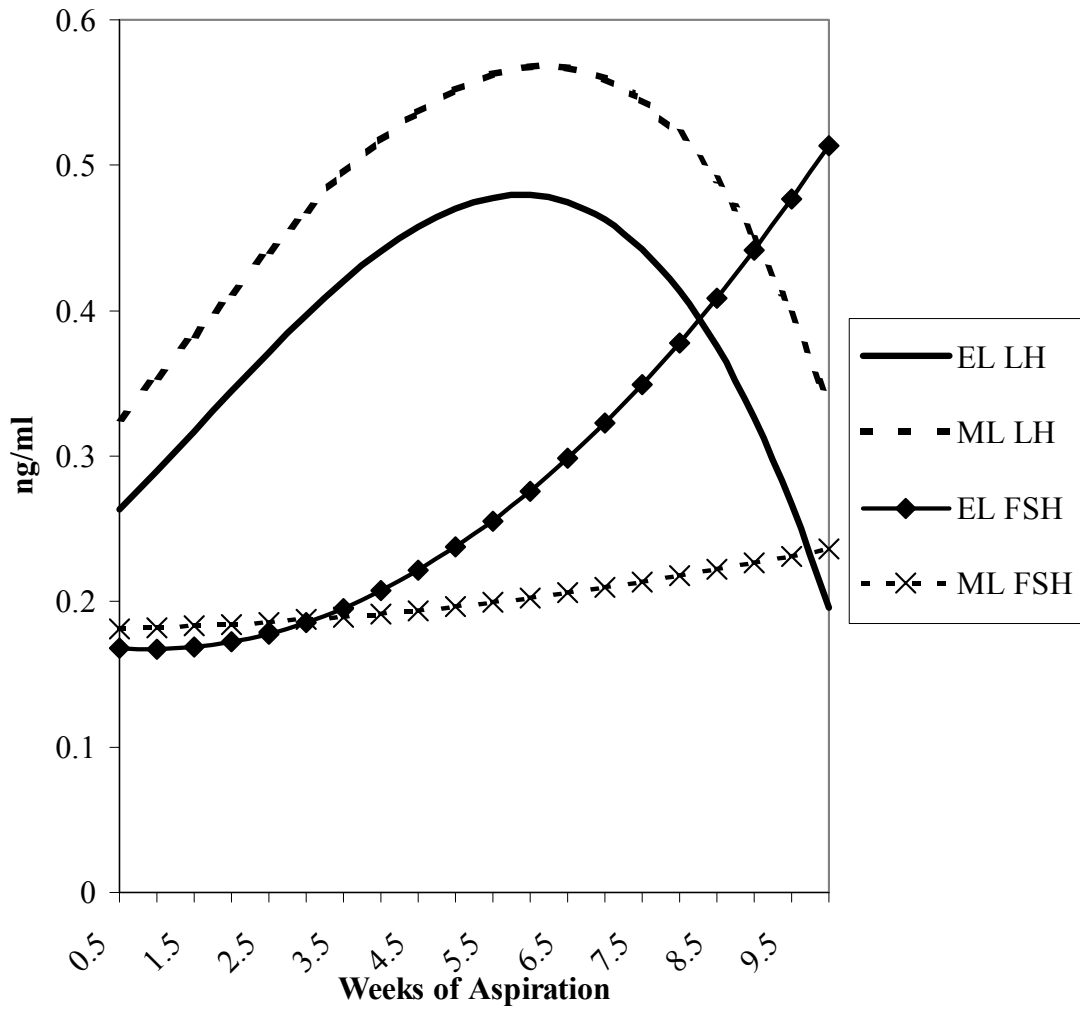


Figure 10. Serum luteinizing hormone (LH) and follicle stimulating hormone (FSH) concentrations in early (EL) and mid lactation (ML) Holstein cow during ten weeks of TVFA that was conducted twice weekly.

Concentrations of steroid hormones were measured in both follicular fluid and serum. There was a significant cubic time by treatment interaction ($P < 0.01$) for serum P4. The serum P4 concentration in EL was 2.25 ± 0.35 ng/ml at the start of oocyte retrievals, and was stable for the remainder of the study (Figure 11). Serum P4 in ML cows fell from a concentration of 2.52 ± 0.33 ng/ml to 0.5 ± 0.33 ng/ml by wk 6 (159 dpp) of oocyte retrievals (Figure 11). Unlike the curvilinear changes of serum P4, follicular fluid P4 concentrations followed a linear pattern (Figure 11). Follicular fluid P4 in EL cows fell only slightly, going from 270 ± 16.7 ng/ml to 245 ± 16.7 ng/ml, whereas follicular fluid P4 in ML cows increased from 100 ± 16.6 ng/ml to 510 ± 16.6 ng/ml (Figure 10). Follicular fluid estradiol was also measured, but no significant differences ($P > 0.05$) could be detected between treatment groups; the mean concentration of follicular fluid estradiol was 1233.5 ± 116.57 pg/ml. There was a significant linear interaction of treatment by time ($P < 0.01$) between EL and ML cows for serum IGF-I concentrations (Figure 12). The EL cows showed a steady decrease in serum IGF-I concentration, going from 30.12 ± 3.29 ng/ml at the start of the study to 23.29 ± 3.29 ng/ml by the conclusion of the study (Figure 12). On the other hand, the ML cows showed a gradual increase in serum IGF-I, going from 41.98 ± 3.02 ng/ml at the start of the study to 49.85 ± 3.02 ng/ml by the end of the study. The concentration of follicular fluid IGF-I was characterized by a linear interaction ($P < 0.01$) of treatment by time (Figure 12). Follicular fluid IGF-I in the EL group was significantly lower ($P < 0.01$) than in the ML group, starting at 145.19 ± 13.11 ng/ml at the first retrieval session and increasing to 154.20 ± 13.11 ng/ml by the last session. Follicular fluid IGF-I in the ML cows started at a higher concentration, 159.66 ± 12.45 ng/ml, and increased more rapidly, reaching 179.26 ± 12.45 ng/ml by the last retrieval session.

There was no significant difference in the number of small follicles during the post aspiration synchronization (PAS). There were however significant differences between treatment groups over time in the number of very large (>12 mm), large (9 to 12 mm), and medium follicles (6 to 8 mm), as well as the occurrence of CL during the PAS. The ML cows had a higher average number of very large follicles and showed a sharp

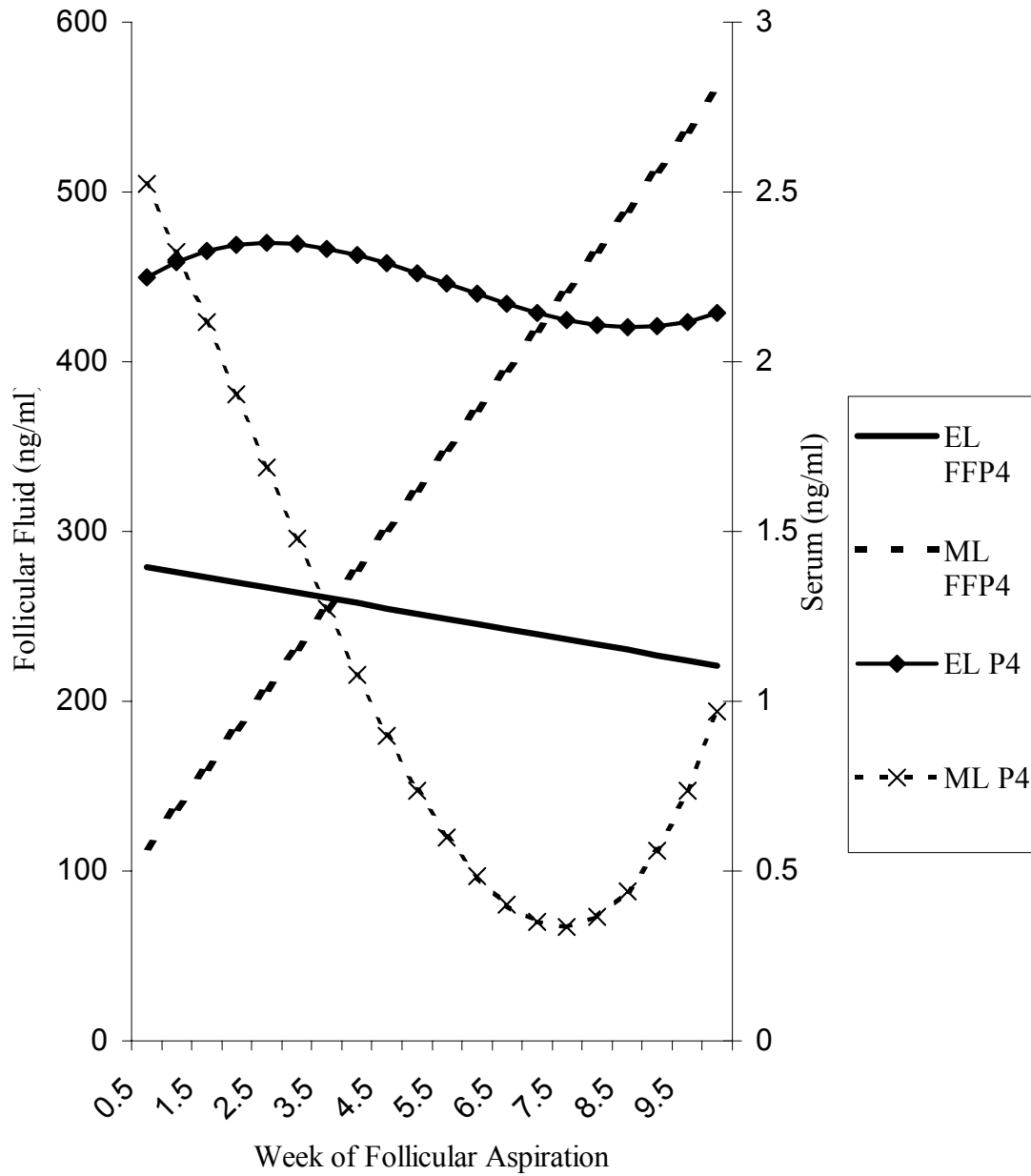


Figure 11. Serum Progesterone (P4) and Follicular Fluid Progesterone (FFP4) in early (EL) and mid lactation (ML) Holstein cows during ten weeks of twice weekly TVFA.

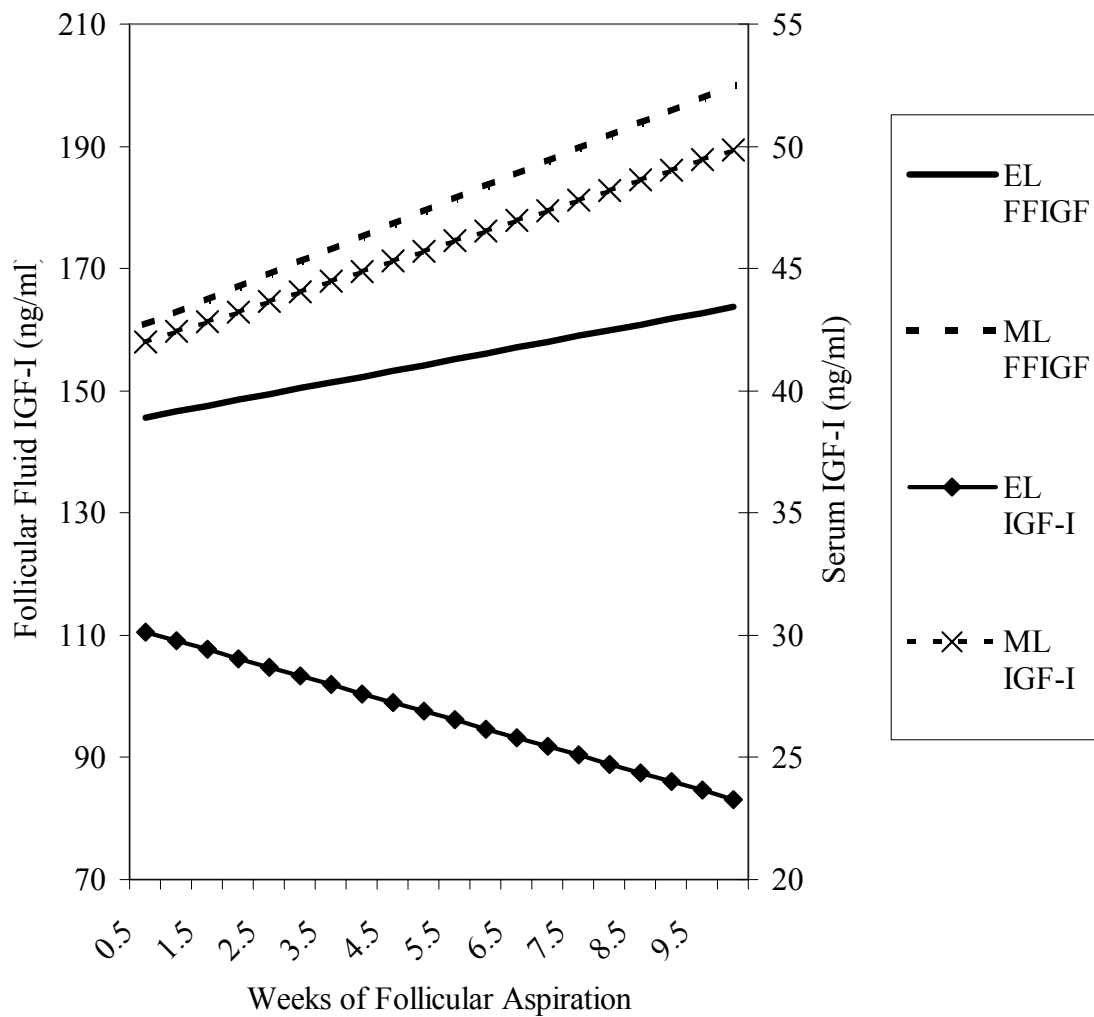


Figure 12. Serum insulin-like growth factor-I (IGF-I) and follicular fluid (FFIGF-I) concentrations in early (EL) and mid lactation (ML) Holstien cows during ten weeks of follicular aspirations.

increase from d 15 to 20 of PAS, while EL cows decreased in the number of very large follicles from d 11 to 20 (Figure 13). Both treatment groups had decreasing numbers of medium and large follicles. The ML cows had fewer medium and large follicles at the start of PAS and their number of medium follicles decreased more rapidly than EL cow (Figure 13). The percentage of cows with a CL increased more rapidly for ML cows than EL cows (0.3 to 40.9 % and 1.4 to 26.5%, respectively) from d 1 to 20 of PAS (Figure 14) .

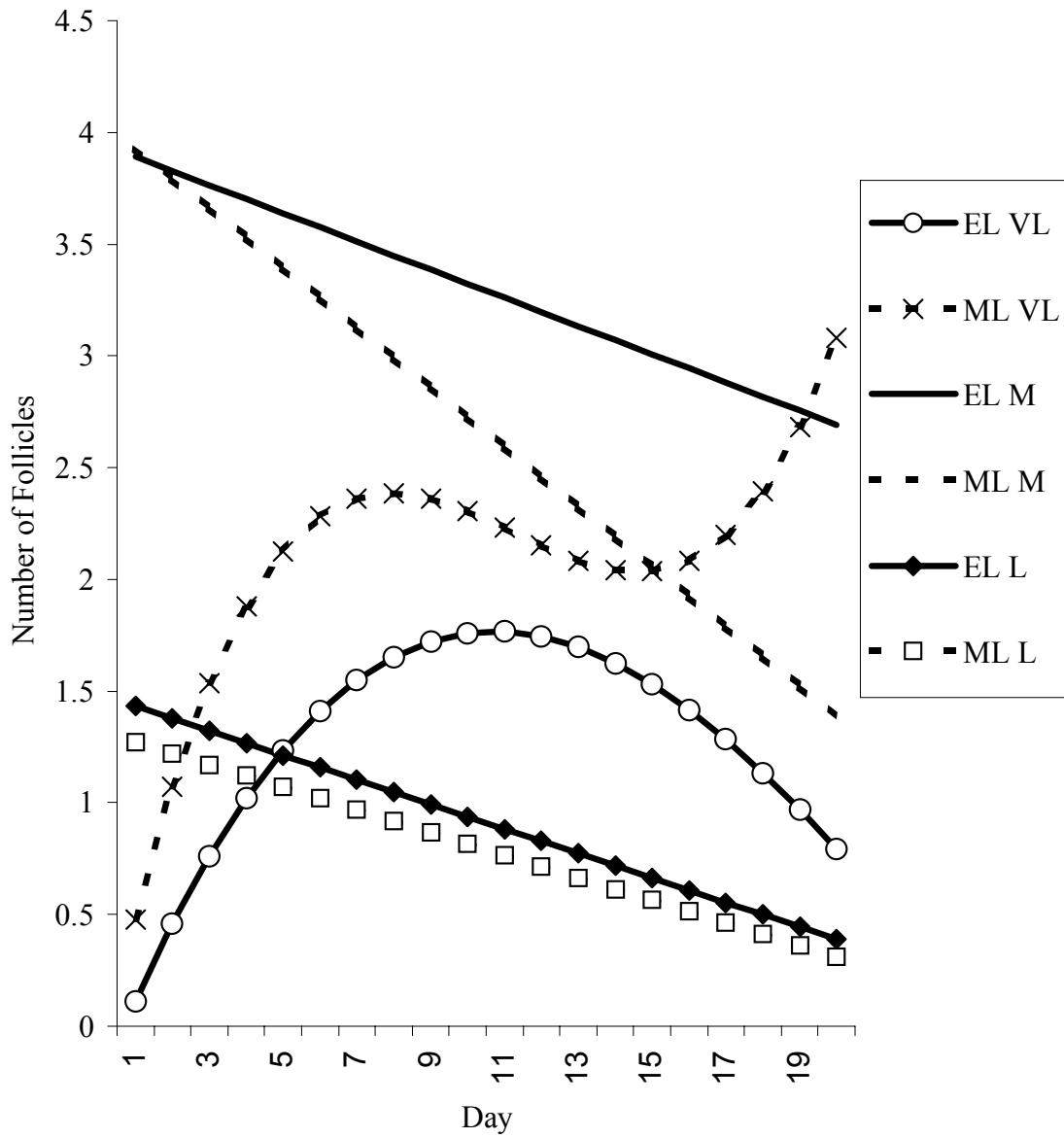


Figure 13. The number of medium (M), large (L), and very large (VL) follicles for early lactation (EL) and mid lactation (ML) cows during post aspiration synchronization.

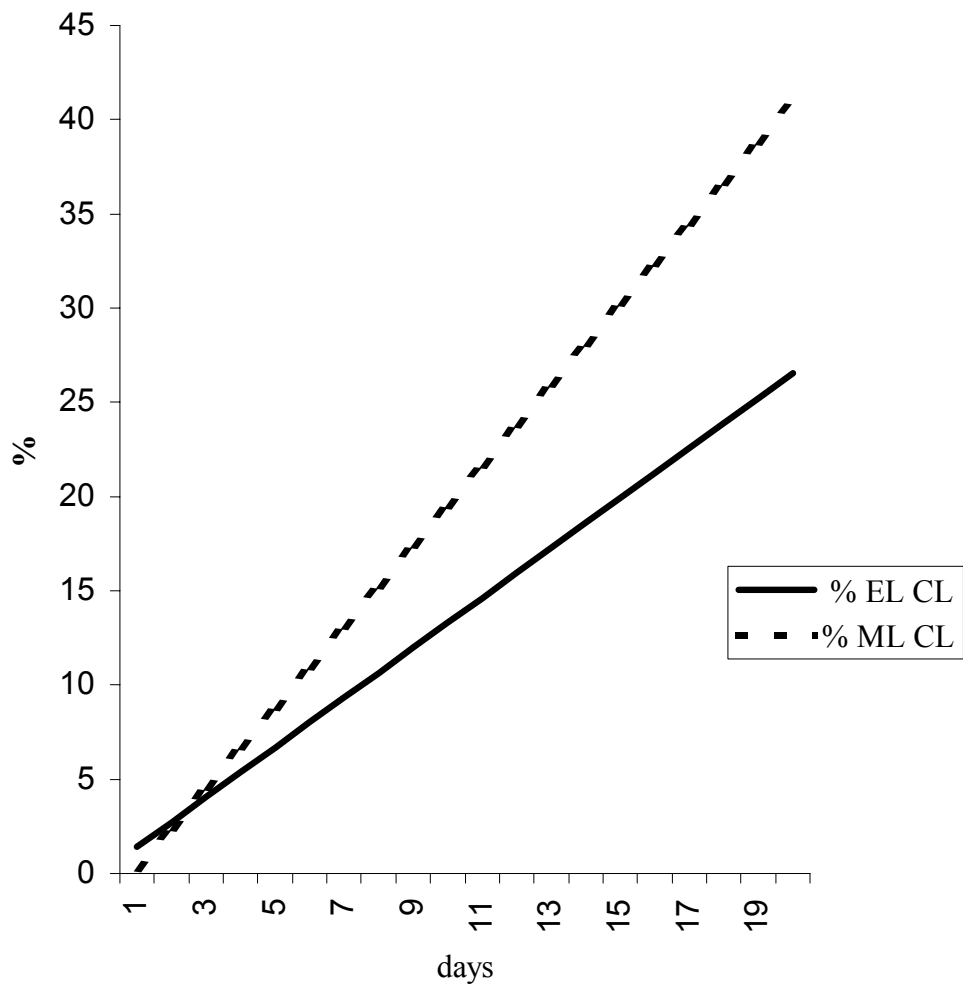


Figure 14. The percentage of cows with CL's for early (EL) and mid lactation (ML) cows during post aspiration synchronization.

DISCUSSION

One of the major objectives of this research was to determine the effects of stage of lactation on reproductive hormones, oocyte quality, and follicular development. Therefore, a group of Holstein cows, uniform in their milk production and representative of the breed, was chosen for this study. The feed data and milk records taken prior to the start of oocyte retrievals on the ML cows and at the end of retrievals for the EL cows confirm that the groups chosen were uniform in their overall production records and typical of the breed's average for milk production. For instance, there was no significant difference between EL and ML cows in EB, milk production, body weight, or BCS at 89 to 98 dpp.

The EL cows of this study returned to positive EB by 85 d postpartum and ML cows maintained a positive EB throughout the 10 wk of follicular aspirations. However, De Vries and Veerkamp (2000) demonstrated that cows could achieve positive EB by 42 d postpartum and remain at a positive EB past 180 d postpartum. Others have reported longer intervals to positive energy balance such as 53 ± 4 d observed by Berghorn et al. (1988), and even as long as 70 d as seen by Villa-Godoy et al. (1988). The cows of this study remained in negative EB for a longer time than in previously reported studies. The feeding system used to measure feed intake may have altered feed intake due to pecking orders and limited access to feed, thus extending the negative EB.

During the time a cow is in negative EB she will lose BW and decrease in BCS (Butler and Smith, 1989; Thatcher et al, 1996). The negative EB of the EL group in this study, due to parturition and the start of lactation caused decreases in both BW and BCS. Furthermore, not only will cows undergo outward physical changes, but metabolic changes occur as well. Expression of insulin, IGF-I, IGF-I binding proteins, and the mobilization of NEFA are some of the physiological changes that occur as a result of a negative EB (Vandehaar et al., 1995; Vicini et al., 1991). These findings were verified by our research as EL cows had decreased serum IGF-I and higher levels of serum NEFA when compared to ML cows. The levels of BHB measured in both treatment groups were typical of cows past 28 d postpartum (Harrison et al., 1990). The changing levels of hormones and metabolites in postpartum cows could be part of the mechanism that controls the reproductive system and ovarian activity during varying phases of EB.

Canfield and Butler (1990) reported that due to the negative correlation between EB and serum NEFA concentrations, NEFA levels may act as a signal to the neural centers that control LH secretion. Furthermore, IGF-I has been shown to have interactions with estradiol in controlling the resumption of the estrous cycle (Richards et al., 1991).

Energy balance affects the rate of folliculogenesis, the days to first ovulation, the functionality of ovarian structures, and the secretion and expression of steroids and gonadotropins in lactating dairy cattle. Lucy et al. (1991) reported shifts in the proportion of different size follicles due to predicted EB within the first 25 d postpartum. Likewise, we found a higher number of follicles in the ML cows as aspiration sessions proceeded, suggesting greater folliculogenesis in cows on a higher energy plane. Zurek et al. (1994) reported that declining EB was the main factor controlling the timing of first ovulation postpartum. De Vries and Veerkamp (2000) report that the highest correlation with start of luteal activity is EB nadir. Furthermore, luteal function or P4 secretion was not maximal during the first estrous cycle and anestrus occurred in 28% of early lactation cows (Staples et al., 1990). We found, as did others, that cows with negative EB (EL group) had lower serum concentrations of LH (Zurek et al., 1994; Canfield and Butler, 1990; Butler and Smith, 1989). However, FSH production remained constant for ML cows, while EL cows had increasing FSH concentrations as aspiration sessions proceeded.

During the course of this study TVFA produced an artificial system in which the negative feedback of the dominant follicle on the ovary was removed, however only the ML cows responded with increases in the number of follicles particularly small size follicles. This suggests that EB or something within the ovary allowed the ML cows to recruit more preantral follicles thus responding more efficiently to the respectively lower levels of FSH. One factor that could have caused this increased efficiency was higher serum levels of IGF-I. Serum IGF-I was reported to influence the time to first ovulation (Butler, 2000) and gonadotropin secretion, particularly the LH pulse frequency (Zurek et al., 1995). Furthermore, higher levels of follicular fluid IGF-I in ML cows may more accurately depict IGF-I's role in ovarian function. Echternkamp et al. (1990) observed a positive correlation between serum and follicular fluid IGF-I. However, we found that both treatment groups increased linearly in follicular fluid IGF-I even though EL cows

had a decrease in serum IGF-I. Concentrations of follicular fluid IGF-I were at higher levels and increased at a greater rate for ML cows. It is noteworthy that follicular fluid was only retrieved from the largest follicle and was not captured at each aspiration session. Furthermore, the follicles collected were the largest follicles present. Estradiol data suggest that some dominance may have been expressed and hormonal concentrations altered. The data suggest the ML cows were more proficient in the production of IGF-I or in their ability to sequester IGF-I within the follicle. The ML cow's higher plane of energy may have this effect on the entire ovary allowing for faster and more efficient growth of all follicle sizes. Moreover, varying levels of IGF-BP between cows within different planes of available energy may play a role in the efficiency of the ovary to produce follicles.

The data obtained during the post aspiration synchronization were similar to the findings of Lucy et al. (1991). There was no difference in the number of follicles, but the higher EB of the ML cows allowed them to grow follicles to a larger size more quickly than the EL cows. Furthermore, ML cows appeared more able to have ovulation occur as the percentage with CL increased more rapidly than for the EL cows in the post aspiration period.

The function of an ovarian follicle is to mature an oocyte to the point of ovulation and to produce the hormones needed for the body to accept the oocyte. Due to variability in collection techniques, the number of oocytes collected did not concur with the total number of follicles. Thus, there was no difference in the total number of oocytes collected between treatment groups. However, there were significant differences in the quality grades of oocytes collected. There was a higher number of poor quality oocytes in the ML cows than in EL cows. This may relate to EB in that, the higher energy state of the ML cows allowed them to grow follicles to larger size faster (Lucy et al., 1990), thus establishing dominance and leading to more atretic follicles faster than in EL cows. This is further supported in the EL cows by the shift in percentage of good plus oocytes to good minus oocytes as days postpartum and their EB increased.

A more dramatic difference between EL and ML cows may not be seen due to a lack of differences between the treatment group's physiological status. Even though there existed a negative EB for the EL cows and a positive EB to the ML cows, the severity of

the difference between these groups may have inhibited our ability to see underlying differences. For instance the lack of a marked difference in BCS of these two groups points to a ML group that does not have an abundant energy supply to support both milk production and increased body fat deposition. This lack of abundant energy may be hindering the ML cows reproductively as well by potentially suppressing the concentrations of hormones such as insulin and IGF-I. Staples et al. (1990) demonstrated that NEFA concentrations only corresponded with EB in cows with scores around 3.0, and not in thinner cows with scores of less than 2.2. The higher EB cows of this study maintained a BCS of 2.3 ± 0.02 , only slightly above the thin cow limits. If NEFA concentrations do play a role in the pituitary's response to energy status, low body condition may effect this control. Thus, the differences noted between groups may have been more pronounced had the EB of the ML cows been elevated.

The results of this study concur with the results of others that have evaluated reproductive performance in early lactation. However, this study also expands upon our knowledge of the cow's transition from negative to positive EB and her reproductive recovery into mid lactation. Our results indicate that early lactation cattle, due to changes in environment, feed intake, management, and milk production are susceptible to experiencing the effects of negative EB. These effects include shifting concentrations of hormones and metabolites, which may have an impact on follicular development and oocyte quality.

CONCLUSIONS

As the dairy industry becomes more and more competitive, knowledge of how to improve the efficiency of dairy cattle is in high demand. Knowledge surrounding the reproductive aspects of dairy cattle is no exception. In this study we have shown significant differences between early and mid lactation dairy cattle, both in their physiological status (EB, NEFA, BHB, BCS, milk production) and reproductive characteristics and capabilities (follicle growth rate, follicle size, hormone levels, CL functionality, and oocyte quality). Furthermore, we have hypothesized potential mechanisms, which may link these characteristics and explain how dairy cattle respond to the dynamic changes that occur during their lactation. Mechanisms involve the use of NEFA levels as a signal to neural centers of the hypothalamus, and changing levels of IGF-I as a controlling factor in cellular activity.

In conducting this study we also chose to use current industry technologies to demonstrate differences researchers and producers may find in their application of these technologies. We used TVFA as a means of oocyte recovery, as it provides a useful tool to scientists and a potential tool for the livestock industry. We demonstrated considerable variability in results obtained from cattle of different stages of lactation. This variability may be of concern to the scientist in research, and has potential economic risk to producers. Further research is needed in this field as others have shown that even genetic merit plays a significant role in the success of these procedures (Snijders et al., 1999). Furthermore, with published results of pregnancy rates of only 5.1% when looking at total oocytes recovered to detected pregnancies, this technology shows considerable room for improvement in its efficiency (Looney et al., 1994). It is hoped that the differences shown in this research may help future researchers fine tune their procedures and take this technology in the proper direction. In summary our research concurs with those who have found variability in reproductive characteristics dairy cattle due to changes in energy balance or differing stages of lactation. More specifically, our results add support to the theories that IGF-I and NEFA levels may be the major controlling factors of the reproductive system during times of nutritional stress.

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Appendix

Table 1. Dairy cattle ration for EL and ML treatment groups

Ingredient	% of ration	Kg of feed/cow (as fed)
Corn Silage	38.3	17.41
Whole Cotton Seed	5.1	2.32
Prolak	1.3	0.59
Soybean Meal	7.0	3.18
Mineral	0.6	0.27
Limestone	0.5	0.22
High Moisture Corn	21.7	9.86
Alfalfa Haylage	25.5	11.59
TMR 55.56 % DM		

Letters (A, B, C) = early lactation cow
Numbers (1, 2, 3) = mid lactation cow

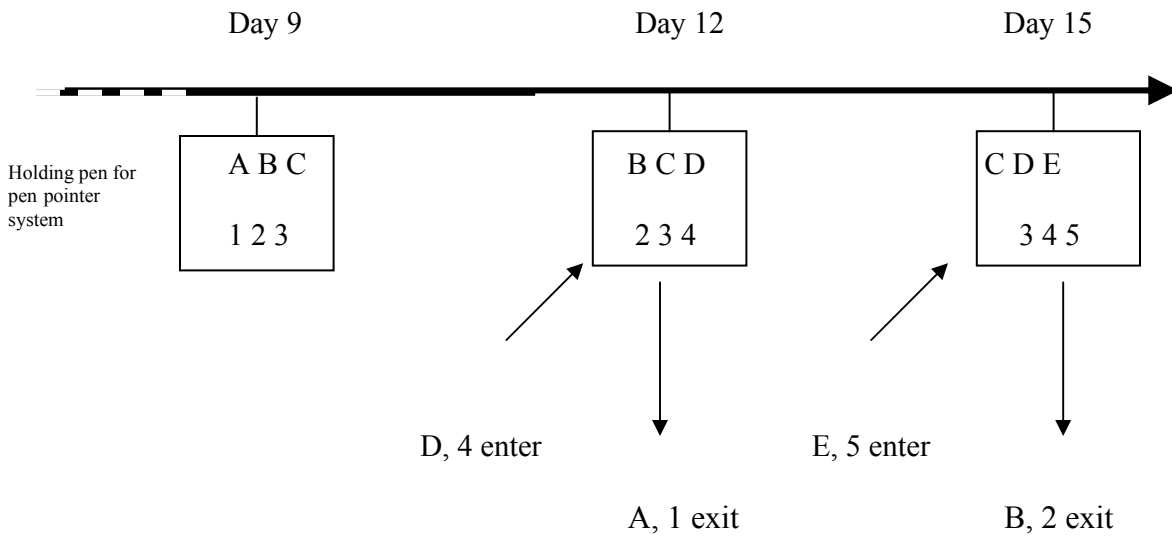


Figure 15. Cow rotation through the pinpointer system for assessment of feed intake.

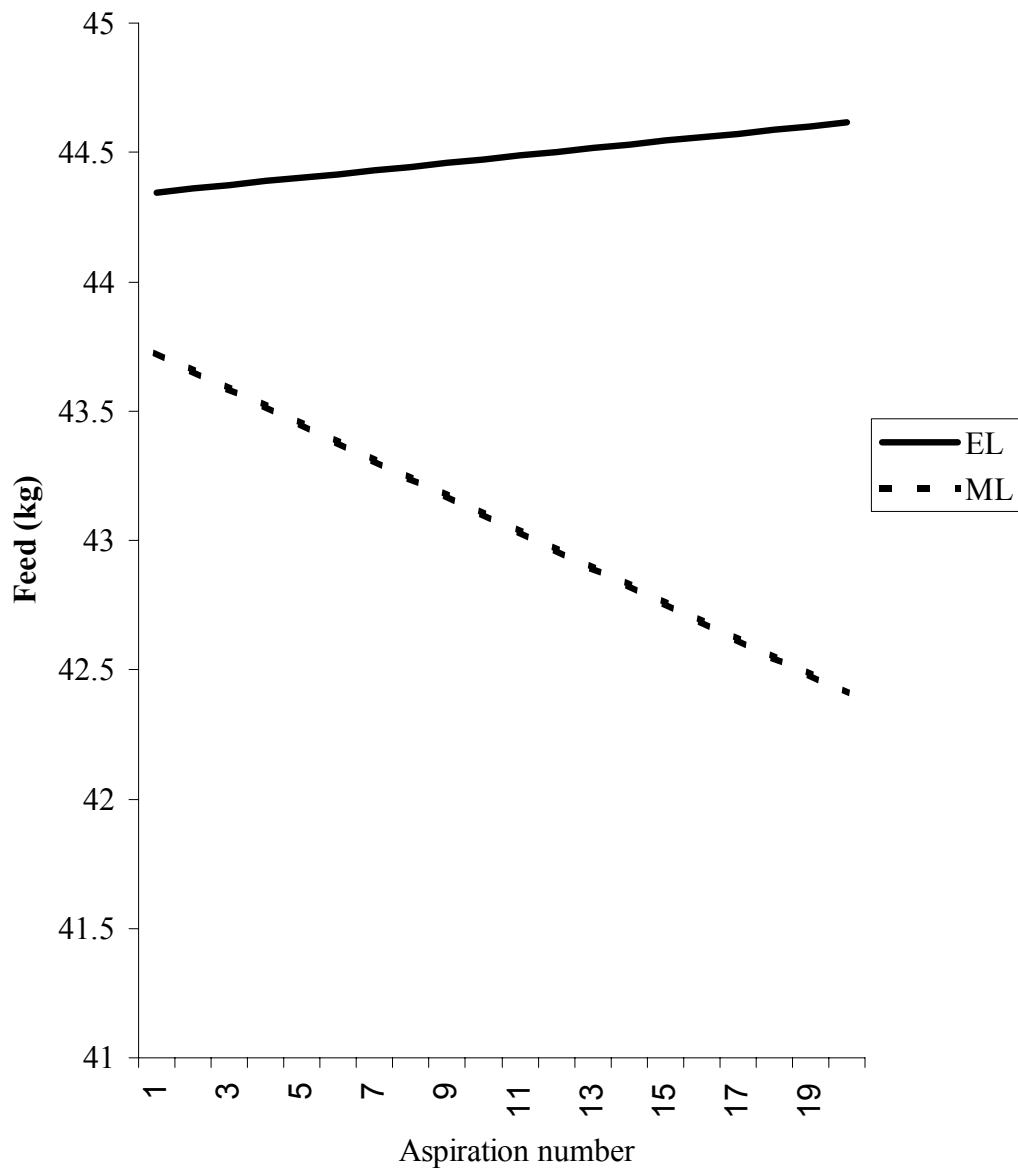


Figure 16. Average daily feed intake for early (EL) and mid lactation (ML) cows during ten weeks of twice weekly TVFA.

Table 2. Analysis of variance for energy balance and milk weight in Early and Mid lactation Holstein cows				
Source	Energy Balance		Milk Weight	
	df	Mean Squares	df	Mean Squares
Treatment	1	883.5**	1	696.7**
Cow (Treatment)	13	94.2**	13	305.9**
Aspiration number * Treatment ^a	2	81.2**	2	650.2**
Aspiration number ² * Treatment ^b	2	119.0**	2	626.2**
Aspiration number ³ * Treatment ^c	2	50.3**	2	215.6**
Residual	188	10.5	201	10.5

^a linear effect; ^b quadratic effect; ^c cubic effect; * P < 0.05; ** P < 0.01.

Table 3. Analysis of variance for body condition score and body weight in Early and Mid lactation Holstein cows

Source	Body Condition Score		Body Weight	
	df	Mean Squares	df	Mean Squares
Treatment	1	0.09	1	11.2
Cow (Treatment)	13	1.01**	13	44557.5**
Aspiration number * Treatment ^a	2	11.11**	2	75546.8**
Aspiration number ² * Treatment ^b	2	1.62**	2	32659.8**
Residual	266	0.05	266	554.0

^a linear effect; ^b quadratic effect; ^c cubic effect; * P < 0.05; ** P < 0.01; ***P < 0.06

Table 4. Analysis of variance for average milk fat and milk protein percentage for Early and Mid lactation Holstein cows

Source	Milk Fat %		Milk Protein %	
	df	Mean Square	df	Mean Square
Treatment	1	0.23	1	0.83**
Cow (Treatment)	13	1.57**	13	0.73**
Aspiration number * Treatment ^a	2	3.61**	2	3.21**
Aspiration number ² * Treatment ^b	2	1.96**	2	2.10**
Residual	193	0.21	193	0.025

^a linear effect; ^b quadratic effect; ^c cubic effect; * P < 0.05; ** P < 0.01; ***P < 0.06

Table 5. Analysis of variance for serum concentrations of non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHB) in Early and Mid lactation Holstein cows				
Source	NEFA		BHB	
	df	Mean Squares	df	Mean Squares
Treatment	1	0.611**	1	61.2**
Cow (Treatment)	13	0.152**	13	12.6**
Aspiration number * Treatment ^a	2	1.308**	2	12.0
Aspiration number ² * Treatment ^b	2	0.288**	2	16.2*
Aspiration number ³ * Treatment ^c	2	0.107*		
Residual	387	0.030	185	4.2

^a linear effect; ^b quadratic effect; ^c cubic effect; * P < 0.05; ** P < 0.01

Table 6. Analysis of variance for size and number of follicles observed in Early and Mid lactation Holstein cows during transvaginal follicular aspiration (TVFA).								
Source	Very Large (>12mm)		Large (9 – 12 mm)		Medium (6 – 9 mm)		Small (2 – 5 mm)	
	df	Mean Squares	df	Mean Squares	df	Mean Squares	df	Mean Squares
Treatment	1	0.28	1	0.24	1	0.02	1	4.1
Cow (Treatment)	13	1.24**	13	1.11*	13	8.09	14	184.5**
Aspiration number * Treatment ^a	2	0.69	2	0.29	2	1.52	2	77.7**
Aspiration number ² * Treatment ^b	2	0.75	2	0.24	2	1.97	-	-
Aspiration number ³ * Treatment ^c	2	0.65	2	0.24	2	2.51	-	-
Residual	273	0.43	273	0.53	273	4.92	283	14.6

^a linear effect; ^b quadratic effect; ^c cubic effect; * P < 0.05; ** P < 0.01

Table 7. Analysis of variance for the total number of oocytes collected during follicular aspirations and the total number of follicles observed.

Source	Total # Oocytes		Total # Follicles	
	df	Mean Squares	df	Mean Squares
Treatment	1	0.19	1	4.15
Cow (Treatment)	13	96.52**	14	232.85**
Aspiration number * Treatment ^a	2	40.23*	2	123.14**
Aspiration number ² * Treatment ^b	2	37.34*	-	-
Aspiration number ³ * Treatment ^c	2	36.38	-	-
Residual	273	12.39	283	14.23

^a linear effect; ^b quadratic effect; ^c cubic effect; * P < 0.05; ** P < 0.01; ***P < 0.06

Table 8. Analysis of Variance for the number of each quality grade of oocyte recovered during transvaginal follicular aspiration (TVFA)								
Source	Good Plus (4)		Good (3)		Good Minus (2)		Poor (1)	
	df	Mean Squares	df	Mean Squares	df	Mean Squares	df	Mean Squares
Treatment	1	0.29	1	0.26	1	0.001	1	6.2
Cow (Treatment)	12	0.63	13	2.00*	13	5.200**	13	40.5**
Aspiration number * Treatment ^a	2	0.45	2	0.20	2	2.392	2	23.9*
Aspiration number ² * Treatment ^b	2	0.33	2	0.13	2	3.072	2	21.0***
Aspiration number ³ * Treatment ^c	2	0.21	2	0.05	2	3.535	2	19.8***
Residual	46	0.84	93	1.03	185	1.472	254	7.4

^a linear effect; ^b quadratic effect; ^c cubic effect; * P < 0.05; ** P < 0.01; ***P < 0.06

Table 9. Analysis of variance for the proportion of quality grades collected at each aspiration session.				
Source	% Good Plus (4)		% Good Minus (2)	
	df	Mean Squares	df	Mean Squares
Treatment	1	19.5	1	1813.0*
Cow (Treatment)	13	269.9**	13	577.8
Aspiration number * Treatment ^a	2	367.2*	2	1413.1*
Aspiration number ² * Treatment ^b	2	562.5**	2	1049.5
Aspiration number ³ * Treatment ^c	2	618.1**	-	-
Residual	272	118.4	274	444.5

^a linear effect; ^b quadratic effect; ^c cubic effect; * P < 0.05; ** P < 0.01; ***P < 0.06

Table 10. Analysis of variance of the proportion of time that a corpus luteum was present at the time of follicular aspiration

Source	df	Mean Squares
Treatment	1	1.0*
Cow (Treatment)	13	1.0**
Aspiration number * Treatment ^a	2	1.5**
Aspiration number ² * Treatment ^b	2	0.6*
Aspiration number ³ * Treatment ^c	2	0.4
Residual	273	0.15

^a linear effect; ^b quadratic effect; ^c cubic effect; * P < 0.05; ** P < 0.01; ***P < 0.06

Table 11. Analysis of variance for serum concentrations of luteinizing hormone (LH) and follicle stimulating hormone (FSH)				
Source	LH		FSH	
	df	Mean Squares	df	Mean Squares
Treatment	1	0.02	1	0.80*
Cow (Treatment)	13	0.58**	13	0.17
Aspiration number * Treatment ^a	2	2.11**	2	0.47*
Aspiration number ² * Treatment ^b	2	0.76*	2	0.45*
Residual	397	0.17	393	0.14

^a linear effect; ^b quadratic effect; ^c cubic effect; * P < 0.05; ** P < 0.01

Table 12. Analysis of variance for the serum concentrations of progesterone (P₄) and insulin-like growth factor-I (IGF-I)

Source	P ₄		IGF-I	
	df	Mean Squares	df	Mean Squares
Treatment	1	81.78**	1	6751.5
Cow (Treatment)	13	32.69**	13	12568.2
Aspiration number * Treatment ^a	2	77.36**	2	1905.9
Aspiration number ² * Treatment ^b	2	10.26**	-	-
Aspiration number ³ * Treatment ^c	2	12.72**	-	-
Residual	395	2.16	342	157.9

^a linear effect; ^b quadratic effect; ^c cubic effect; * P < 0.05; ** P < 0.01

Table 13. Analysis of variance of follicular fluid concentrations of progesterone (P₄), insulin-like growth factor-I (IGF-I), and estradiol (E₂)

Source	P ₄		IGF-I		E ₂	
	df	Mean Square	df	Mean Square	df	Mean Square
Treatment	1	88.1	1	25,008**	1	17,292,188**
Cow (Treatment)	2	42.4	12	5,780**	12	16,067,579**
Aspiration number * Treatment ^a	2	94.1*	2	8,415**	2	1,192,688
Aspiration number ² * Treatment ^b	-	-	-	-	2	1,182,828
Aspiration number ³ * Treatment ^c	-	-	-	-	2	1,259,823
Residual	180	24.0	161	1,284	161	1,117,303

^a linear effect; ^b quadratic effect; ^c cubic effect; * P < 0.05; ** P < 0.01

Table 14. The analysis of variance for the number of medium, large, and very large follicles, and for the occurrence of CL during post aspiration synchronization.

Source	Medium Follicles		Large Follicles		Very Large Follicles		Occurrence of Corpus Luteum	
	df	Mean Squares	df	Mean Squares	df	Mean Squares	df	Mean Squares
Treatment	1	4.4	1	1.51	1	1.3	1	0.00027
Cow (Treatment)	9	5.8	9	1.02	9	6.2**	9	0.23467
Treatment * day ^a	2	21.6*	2	5.62**	2	14.2**	2	0.62946**
Treatment * day ^{2b}	-	-	-	-	2	10.4**	-	-
Treatment * day ^{3c}	-	-	-	-	2	7.5**	-	-
Residual	149	4.8	149	0.85	145	1.1	149	0.12915

^a linear effect; ^b quadratic effect; ^c cubic effect; * P < 0.05; ** P < 0.01

VITA

Andrew William Pryor Jr. was born in Goochland County on August 16, 1974. He completed High School in Virginia public schools at Goochland High School in June of 1992. He then came to Virginia Polytechnic Institute and State University in the fall of 1992. He graduated with honors and a Bachelors of Science degree in Dairy Science, with two minors, Animal Science and Biology, in May of 1996. He then subsequently began a Masters program in Dairy Science in the fall of 1996. In the Summer of 1998 Andy took employment with MCV Physicians as a embryologist. After one and half years of employment as a embryologist, he returned to Goochland to assist his father in the management of the family farm. In the Spring of 2002, he returned to VPI & SU and completed his Masters program.

Refereed Publications

Chauhan, M. S., S. Nadir, T. L. Bailey, **A. W. Pryor**, S. P. Butler, D. R. Notter, W. H. Velandar, and F. C. Gwazdauskas. 1999. Bovine Follicular Dynamics, Oocyte Recovery, and Development of Oocytes Microinjected with a Green Fluorescent Protein Construct. *J. Dairy Sci.* 82:918-926.

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Pryor, A. W., T.L. Bailey, S. Nadir, M.H. Irby, R.E. Bethard, and F.C. Gwazdauskas. 1998. Effects of stage of lactation on follicular dynamics and oocyte quality following transvaginal follicular aspiration. Virginia-Maryland College of Veterinary Medicine Tenth Annual Research Symposium.

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