EFFECTS OF EXPERIMENTAL FASCIOLIASIS ON PUBERTY AND COMPARISON OF MOUNTING ACTIVITY BY RADIOTELEMETRY IN PUBERTAL AND GESTATING BEEF HEIFERS

A Thesis

by

MELISSA JEANNE PACZKOWSKI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2004

Major Subject: Physiology of Reproduction

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Approved as to style and content by:

David Forrest (Chair of Committee)	Thomas Craig (Member)
James Thompson (Member)	Derry Magee (Member)
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ABSTRACT

Effects of Experimental Fascioliasis on Puberty and Comparison of Mounting Activity by Radiotelemetry in Pubertal and Gestating Beef Heifers.

(August 2004)

Melissa Jeanne Paczkowski, B.S., Texas A&M University

Chair of Advisory Committee: Dr. David Forrest

Angus-sired heifers were allotted by age (mean=4 mo), BW (mean=135 kg), and sire (n=4) to either a control (n=10) or infected group (n=11; 600 metacercariae of *Fasciola hepatica,* intraruminally) to test our hypothesis that puberty is delayed by experimental fascioliasis. Blood samples were collected biweekly for analysis of steroid hormone concentrations. At 2-wk intervals, BW was recorded, and samples were collected for analysis of liver enzymes and serum proteins and fecal egg counts. A radiotelemetry system (HeatWatch®) was used to detect estrus and ovulation was confirmed by an elevation in serum progesterone (P_4) after estrus. Heifers were artificially inseminated (AI) at the second observed estrus. Serum γ-glutamyl transpeptidase (GGT) and aspartate aminotransferase (AST) increased ($p<0.0008$) between day 0 and 112 in the infected group. Serum estradiol (E_2) and P_4 concentrations did not differ ($p>0.1$) between treatment groups. Mean age at puberty was 10 days later ($p>0.1$) in the infected group. Conception rate did not differ between control and infected heifers.

The HeatWatch® data were used to compare mounting activity during estrus in pubertal and gestating heifers. Mean duration of estrus was longer ($p<0.01$) for the second than for the pubertal estrus, though total mount duration and number of mounts did not differ. Number of mounts at second estrus was greater ($p<0.05$) for heifers that conceived (n=9). Mean duration of estrus and total mount duration at second estrus were not associated with pregnancy outcome. Estrus events were detected in all nine heifers during pregnancy (total=73). A majority (75%) of the interestrus intervals during gestation was <17 d. Number of mounts ($p=0.035$) and total duration of mounts ($p=0.022$) at second estrus were predictive of number of mounts during gestation.

Experimental infection of *Fasciola hepatica* did not alter serum steroid hormone concentration or delay pubertal development in heifers. Estrus duration was longer for the second estrus compared to the pubertal estrus, and the number of mounts received during the second estrus was greater in heifers that did conceive to AI. Estrus events were detected in each heifer during pregnancy; however, a normal interestrus interval occurred in only 10% of the estrus events.

This work is dedicated to my parents:

The late Mary Ann Hejl Paczkowski, for everything I accomplish is for you,

and

Walter Richard Paczkowski, Ph.D., for being everything to us.

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CHAPTER I

INTRODUCTION

Livestock reproduction is an important aspect of the meat industry but was overlooked for years as nutrition and growth became the main focus for producing leaner cuts of meat for less. As Zajac et al. [1] stated, "fertility in the beef herd has been shown to be the most important single factor in determining profitability in a beef cattle operation." Replacement heifers increase profitability of beef operations by enhancing the genetic pool, by allowing producers to select for specific traits with the use of artificial insemination, and by allowing producers to cull cows with poor reproductive performance.

The onset of puberty in heifers is a highly complex and timed process that is influenced by nutrition, genetics and endocrinology. Studies have indicated that poor nutrition results in delayed puberty and reduced fertility, as determined by decreased pregnancy rates [2, 3]. Heifers need to conceive by 15 months of age in order to calve by 24 months. Laster et al. [4] demonstrated breed influences puberty as a higher percentage of Angus heifers reached puberty by 15 months of age compared to their Hereford contemporaries. Jones et al. [5] reported breed type influences age at puberty with Angus and Simmental heifers reaching puberty at a younger age. Pubertal development is dependent upon the maturation of the endocrine system, primarily the hypothalamic-pituitary-ovarian axis. During the peripubertal period, the inhibitory

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This thesis follows the style and format of Theriogenology.

feedback of estrogen decreases allowing the release of gonadotropins, essential for follicular growth and ovulation. Proper reproductive management which takes into consideration factors affecting the attainment of puberty can decrease age at puberty, resulting in an increased lifetime production of calves.

Estrus detection methods were developed to aid producers in determining the appropriate time for artificial insemination to increase conception rates. Methods such as the K-mar, Bovine Beacon, and paint markers provide efficient means of detection; however, accuracy may be compromised as false mounting can occur leading to inaccurate insemination times and reduced conception rates. These methods are also dependent upon the frequency of observation to determine the onset of estrus. Use of systems such as time-lapse video recorders or radiotelemetry, allows for efficient and accurate detection of estrus by allowing for continuous monitoring of mounting activity. Radiotelemetry offers ease of identification of females in estrus, minimizes labor, and data retrieved is less tedious than for time-lapse recorders. Therefore, radiotelemetry fulfills the requirements for accurate estrus detection systems as characterized by Senger [6].

Statement of Problem

Experiment 1

Parasitic infection affects productivity of livestock operations by endangering the health and performance of animals. *Fasciola hepatica*, also known as the liver fluke, infects ruminants after ingestion of metacercariae and causes liver trauma as the flukes

migrate through the parenchyma and tissue. Fascioliasis, the disease caused by *Fasciola hepatica* infection, has been correlated with depressed appetite and weight gain, poor milk production, increased mortality, and liver condemnation resulting in economic losses in several countries. Few controlled studies have quantified the effects of experimental infection on pubertal development, though it was hypothesized that *Fasciola hepatica* would alter age at puberty due to reduced metabolism of steroid hormones by the liver. Fleming and Fetterer [7] and López-Díaz et al. [8] studied the effect of *Fasciola hepatica* infection on pubertal development in rams and heifers and their findings further supported this hypothesis. López-Díaz et al. [8] observed elevated concentrations of estradiol in infected heifers while Fleming and Fetterer [7] reported normal concentrations of testosterone in prepubertal rams. Though contrasting results were observed, Fleming and Fetterer [7] went on to report decreased ability of the liver to metabolize exogenous testosterone, indicating the metabolic clearance rate was altered and suggested compensatory mechanisms were maintaining normal ranges of circulating endogenous testosterone.

The current study utilized an experimental design similar to that of López-Díaz et al. [8] in order to test the hypothesis that *Fasciola hepatica* infection delays the onset of puberty and increases the concentration of estradiol in the circulation. Improved estrus detection systems, sensitive steroid hormone analysis, increased sample size, and liver enzyme analyses were employed in the present study to correlate level of infection with age at puberty and with serum estradiol and progesterone concentrations.

Experiment 2

The HeatWatch® system was developed for continuous monitoring of estrus behavior, enabling the producer to view data on the number of mounts received, and mount and estrus duration. Compared to conventional methods of visual inspection, both methods have high accuracy (low incidence of misdiagnosed estrus), though HeatWatch® is more efficient, detecting 100% of the estrus events in cattle compared to only 73% detected by visual inspection [9]. Stevenson et al. [9] stated heifers which received few mounts or had a short duration of estrus were more likely to be missed by visual inspection than by HeatWatch®. As previously mentioned, producers intending to breed by 15 months of age need to be mated soon after puberty (first to fifth estrus events); however, no reports were found on mounting activity of pubertal heifers. At this critical time, if heifers receive few mounts or are in estrus for a shorter duration during their pubertal estrus, they may not be detected by visual inspection.

Estrus, and the accompanying ovulation of a Graafian follicle, occurs on average every 21 days in non-pregnant cattle, though it has been reported that cattle have displayed signs of estrus throughout gestation without the occurrence of ovulation. Erb and Morrison [10] reported that 5.6% of estrus events in dairy cattle occurred 21 days post-conception, adding complications to breeding programs. Cattle misdiagnosed as non-pregnant due to displayed signs of estrus after conception, could abort if rebred or if synchronization protocols were used in the herd, primarily those that involve prostaglandin F2α.

The second study was designed to quantify the mounting activity of heifers during their pubertal and second estrus events by comparing number of mounts, duration of mounts, and estrus duration. Mounting activity and pregnancy outcome were analyzed to determine whether mounting activity at the time of insemination was correlated with pregnancy. Heifers that conceived to artificial insemination were monitored to determine the rate of occurrence and interestrus interval of estrus activity post-conception. Mounting activity was analyzed to determine if characteristics of estrus activity at the time of insemination were predictive of the mounting activity during post-conception estrus events.

CHAPTER II

LITERATURE REVIEW

Endocrine Mechanisms and External Factors Controlling Puberty and Normal Cyclicity in Cattle

Defining Puberty

Puberty can be defined in several ways: first ovulation of a dominant follicle resulting in progesterone concentrations greater than one nanogram per milliliter, first observed estrus behavior, or the ability of an animal to reproduce oneself. The latter definition is more accurate as research has indicated that first ovulation is not always followed by a behavioral estrus or by an adequate luteal phase (discussed further in this chapter) and observed estrus can occur without ovulation of an oocyte. Genetic and environmental factors influence the onset of puberty, suggesting "puberty occurs at a specific physiological, as opposed to chronological age" [11].

"Fertility in the beef herd has been shown to be the most important single factor in determining profitability in a beef cattle operation" [1]. Heifers reaching sexual maturity earlier than their contemporaries have a greater likelihood of producing more calves in their reproductive life. Lesmeister et al. [12] reported that early calving heifers (as two-year-olds) continuously calved earlier in their reproductive life ($p<0.05$), produced more kilograms of calf ($p<0.01$), and had a higher annual calf production than heifers calving as three-year-olds. Pope [13] reported that heifers calving as two-year-olds produced 0.7 more calves in 6.5 years compared to three-year-olds. Early calving heifers also produced heavier calves at weaning ($p<0.01$, 488 lbs. vs. 370 lbs) [12] and

cows that calved earlier (days 1 to 20, January 1 through 20) were less likely to fail to calve in the subsequent season than those that originally calved late (days 21 to 220) [14]. Marshall et al. [15] described that "early calving dams tended to be more efficient because a greater proportion of their annual production cycle was spent in a productive (lactating) mode, diluting maintenance costs as a fraction of total costs" indicating the earlier females reach puberty and conceive, the more productive and cost efficient it will be in her reproductive life.

Factors Affecting Puberty: Breed and Nutrition

As stated previously, genetic and environmental factors, primarily breed type and nutritional status, influences the onset of puberty. In a comparison of breed types, Jones et al. [5] reported that Angus (321 ± 7 d) and Simmental heifers (361 ± 5 d) on average reached puberty earlier than their Charolais (420 ± 27 d) and Braford counterparts (497 ± 19 d; $p<0.05$). Due to an earlier puberty date, Angus heifers weighed less at puberty (310 ± 8 kg) compared to the other purebred heifers ($p<0.05$; Simmental 359 ± 10 kg < Charolais 403 ± 19 kg ≈ Bradford 424 ± 13 kg). Early onset of puberty and early calving is advantageous to the commercial producer by increasing the pounds of calf produced by each heifer; to produce a calf from a heifer around 24 months of age, the heifer must reach puberty by 15 months. Breed type influenced the percentage of heifers that reached puberty by 15 months ($p<0.005$); for example, 91.7 ± 7.1% of Angus purebred heifers reached puberty by 15 months of age compared to Hereford purebred heifers (48.1 ± 6.8% by 15 months) [4].

Different planes of nutrition have shown to significantly impact age at puberty; however, it has no impact on weight at puberty [2]. Heifers were separated into four treatment groups: continuous high plane of nutrition from birth to first calving (HH), high plane of nutrition for 44 weeks followed by a low plane until two months prior to calving (HL), continuous low plane of nutrition from birth to calving (LL), and low plane of nutrition for 44 weeks followed by a high plane until two months prior to calving (LH). Weight at puberty was not significantly different between the nutritional planes (HH: 565 lb., HL: 548 lb., LL: 525 lb., LH: 567 lb.); however age at puberty was significantly affected by nutritional status (HH: 372 d, HL: 552 d, LL: 474 d, LH: 440 d; $p<0.001$) [2]. A recent study [16] reported that Zebu heifers fed high (19.17%), medium (13.37%) or low (8.3%) amounts of protein in the feed did affect weight at puberty (207.1 kg, 187.0 kg, 161.7 kg, respectively; $p<0.05$).

Short and Bellows [3] fed Angus x Hereford and Hereford x Angus heifers on three planes of nutrition (Low: 0.28 kg/d ADG, Medium: 0.45 kg/d, High: 0.52 kg/d) to determine the impact on age at puberty, percentage of heifers reaching puberty before and during the breeding season, and pregnancy rates. Heifers fed at low and medium nutritional planes were delayed in the onset of puberty compared to the heifers on the high nutritional plane (L: 433 d, M: 411 d, H: 388 d; M vs. H and L vs. M and H, $p<0.01$). At the beginning of the breeding season, only 7 and 24% of the heifers in the Low and Medium groups, respectively, reached puberty (H, 83%; M vs. H, $p<0.01$) and only 80% of the heifers in the Low group reached puberty before or during the breeding season (M: 97%, H: 100%; L vs. M and H, $p<0.01$). Pregnancy rates were reduced in

heifers in the Low group (L: 63%, M and H: 90%, L vs. M and H, $p<0.01$) indicating that poor nutrition reduces fertility [3].

Similar outcomes were reported [17] when low levels of feed (0.43 kg/d ADG) resulted in only 61.3 ± 3.4% of heifers reaching puberty by the beginning of the breeding season compared to heifers fed high levels of nutrition (0.62 kg/d ADG, 70.9 ± 3.4% reached puberty; $p<0.05$). Heifers receiving the lower ADG had on average less milk production ($p=0.006$) and produced calves that weighed less from 54 d to 153 d post-calving ($p<0.04$).

Hormonal Control of Cycling Females

The hypothalamic-pituitary-ovarian axis is the hormonal control system for the reproductive cycle in females. The hypothalamus acts as a "puppeteer" of the endocrine system; its major function involves receiving and integrating messages from the body to control various aspects of hormonal function, as a puppeteer would control the functions and movements of a marionette. In reproductive physiology, the hypothalamus focuses on the release of Gonadotropin Releasing Hormone (GnRH) into the hypothalamo-hypophyseal portal system which connects the hypothalamus and the anterior pituitary. Gonadotropins, Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH), are secreted from the anterior pituitary in response to GnRH pulses to promote follicular growth, and induce ovulation and luteinization of the follicle, respectively [18].

The effect of LH and FSH are concentrated on the ovary in the female, particularly the thecal and granulosal cells of the ovarian follicle which contain receptors specific for LH and FSH, respectively. The hormone-receptor complex in the theca

interna cells initiates the steroid hormone pathway where cholesterol is enzymatically converted to pregnenolone in the mitochondria via cytochrome P450 side chain cleavage ($P450_{SCC}$, CYP11A) [18, 19]. Outside the mitochondria, biosynthesis of estrogens involves the conversion of pregnenolone to progesterone and testosterone [18]. The conversion of testosterone to estradiol requires cytochrome P450 aromatase ($P450_{AROM}$), which is not naturally found within the thecal cells. Aromatase is localized within the granulosal cells of the follicle, requiring testosterone to passively diffuse out of the thecal cells and into the granulosal cells to be converted to estradiol [18].

The estrous cycle in females refers to the interval from one estrus, or period of sexual receptivity, to the next (approximately 21 days in beef cattle) and is controlled by the hormones secreted by the hypothalamus, anterior pituitary, and ovary. The estrous cycle is divided into two phases, the follicular and luteal phase, based on hormonal concentrations of estradiol and progesterone.

The systems in the body are controlled by a series of positive and negative feedback mechanisms to control homeostasis or the resting and control points of a system. During proestrus, follicles undergo a process of recruitment and selection until one dominant follicle is produced. Many follicles are randomly recruited for development and release estradiol to up regulate the release of FSH from the anterior pituitary in a positive feedback manner. The majority of the smaller follicles will undergo apoptosis, or programmed cell death, during recruitment and selection until a dominant follicle is formed, the largest and most viable follicle which is preparing to ovulate (dominance). Inhibin is secreted by the dominant follicle to down regulate the

release of FSH by a negative feedback mechanism to reduce the development of smaller follicles, causing them to undergo apoptosis; ensuring only one follicle will ovulate. The increased concentrations of estradiol enhance LH release to create the hourly pulses and the preovulatory surge (>10 fold increase) of LH required for ovulation at the end of estrus. After ovulation, the thecal and granulosal cells collapse and reform to produce the corpus luteum (CL), composed of progesterone-secreting luteal tissue. Staining for $P450_{SCC}$ in mature corpora lutea demonstrated a high intensity for the enzyme which is required for the conversion of cholesterol to progesterone [20]. Progesterone down regulates the hypothalamic secretion of GnRH and pituitary secretion of LH to prevent ovulation of further developing follicles [18].

The "Gonadostat Theory" in Prepubertal Females

The hypothalamic-pituitary axis has been researched for more than 40 years to determine the events that occur in immature females as puberty approaches that allow the axis to switch from an inactive to an active state. In prepubertal rats, the hypothalamus-pituitary axis was shown to be fully functional by Ramirez and McCann [21] by measuring ovarian ascorbic acid depletion (ascorbic acid is produced by the CL and depleted by concentrations of LH in plasma) [22]. Extracts from hypothalamic tissue from immature and adult males were administered to immature and adult female rats, resulting in similar levels of ovarian ascorbic acid deletion. These data were then confirmed with ovariectomized immature and adult females, primed with progesterone and estrogen injections to enhance the negative feedback in order to block LH secretion from the anterior pituitary. Administration of stalk-median eminence extract from

immature and adult male and female rats resulted in similar LH-releasing capabilities (immature rats: 18.2 ± 1.7% ascorbic acid depletion, adult rats: 18.7 ± 2.3% ascorbic acid depletion) [21].

Gonadectomized immature and adult rats were injected with estradiol benzoate (control rats were administered sesame oil) to determine the effects of estradiol on plasma LH concentrations based on ovarian ascorbic acid depletion. At low doses of estradiol (0.002 μg/100g/day), immature rats had slightly reduced ovarian ascorbic acid depletion compared to the adult and control rats (immature rats: ~15% depletion, adult and control rats: ~17% depletion; $p<0.02$). After administration of 0.04 μg/100g/day of estradiol, ovarian ascorbic acid depletion was not detectable, indicating that LH was not present in the plasma, while concentrations remained high in adult rats, albeit lower than the control (immature rats: ~0% depletion, adult rats: ~14%, control rats: ~17%). Estradiol concentrations of 0.12μg/100g/day resulted in less than 5% ascorbic acid depletion in both immature and adult rats, indicating that the high concentrations of estradiol was over physiologic levels and suppressing LH release. These results indicate in immature rats the plasma concentration of LH is reduced due to low concentrations of estradiol exerting a negative feedback on the hypothalamus [21].

The "gonadostat theory" states that while the hormonal mechanisms are in place, the hypothalamus has a heightened sensitivity, shown to be about two to three times greater in immature rats compared to adults, due to the negative feedback from the low amounts of estrogen produced by the follicles [21]. This increase in sensitivity causes a decrease in LH synthesis and secretion by the pituitary, observed as reduced levels of

hypophysial and plasma LH, thus inhibiting the normal hormonal actions required for initiation of the estrous cycle [21, 23].

During the peripubertal period, the sensitivity of the hypothalamus is reduced allowing increased amounts of GnRH to be released and induce follicular development and ovulation, thus initiating puberty [21]. Day et al. [24] observed that there was a decline in the number of estradiol receptors located in the anterior hypothalamus (4.27 fmol/mg at 88 d, 2.54 fmol/mg at 46 to 25 d prepubertal; $p<0.05$), medial basal hypothalamus (4.69 fmol/mg at 88 d, 3.20 fmol/mg at 46 to 25 d prepubertal; $p<0.08$), and anterior pituitary (128.37 fmol/mg at 88 d, 99.35 fmol at 46 to 25 d prepubertal; $p<0.05$) suggesting a possible mechanism for the decrease in the sensitivity of the hypothalamus to estradiol. The concentration of GnRH receptors located in the anterior pituitary in the peripubertal and pubertal periods was not significantly different (93.70 ± 6.76 fmol/mg in prepubertal heifers, 109.27 ± 15.11 fmol/mg in postpubertal heifers; $p>0.50$), indicating that the changes in sensitivity were influenced by changes in estradiol receptor concentration rather than changes in GnRH receptivity by the anterior pituitary [24]. Approximately eight days prior to the onset of puberty, estradiol concentrations peak (6.3 ± 1.3 ng/l; $p<0.02$), indicating that the inhibitory activity of estradiol on the hypothalamus is reduced [25].

Ovarian Steroid Concentrations: Prepubertal, Peripubertal, and Pubertal

In the prepubertal heifer, peripheral plasma estradiol concentrations fluctuate but remain at basal levels, less than 4 ng/l, which is attributed to the secretion from growing antral follicles. Concentrations increased during proestrus and estrus (maximum

concentration approximately 6.3 ± 1.3 ng/l), indicative of the preovulatory peak, as seen in normal cycling females prior to ovulation [25]. Similar findings were reported by Moran [26] with estradiol concentrations remaining less than 4 pg/ml prior to puberty [11] while Gonzalez-Padilla et al. [27] reported peripubertal concentrations of less than 20 pg/ml.

Progesterone concentrations remain below one nanogram per milliliter in prepubertal heifers and rise as the first oocyte is ovulated, luteal tissue is formed and progesterone is secreted [28, 29, 30]. As stated previously, puberty can be defined in several ways including time of first ovulation and time of first observed estrus, neither of which may be an accurate definition. First ovulation is generally followed by a transient increase in progesterone; however, this increase in concentration is shorter than the typical luteal phase, 15-17 days, and is not preceded by a behavioral estrus [31]. Heifers can show one or two transient increases in progesterone between ten and twenty days prior to the first observed estrus indicating active luteal tissue. Progesterone levels were observed at concentrations lower than that of normal luteal function, 2.0 to 6.0 ng/ml compared to greater than 6.0 ng/ml, and were elevated for 7 ± 2 days [28, 32, 33]. Gonzalez-Padilla et al. [27] originally hypothesized that the first increase in progesterone was produced by the adrenals since there was no hormonal evidence in the blood that would implicate ovarian function, while the second increase was of ovarian origin caused by the luteinization of follicles to form a CL after the priming peak of LH. Gross analysis of the ovaries indicated the elevations were of ovarian origin produced

from luteinized follicles or from a CL that had formed within the ovarian tissue and were undetectable during a rectal examination [27].

Estrus Behavior, Detection, and Activity for Artificial Insemination

Behavior and Detection

During the estrus phase of the reproductive cycle, the female is sexually receptive to the male and will stand to be mounted; in beef cattle this averages 15 to 18 hours. The female will display behavioral signs of estrus which can be used to indicate the appropriate time for artificial insemination with fresh or frozen-thawed semen. Well documented behavioral estrus signs include standing to be mounted, mounting of other females, swollen vulva, and cervical mucus discharge [34]. The onset of behavioral estrus is correlated to the steady rise and peak (range from 4.5 to 10.4 pg/ml) of estradiol concentrations during proestrus as well as the rapid decrease in concentration approximately 12 hours after the end of behavioral estrus [35].

Esslemont et al. [36] studied the behavioral activities (mounting, chin-resting, licking, rubbing, etc.) of heifers over 24 days to determine the ratio of activity frequency during estrus to the frequency during the 24 day study. Heifers standing to be mounted, categorized by whether the bottom female received pelvic thrusts from the top heifer, resulted in the highest ratio of frequency (approximately a 1300 ratio with thrusts, 800 without thrusts) compared to the other activities (the second highest, females receiving disoriented mounts, less than 100 ratio). Based on these results, “it is evident that standing behavior is very highly diagnostic of oestrus” [36].

Standing to be mounted by either a male or another female was determined the most reliable indicator of a female being in estrus; however, mounting activity is influenced by environmental conditions, mainly location, time of day, and total number of females in estrus. In a comparison of barn-housed, drylot, or pastured dairy cows by visual inspection [37], location influenced the number of mounts received during estrus and the interestrus period (barn-housed: 8.7 ± 0.4 mounts, 36.7 ± 2.8 d interestrus; drylot: 6.1 ± 0.2 mounts, 29.5 ± 3.8 d interestrus; pasture: 5.5 ± 0.2 mounts, 29.5 ± 3.8 interestrus; $p<0.05$). Hurnik et al. [38] utilized a time lapse videorecorder and reported mounting events increased during nocturnal hours, suggesting the cause to be a decrease in distractions by human presence (6.0 ± 0.2 m/h in the morning vs. 7.7 ± 0.3 m/h in the evening, $p<0.05$ as determined by visual inspection) [37]. In the same study, Hurnik et al. [38] demonstrated that mounting activity increased when increased numbers of females in estrus were present (number of cows in estrus, one: 11.2 mounts, two: 36.6 mounts, three: 52.6 mounts, four or more: 49.8 mounts).

Senger [6] reported inadequate estrus detection resulted in an annual loss of over $300 million in the dairy industry indicating that accurate estrus detection was essential for proper reproductive management. Misdiagnosis of estrus (artificial inseminations of cows not in estrus occurs up to 30% of total inseminations) [39] or failing to observe females in estrus results in a loss of profit due to increases in semen expenses and labor costs. More importantly, the failure to detect estrus means a 21 day delay for females to be artificial inseminated, resulting in decreased pregnancy rates, an increase in the

number of females open at the end of the breeding season, and decreased number of calves produced per year.

Management considerations for selecting an appropriate detection system is based on herd size, location, labor, and most importantly, cost. Estrus detection methods can be as simple as visual inspection of the herd for estrus behavior twice a day for 30 minutes or more, to current sophisticated technology such as radiotelemetry requiring pressure-sensitive transponders and scientific computer software programs. Estrus detection technologies should offer: "continuous (24 h/d) surveillance of the cow, accurate and automatic identification of cows in estrus, operation for the productive lifetime of the cow, minimize labor requirements and high accuracy in identifying appropriate physiological or behavioral events" [6].

Radiotelemetry utilizes electronic transmission for continuous monitoring of estrus activity. The HeatWatch® system operates via miniaturized radiowave transponders linked to pressure sensors encapsulated within plastic cases. The transponders are housed within water-resistant patches and glued to the tail head of the cow [40]. Cow identification number, unique transponder identification number, time and date of mount, and duration of mount are recorded when the pressure sensors are depressed for more than one second. This information is transmitted to a receiver located within one mile of the herd and stored in a buffer until accessed by the HeatWatch® software. Estrus is defined as three mounts with a duration of one second within a four hour interval, based on the software's default setting which can be altered to include more stringent parameters [40, 41, 42].

Visual inspection is the traditional form of detection and is highly accurate; however, the efficiency in detecting females in estrus is approximately 50-70% [43]. During visual inspection twice a day for 45 minutes each, estrus activity was not detected in 11 out of 41 heifers (37%) as determined by mounting activity recorded by radiotelemetry [9]. Based on monitored data, heifers that received fewer mounts (19.3 vs. 60.5; $p<0.001$) or whose estrus duration was shorter (8.4 vs. 15.6 h; $p<0.001$) were not detected by visual inspection. The efficiency of estrus detection was greater when utilizing radiotelemetry (100%; $p<0.05$) compared to visual inspection (73%) [9]. In a similar study, visual inspection of the herd was done twice a day for 30 minutes and reported efficiency rates for estrus detection of 54.7 and 54.4% compared to rates of 71.7 and 86.8% (depending on synchronized or spontaneous estrus, respectively) for HeatWatch® [41]. Data from these experiments reported continuous monitoring of estrus behavior determined by radiotelemetry is more efficient than visual inspection and suggested that longer duration of visual inspection is more efficient in detecting females in estrus (54% for 30 minute inspection vs. 73% for 45 minute inspection) [9, 41].

Radiotelemetric detection systems have demonstrated differences in estrus activities (number of mounts, duration of mounts and estrus duration) between dairy and beef herds. Dairy cows receive on average between six and twelve mounts per estrus event with an average mount duration of 2.5 to 3.36 seconds (or average total mount duration from two herds of 29.0 seconds) [44] and estrus duration of 5.1 ± 3.8 to 10.6 ± 6.8 hours [40, 41, 44]. In beef cows, however, number of mounts received during estrus

on average was 50.1 ± 6.4 mounts with estrus duration ranging from 2.6 to 26.2 hours [9, 42].

Estrus Behavior in Gestating Females

Estrus behavior observed after conception is problematic in today's industry if estrus synchronization protocols are used, primarily those that incorporate prostaglandin F2α which are designed to lyse the CL. Inaccurate pregnancy detection for animals displaying estrus behavior after conception can lead to abortion, culling and slaughter. In previous studies, ewes displayed estrus behavior after conception with an occurrence rate of 22% and 62% in Western and Rambouillet ewes, respectively, with interestrus intervals ranging from three to 40 days post-insemination. On average, interestrus intervals were 21.61 ± 2.11 days, significantly different from the typical estrous cycle length for ewes [45]. The occurrence of estrus behavior in gestating Holstein-Friesian cows over a 30 year study was 5.6% of the 6,751 pregnancies ending in successful calving. Estrus behavior increased to 18.3% out of 1,905 pregnancies in cows that had calved one or more times in her reproductive life with 17.5% of those females displaying estrus during more than one reproductive period. Interestrus interval was on average 43 ± 1.9 days, while only 5.6% of estrus events occurred at 21 days [10]. During visual inspection of dairy and beef herds, it was found that 5.7% of the pregnant cows displayed behavioral estrus throughout gestation which were indistinguishable from estrus behavior from non-pregnant cows, with the exception the estrus duration was shorter (on average 5.6 hours) [46].

Artificial Insemination in Cattle

Artificial insemination (AI) involves depositing spermatozoa into the female reproductive tract by unnatural means. The rectovaginal technique of AI for cattle involves guiding the annular rings of the cervix over the AI gun and depositing the frozen/thawed semen into the uterine body. Pregnancy rates after AI or natural service is not significantly different in dairy cattle (57.5 and 58.0%, respectively) [47], while other studies have reported pregnancy rates after AI in dairy herds as low as 40% [40] and as high as 65% [44]. Insemination in beef cattle yielded higher pregnancy rates (between 84.2% and 93%) after first service [42, 48]. Pregnancy rates were higher in pubertal heifers inseminated on the third estrus than on the pubertal estrus (78% vs. 57%; $p<0.05$) with the probability of becoming pregnant increasing with age ($p<0.05$) [49].

Appropriate timing of insemination has been proven critical for pregnancy rates. Herman [50] recorded the pregnancy rates and average number of inseminations of dairy cattle after artificial insemination 4-12 h, 12-14 h, 24-48 h, and 48-60 h after the onset of estrus, determined by visual inspection. Cows bred 4-12 h after the onset of estrus had the highest pregnancy rate (536 settled/920 inseminations, 58% pregnancy rate) and required fewer inseminations (1.71 inseminations) before conceiving. Trimberger and Davis [51] repeated Herman's experimental design and further classified the stage of estrus at the time of insemination and recorded first service pregnancy rates in dairy cattle. Pregnancy rates were highest when females were bred during the middle of estrus (82.5%) and then decreased to 62.5% when bred six hours after the end of estrus. Breeding more than six hours after the end of estrus resulted in low pregnancy rates (12

hours, 32%) [51]. Similar findings were reported using the HeatWatch® system in dairy cows; conception rates were highest four to 12 hours after the onset of estrus (approximately 51%) and then decreased 16 to 20 hours after insemination (28.1%) [40].

Appropriate timing of insemination is based on the time of ovulation and the approximate life spans of spermatozoa and ova. Using the HeatWatch® system, dairy cattle were monitored for mounting activity and were evaluated by ultrasound at 12, 20 and 24 hours after the onset of estrus, and then every two hours until ovulation. Based on ultrasonography of follicular development and luteal formation, ovulation occurred 27.6 ± 5.4 hours after the onset of behavioral estrus, with 78% of the dairy cows ovulating before 40 hours [52]. Viability of the oocyte after ovulation is relatively short, approximately six to eight hours [40, 53]. Studies indicate that spermatozoa require more than 8 hours before becoming capable of fertilizing ova [54] and are viable for more than 24 hours [55]. Beshlebnov [56] reported spermatozoa were 50% progressively motile within the female tract after 24 hours and observed the spermatozoa were viable and capable of fertilizing up to 30 hours after artificial insemination.

The AM/PM rule of breeding cattle states that females observed in estrus in the morning should be inseminated in the evening, and females observed in estrus in the evening should be inseminated the following morning, approximately 12 hours after the onset of estrus. This rule was developed based on the previous studies for the optimum time of inseminating resulting in the highest pregnancy rates, approximately 4-12 hours after the onset of estrus, as well as the lifespan of the spermatozoa and ova. Saacke [57] diagrammed pregnancy results based on time of insemination, while incorporating sperm

and egg lifespans. Based on the combined data, inseminating too early will result in reduced fertilization capabilities of spermatozoa by the time ovulation occurred leading to low fertilization rates but high embryo quality. Inseminating too late, close to ovulation, however, will result in an aged ovum when the spermatozoa attained fertilization capabilities leading to high fertilization rates but low embryo quality [57].

Fascioliasis in Livestock Species

Fasciola hepatica: Hosts and Life Cycle

Infections of *Fasciola hepatica,* belonging to the family Fasciolidae, commonly known as the liver fluke, has been deemed the most significant trematode infecting domestic livestock [58]. *Fasciola hepatica* is an obligate parasite, needing two hosts for development and survival [59]. The pathogen requires an intermediate host, a species of snail in the genus *Lymnaea,* for maturation of the miracidium before infecting its final host, cattle, sheep and humans, for final development and reproduction [58].

There are seven species of *Lymnaea* important for transmission of *Fasciola hepatica* depending on region of habitation. *Lymnaea* snails are amphibious and require water for breeding, but primarily live on dry land preferring a slightly acidic environment. Even in droughts, this genus can survive for up to a year under mud coverings in an aestivated, or metabolically inactive, state. Snails thrive in temperatures up to 26°C, however temperatures exceeding this results in cessation of development [58].

Hatching of the miracidium, or embryo of *Fasciola hepatica*, from eggs excreted by the final host, requires proper hypertonicity and light, for secretion and release of enzymes essential for hatching, and temperature. Optimum temperature for hatching is between 22 and 26°C, coincidently similar to that of its intermediate host. Upon hatching, the miracidium is viable for 24 hours; however, its infective lifespan is only an hour and therefore must find an intermediate host within this time to be capable of penetrating the snail. Within the snail, the miracidium develop into cercaria in approximately five to seven weeks. Cercariae are excreted from the mouth of the snail in water and encyst on vegetation and form metacercariae ready for ingestion by the final host. The metacercariae can remain viable even below freezing temperatures for over three months [58, 60, 61].

Within 24 hours after ingestion, the metacercariae excyst and penetrate the wall of the intestines and migrate within the peritoneal cavity for three to four days. The metacercariae, considered an immature fluke, migrate through the liver tissue for approximately six weeks before entering a bile duct and maturing into an adult, fully capable of reproducing. Adult flukes are hermaphroditic and produce eggs which are released into the intestines and excreted in the feces of the host [58].

Liver Trauma and Clinical Signs

Severe damage to the parenchyma can be visualized three weeks post-infection (pi.) caused by abrasions from the spines of immature flukes during the migratory phase of development in the liver tissue [62]. Hemorrhagic plaques, or raised lesions, develop on the parenchyma three weeks pi. during the initial penetration and migration through

the liver tissue [59, 63]. Fibrosis, causing irregular contraction of the tissue, results as the liver undergoes repair around eleven weeks pi. when the initial onslaught from the flukes has ceased and have now entered the bile ducts [58]. Enlargement, calcification, and obstruction of the bile ducts occur between the seventh week and 23rd week pi. as the flukes finish migration and development and begin reproducing [63]. *Fasciola hepatica* adults secrete toxins within the final host which alters the structure of the bile duct and liver tissue walls, leading to distension and thickening of the bile ducts and lobules [60].

Fascioliasis, the disease caused by *Fasciola hepatica* infection, results in decreased body weight (described in further detail below) and other clinical signs. Animals continuously infected with low doses of *Fasciola hepatica* develop a chronic infection that is generally less severe (in terms of mortality losses and clinical signs) than acute forms [62, 64]. Infections of greater than 1000 metacercariae are generally required to initiate visible symptoms of fascioliasis in calves. Mortality losses have been observed in cattle infected with infections of 10,000 metacercariae [62].

Adult flukes feed on the blood of the final host [58, 60] at a rate of 0.2 to 0.5 ml per day per fluke [62] which leads to severe anemia. Hypoalbuminaemia (reduction in albumin levels produced by the liver) and hyperglobulinaemia (increase in immunoglobulins synthesized by leucocytes) are common signs; extensiveness depends on the burden of the infection [58, 62, 64, 65]. With the obstruction of the bile ducts, jaundice ensues from the increased concentration of bilirubin [66], the yellow bile pigment produced as the by product of degenerating heme groups in red blood cells [67].

Intoxication from the secretion of toxins by the flukes alters the bile duct walls and liver tissue and can enter the vascular system, resulting in leukocytosis, anemia, emaciation, and excitation of the central nervous system [60]. In chronic infections, anorexia, depression, diarrhea, and gaseous swelling can occur, and in severe cases, fever and death [60].

Damage to the liver tissue increases the auto-immune response of the host and directs eosinophils to the site of infection. In sheep infected with 150 metacercariae, eosinophil concentrations were increased by two weeks pi. and were significantly higher by six weeks ($p<0.05$), showing a correlation between eosinophilia and the intensity of the fluke burden (~1400 eosinophil cells/mm^3) [68]. In chronically infected cattle, lymphocyte concentration was reported to be highly correlated with fluke burden when measured at two and four weeks pi. and at slaughter, eight weeks pi. ($p<0.01$, $R= 0.751$) [69].

Hepatic Enzyme Concentrations and Protein Analysis and Their Use in Diagnosis

Due to its prominent existence and infection in cattle, accurate diagnosis of fascioliasis is necessary to reduce the health and economical problems that can ensue. Fecal analysis is the most conventional method for diagnosing infection; however, it has two main drawbacks. Due to the length of the life cycle for *Fasciola hepatica*, eggs are not detected in the feces until 10 to 21 wk post-infection [8, 70, 71, 72], after the immature fluke have reached the bile ducts, matured and reproduced. Fecal analysis for *Fasciola hepatica* eggs results in an under diagnosis of the true infection [71, 73]. As the number of adult flukes in the liver increases, the number of eggs produced by each

adult decreases [71] and the number of eggs per gram reported reflects only the population of adult flukes [71, 73]. In a comparison of fecal analysis, both flotation and sedimentation analysis resulted in approximately 30% of the total eggs per gram being accounted for in each sample, indicating a low sensitivity for these experimental methods [74].

Serum enzyme concentrations and/or activity may be increased in response to liver trauma. Damaged hepatic cells release enzymes into the blood circulation and plasma analysis for these hepatic concentrations can be utilized as an alternative diagnosis for *Fasciola hepatica* infection [72]. The use of aspartate aminotransferase (AST, previously known as glutamic oxaloacetic transaminase, SGOT), glutamate dehydrogenase (GD), and γ-glutamyl transpeptidase (GGT) concentrations in serum are common enzymes that have been examined for their use in diagnosis of *Fasciola hepatica* infection.

Increases in AST[1] and GD concentrations in blood serum have been associated with the migratory phase of infection and resultant parenchymal damage [71, 72, 75] whereas increases in GGT have been correlated to hepatobiliary damage [71, 75, 76]. Increased concentrations of AST in blood serum have been related to cellular tissue damage, such as skeletal tissue and cardiac muscle, possibly induced by handling, indicating a lack of liver specificity and a drawback for analysis of liver trauma [71, 75, 77]. GGT and GD concentrations are considered liver specific and will provide a better determination of the extent of liver trauma [75, 77, 78].

[1] Normal blood serum concentrations for AST and GGT are 47-138 U/l and 11-39 U/l, respectively (Texas Veterinary Medical Diagnostic Laboratory System, Texas A&M University, College Station TX 77841)

Friesian calves were experimentally infected with two doses of 500 metacercariae by oral drenching and bled weekly to determine serum concentrations of GD, GGT, sorbitol dehydrogenase (SDH), ornithine carbamoyl transferase (OCT), lactate dehydrogenase (LDH) and AST. The mean ratio of activity for the six enzymes of infected calves to controls was greatest for GD and GGT (17.6 and 13.8, respectively) with mean concentrations of 643 and 317 U/l, respectively, suggesting these two enzymes may be accurate diagnostic aids. The mean ratio of activity was lowest for LDH and AST indicating their lack of liver specificity and suggesting their inadequacies in diagnosing *Fasciola hepatica* infection (2.2 and 2.1, respectively) [77].

When calves were infected with three doses at four week intervals for a total load of 1200 metacercariae, AST concentrations were increased by 66 days pi. ($p<0.01$) and reached maximum values (twice the pre-infection levels) by day 185 ($p<0.01$). GGT concentrations were significantly higher 91 days pi. ($p<0.01$) and remained four to seven times higher than pre-infection levels until day 185 ($p<0.05$) and in some individuals, concentrations were up to 20 times higher [75]. Similar findings were observed by Wyckoff and Bradley [71] with Brahman calves infected with 1000, 100 or 10 metacercariae in three doses, for a total infection load of 3000 (group 1), 300 (group 2) and 30 metacercariae (group 3). AST concentrations were significantly increased by four weeks pi. ($p<0.05$) and remained elevated to 16 weeks, with maximum concentrations occurring between weeks five and 12 for group 1. AST values for the lesser infected calves were not significantly greater, except on occasion in group 2. For all infection groups, GGT concentrations increased by 9 weeks pi., however, only

concentrations for groups 1 and 2 were significantly increased ($p<0.05$). This study demonstrated the relationship between infection level and liver damage by the relative differences in GGT concentrations between groups 1, 2 and 3 [71]. In the three previous studies, experimental infections were administered in few doses at high concentrations of metacercariae. To simulate a "natural" infection, six month old beef calves were administered 5 metacercariae every other day for 80 days. Even with a "natural" infection load, concentrations of AST and GGT followed suit with the trends observed from the high dose experiments [76], suggesting that hepatic damage is consistent between natural and experimental conditions. Alkaline phosphatase (ALP) concentrations were analyzed and were not significantly different between the control and infected calves, consistent with Anderson et al.'s [77] findings. At slaughter, livers from the infected calves were examined for fibrosis, biliary hyperplasia, and hyaline and eosinophilic deposits and grouped according to GGT responses. In all infected calves, slight to marked fibrosis (5 slight, 7 moderate, and 7 marked) was observed [76].

Elevated concentrations for liver enzymes resulting from *Fasciola hepatica* infection are consistent across species. Water buffalo infected with 60 metacercariae daily for 20 days exhibited increased ($p<0.05$) GD (6-21 wks pi.), GGT (8-26 wks pi.) and AST concentrations (6-23 wks pi.) [72]. At low doses of infection in sheep (25 metacercariae daily for 6 days), GD and GGT concentrations increased after 20 and 40 days pi., respectively ($p<0.05$) [79]. "Natural" infections in sheep (3, 8 or 14 metacercariae for 5 days for 22 weeks) resulted in significant dose responses in GGT

and GD at 23, 12 and nine weeks and 32, 24 and 12 weeks pi, respectively. AST concentrations were not correlated with dosage level [78].

In addition to hepatic enzyme analysis, serum protein concentrations can be measured to diagnose fascioliasis. After infection with two doses of 500 metacercariae, albumin concentrations were significantly decreased from six to 17 weeks pi. ($p<0.01$), and globulin concentrations were significantly increased ($p<0.01$) from 12 to 17 wks pi. and at 23 wks pi. ($p<0.001$), albeit all concentrations remained within normal limits[2]. Bilirubin concentrations were not significantly increased [77]. Minor differences between infected and non-infected calves for albumin and bilirubin concentrations were also reported by Wyckoff and Bradley [71].

Prevalence

Fasciola hepatica is a trematode parasite that has been affecting beef productivity for years. The number of cattle affected by *Fasciola hepatica* has been steadily increasing in some parts of the world. Diagnosis of fascioliasis in England and Wales increased from 50 to almost 400 cases annually in the last decade [80]. Abattoirs in the United States have reported up to 140 flukes per condemned liver [65] and liver inspections of cattle from Argentina in 1979 and 1980 revealed 13% of slaughtered cattle were infected with *Fasciola hepatica* [81].

In the United States, fascioliasis affects livestock in the southern states of Florida, Louisiana, and Texas, the northwestern states of Oregon, Washington, Idaho,

[2] Normal concentrations for albumin, globulin and bilirubin are 3.1-4.3 g/dl, 2.5-6.1 g/dl, 0.1-0.5 mg/dl, respectively (Texas Veterinary Medical Diagonostic Laboratory, Texas A&M University, College Station, TX, 77841)

and Montana, and the western states of California, Nevada, and Utah [61]. As stated previously, optimum temperature for snail and *Fasciola hepatica* development is 26 °C (78.8 °F) making livestock in southern and western states highly susceptible to infection. Wet periods recorded high mortality rates caused by fascioliasis and periods of draught may reduce the incidence of infection, however, infected snails can prevail in draughts for up to a year [61]. High prevalence has been recorded on irrigated lands which may prolong the productivity of the parasite. Young calves appear to be more susceptible to *Fasciola hepatica* infection than adult cattle. Calves experimentally infected at 52 to 66 days of age consistently had fewer eggs per gram in feces samples compared to calves infected at 1 to 27 days of age; however, upon slaughter, the older group on average had a significantly higher fluke count (21.25 flukes vs. 9.88 flukes; $p<0.01$) [82]. Bull calves, 60 to 90 d of age, experimentally infected with 1200 metacercariae had fluke counts ranging from two to 96 (average 53.7 flukes) and had values for liver enzymes, indicating trauma (AST and GGT), two and three to 20 times higher than normal values, respectively [75]. Adult livers obtained from slaughterhouses contained less than 33 flukes and liver enzymes were not significantly increased [83].

In a few experimental studies of *Fasciola hepatica* infection, reports on cattle resistance have been observed based on the level of infection received. In experimentally infected cattle, higher levels (5000 and 15,000) of metacercariae resulted in flukes becoming caught within the liver parenchyma and reducing the number of immature flukes reaching the bile ducts [84]. Dwinger et al. [81] found in a field study that fewer flukes were found in the liver of severely infected cattle (more hepatic

damage) than the moderately infected (22 flukes present vs. 32.5 flukes, respectively). In sheep and cattle it was suggested that fibrosis of the liver in severe infections may impede the migration of immature flukes and expel adult flukes from the bile ducts resulting in an acquired resistance [84, 85].

Economical Implications: Direct and Indirect Losses

Chick [86] divided the economical significance of *Fasciola hepatica* infection in cattle into two primary categories, direct and indirect costs, based on completed questionnaires from Australian cattle graziers. Direct costs can be classified as costs the producer loses to maintain normal productivity of the herd, including drenches, labor, anthelmintic treatments, and losses at slaughter, mainly in liver condemnation from *Fasciola hepatica* infection. Only approximately 50% of the graziers questioned acknowledged a *Fasciola hepatica* infection in their herd and were using regular control action, estimated to cost $2.40 per head. Labor for drenches was included in normal farm management and expenses were estimated to be close to zero [86]. Losses from treatment and prevention of cattle in Nigeria due to fascioliasis was estimated N30,000[3] (total annual loss, estimated N5 million) [87].

With only a 0.4% prevalence rate during liver inspection in Greece, *Fasciola hepatica* infection averaged cost of loss was 6500 GDR[4] from condemned livers,

[3] Exchange Rate in 1980 for Nigerian Naira- N0.5587. Information obtained from the United States Department of the Treasury Library.

[4] During the third quarter in 2002, the monetary unit of Greece (GDR) was replaced by the Euro. Exchange rates in 2002, 1st quarter- 382.39 GDR and 2nd quarter - 392.84 GDR, 3rd quarter- 1.0730 EURO and 4th quarter - 1.0200 EURO. Information obtained from the United States Department of the Treasury, Financial Management Service, Contact: Andrea Pearson

calculated from the average liver weight, 5 kg, by the market value per liver, 1300 GDR [88]. Theodoropoulos et al. [88] estimated the cost: benefit ratio of anthelmintic treatment by assuming (1) infected animals had not received proper treatment during production, (2) the loss of condemnable organs could have been avoided if treated, (3) treatments were administered twice annually, (4) average dosage of 150 kg treatment for cattle, and (5) cattle would be treated four times before slaughter. The estimated cost: benefit ratio of anthelmintic treatment for *Fasciola hepatica* infection in calves less than three years of age was 113:1 indicating a substantial loss in profit for treated calves; however, the use of treatments could reduce overall indirect costs from production losses. Treatment of *Fasciola hepatica* in beef heifers simultaneously infected with gastrointestinal nematodes (GIN, active GIN infection remaining after treatment) increased the average gross return per heifer from $507 (untreated heifers) to $516 (calculated from the estimated sale value of the heifer and pregnancy rate) [89].

Foreyt and Todd [90] reported annual losses in the United States from the 1.2 to 1.5 million liver condemnations from *Fasciola hepatica* infection to be approximately $10 million. In 1981, the 1.4 million liver condemnations from cattle in the United States, equaled about 4.4% of all slaughtered animals, resulting in an annual loss of $7.2 million (average liver price $5) [91]. The American Association of Veterinary Parasitologists [92] reported approximately 1.5 million cattle livers were condemned in the United States in 1983, indicating that numbers of condemnations were gradually increasing each year [65]. A 10 year study of slaughtered cattle in Kenya reported that 8% of the animals slaughtered were infected with *Fasciola hepatica*, totaling 1,283,793

kg of condemned livers [93]. Annual and decennary monetary losses were calculated from the average weight of bovine livers, 3 kg, and market price, $2.00 in 2002. Kithuka et al. reported annual losses ranging $0.2 to 0.3 million and decennary losses of $2.6 million from liver condemnations alone.

Overall losses in sheep from the Ethiopian highlands in 1993 were estimated to be 48.4 million Ethiopian Birr per year[5]. Losses from mortality, productivity (weight loss and reproductive wastage) and liver condemnation equaled 46.5, 48.8 and 4.7% of the total estimated loss [94].

Indirect losses in profits are primarily categorized as losses from reduced productivity, such as growth rate, milk production, fertility, and mortality [86]. Chick found that experimental infection with 600 or 1200 metacercariae of *Fasciola hepatica* resulted in decreased growth rate of 11.7 and 13.9%, respectively. Dargie's review [65] of production impacts from *Fasciola hepatica* infection in 1987, described previous findings on weight loss, primarily a notable less than 0.03 kg/week with low infection levels (45 flukes) and 0.13 to 0.30 kg/week with high infection levels (87 to 500 flukes). Loyacano et al.'s (89) comparison of beef heifers simultaneously infected with GIN and *Fasciola hepatica,* found an 8 kg difference in total weight gain for heifers treated for *Fasciola hepatica* compared to untreated heifers.

Decreases in milk production have been noted in cattle infected with *Fasciola hepatica.* Ross [95] indicated that infections increased milk productivity loss depending on the severity of infection (8% loss from 100 flukes, greater than 23% loss from more

[5] Exchange rate in 1993 for Ethiopia-Birr- 5.175 Birr. Infromation obtained from the United States Department of the Treasury, Financial Management Service, Contact: Andrea Pearson

than 500 flukes). Taking into account percent of animals infected with an 8% production loss per head and current milk prices in 1970, Ross [95] estimated a loss of £75[6] for 50 head of cattle in three months when the prevalence of infection was high (January to April).

"One of the most important impediments to efficient livestock production is poor reproductive performance due to infertility, abortion, and embryonic and neonatal death" [96]. A herd of approximately 100 Angus cattle naturally infected with *Fasciola hepatica* with clinical signs (emaciation, anemia, hypoproteinemia, hypocalcemia, hypophosphatemia and hypoalbuminemia) in Idaho demonstrated reduced reproductive performance with a calf crop less than 50% [97]. Beef cows experimental infected with 1000 *Fasciola hepatica* and 350 *Fascioloides magna* metacercariae produced on average fewer calves compared to the non-infected cows (80% vs. 95% calf crop, respectively) [97].

Prenatal invasion of the fetus by *Fasciola hepatica* was reported by Rees et al. [98] after the slaughter of newborn calves (one to three weeks of age) whose dams were naturally infected. Livers of the newborns showed gross lesions, acute inflammation, fibrosis of the bile ducts, and accumulation of neutrophils and eosinophils. A total of 108 flukes was collected from 84 livers, and the flukes were estimated to be approximately 10-weeks-old, indicating invasion and infection of the fetus occurred in utero [98]. Abortions during all stages of gestation (one to eight months) were associated with natural infections of *Fasciola hepatica* in 25% of the pregnant dams.

[6] Exchange rate in 1970 for British Pounds- £0.4171. Information obtained from the United States Department of the Treasury Library.

Cause of the abortion was suggested to be the production and/or release of toxins from the flukes which affected fetal circulation, damage to the fetus during prenatal invasion, or hypoglycaemia of the dam from liver trauma [99].

Mortality rates of ewes naturally infected with *Fasciola hepatica* in 1966 to 1967 were reported to be 14.3% from September and December (pregnancy unclassifiable) and 22.8% from January to April (7% pregnant and 15.8% barren; mortality rate $p<0.01$ from pregnant vs. barren ewes). Level of infection, based on mortality rate, and fertility, based on pregnancy rate, of infected ewes were negatively correlated ($r= -0.83$; $p<0.001$) [100]. Mortality rates in Nigeria in 1980 from cattle infected with *Fasciola hepatica* were an estimated 1% (approximately 1 million of the 10 million cattle population) [87].

Effects of Experimental Infection on Puberty

Few studies have indicated the effect of *Fasciola hepatica* infection on fertility in livestock; though even fewer studies have explored the possible impact that infection could have on pubertal development. Fleming and Fetterer [7] hypothesized liver trauma from *Fasciola hepatica* infection would alter the normal function of steroid catabolism, thus altering peripheral concentrations of steroids resulting in delayed puberty, short estrous cycles, and infertility of ewe lambs. Prepubertal rams were infected orally with 50 *Fasciola hepatica* cysts and bled weekly to determine the concentration of circulating testosterone detected by radioimmunoassay. Concentrations for infected rams were not significantly different from their non-infected contemporaries ($p>0.05$). During the postpubertal period, the rams were administered a challenge dose of 50 mg of testosterone to determine metabolic clearance rate. The infected rams

displayed a decreased ability to clear exogenous testosterone concentrations from circulation ($p<0.05$). Hepatic damage from moderate fluke levels affected the catabolism rate of exogenous testosterone (metabolic clearance rate) but showed compensatory action to maintain endogenous concentrations within normal limits as compared to the non-infected rams, possibly from the negative feedback of testosterone on LH frequency [7].

López-Díaz et al. [8] explored the possibility of an altered metabolic clearance rate of steroidal hormones in the blood serum of prepubertal Friesian heifers experimentally infected with 600 metacercariae of *Fasciola hepatica* which may delay the onset of puberty. At four months of age, thirteen heifers were designated to the infected (n=6) and control group (n= 7) based on weight and age. Fecal samples were collected and analyzed starting at 8 week pi. and analyzed every 15 weeks to assess the level of infection received by each heifer. Blood samples were collected biweekly and analyzed by a solid phase ^{125}I assay and competitive enzyme linked immunosorbent assay (ELISA) for estradiol 17β (E_2) and progesterone (P_4) concentrations, respectively. Estrus activity was detected via heat-mount detectors (Kamar Inc., Steamboat Springs, CO) checked biweekly where a positive response indicated the heifer was in estrus within the previous three to four days. The onset of puberty was determined by a positive response reading and confirmed by a second positive reading indicating the female was cyclic [8].

In the experimental heifers, *Fasciola hepatica* eggs were not detected via fecal analysis until 12 weeks pi. Average weight at puberty (age = 286 d) was substantially

different between the infected and control heifers (I: 215.4 ± 5.7 kg, C: 230.7 ± 9 kg; $p>0.05$), albeit the sample size was insufficient to reject the null hypothesis at $p<0.05$. Estrogen and progesterone concentrations were higher prior to first estrus (I: 10 ± 3 pg/ml E_2 and 0.26 ± 0.1 ng/ml P_4, C: 4.4 ± 0.8 pg/ml E_2 and 0.93 ± 0.3 ng/ml P_4; $p<0.05$) and estrogen concentrations were continuously higher in the postpubertal period ($p<0.05$). *Fasciola hepatica* infection in prepubertal heifers resulted in a 39 day delay in puberty (I: 325 ± 16 d, C: 286 ± 10 d; $p<0.05$). López-Díaz et al. [8] hypothesized the delay in puberty was resultant from an alteration in the metabolic clearance rate of estrogen induced from the trauma of the migrating flukes as was seen in the prepubertal ram study by Fleming and Fetterer [7]. Consistent with the "gonadostat theory," the increased estrogen concentrations suppress the release of GnRH thus inhibiting follicular development and ovulation [8].

Though well detailed, the experiment by López-Díaz et al. [8] had several shortcomings. The Kamar® system utilized for estrus detection involves a pressure-sensitive patch glued to the tail head of the animal. Unlike the HeatWatch® system which allows for multiple readings, once depressed, the pressure-sensitive patch changes from white to red, thus allowing for only one reading and several instances for false-positives. The heifers were checked twice weekly for positive responses, indicating the onset of estrus had occurred within the three to four days since the last check. False positives from heifers false mounting or rubbing could not be accounted for using this style of estrus detection if the heifers were not monitored more frequently to associate a positive response with behavioral estrus. Secondly, the onset of puberty was defined as

the occurrence of two positive responses without the authors' mention of utilizing progesterone concentrations for confirmation.

As previously mentioned, average weights between infected and control heifers were noticeably different but were not significant at the 0.05 level. As stated by the authors, the difference failed to reject the null hypothesis because of insufficient sample size (six infected and seven control heifers). The authors inconsistently claim significant levels less than 0.05 for the delay in puberty and estrogen and progesterone concentrations although the experimental design and sample size remain unchanged. With no change in experimental design, the insufficient sample size would need to be accounted for in the analysis of puberty and steroid hormone concentrations as well. The authors may perhaps be leading to the assumption that the significance levels obtained could be indicative of significant effects from *Fasciola hepatica* infection; however, experimental infection with a larger sample size should be analyzed.

CHAPTER III

EFFECTS OF EXPERIMENTAL FASCIOLIASIS ON PUBERTY

Introduction

Fasciola hepatica infection alters normal liver function in cattle indicated by elevations in hepatic enzymes released into circulation as hepatic cells are damaged, and can be used in diagnosing infection earlier than the conventional fecal analysis [72]. Reports demonstrate the severity of infection is dependent on dosage of metacercariae [78]. AST and GGT concentrations increased 66 and 91 days post-infection in calves infected with 1200 metacercariae from parenchymal and hepatobiliary damage, respectively [75]. In contrast, infection loads of 3000 metacercariae in Brahman calves resulted in an earlier elevation in AST and GGT concentrations at only 4 and 9 wks post-infection [71]. "Natural" infection of calves administered low doses of metacercariae over an extended period of time resulted in similar elevations in AST and GGT concentrations [76], suggesting that findings from experimental infections mimic those normally observed in natural infections.

Numerous studies have reported the impacts of *Fasciola hepatica* infection on the economy by investigating the effect of fascioliasis on liver condemnation, weight gain, milk production, fertility and mortality. In contrast, few studies have investigated the impact of fascioliasis on pubertal development, a critical time in the production of livestock. Fleming and Fetterer [7] observed concentrations of circulating testosterone within the normal range in prepubertal rams infected with *Fasciola hepatica.* However, a decreased ability to clear exogenous testosterone in the infected rams suggested that the

metabolic clearance rate of the hormone in the liver might be impaired. López-Díaz et al. [8] observed an increase in circulating estrogen concentration and a 39-day delay in the onset of puberty in prepubertal heifers infected with *Fasciola hepatica.*

This experiment was designed to characterize the patterns in circulating steroid hormone concentrations and pubertal development after infection of 4-mo-old heifers with 600 metacercariae of *Fasciola hepatica*. We used a larger sample size (eleven infected and eleven control heifers) to extend the observations of López-Díaz et al. [8] by incorporating hepatic enzyme and plasma protein analysis and improved estrus detection technology.

Hypothesis: *Fasciola hepatica* infection of 4-mo-old beef heifers will result in alterations of serum concentrations of ovarian steroid hormones and delay puberty.

Objectives:

1. Quantify the effects of *Fasciola hepatica* infection on: body weight, hepatic enzyme, ovarian steroid hormone, and protein concentrations in serum, and
2. Determine the effects of *Fasciola hepatica* infection on the age and BW at puberty and first service conception rate.

Materials and Methods

Treatment Groups

Twenty-two Angus-sired heifers (born within a 28-d period), known to be free of *Fasciola hepatica,* were housed as a single group on a concrete surface. Upon arrival to

the research facility (May 30, 2002), preliminary weights were obtained and heifers were administered CattleMaster 4+VL5, Vision 8 with SPUR, and Ivomec® Eprinex Pour-On

Table 1. Feed composition and percentage.

Ingredient Name	Percentage
Corn	23.6%
Cottonseed Meal	15%
Beef Vit 6905	0.05%
Beef TM 6962	0.05%
Soybean Hulls	30%
Cottonseed Hull	25%
Ground Lime	0.8%
Salt Mixing	0.5%
Molasses Summer Blend	5%

(150kg dose to ensure heifers were free of parasitic infections). Vaccinations were repeated 4-wk later. Heifers were allotted into two groups (control and infected) based on age, BW at 4 mo (± 2 wks) of age (average of 135 kg), and sire (n= 4). On day 0 (June 13, 2002), the infected group was administered 600 metacercariae of *Fasciola hepatica* (Baldwin Aquatics Inc., Monmouth, OR) intraruminally in a single dose. Heifers were individually fed a concentrate (Table 1) twice daily and hay to achieve an average daily gain of 0.68 kg per day. On day 82, the heifers were assigned individual feeding chutes (Calan gate chutes) with unique electromagnetic access to ensure adequate and consistent consumption amongst the herd. Chutes were assigned by preference after being observed for 53 days and trained to eat from their respective chutes.

Infection Levels

Fecal samples were collected twice monthly from each heifer to monitor fecal egg counts using two methods. The Wisconsin Double Centrifugal Sugar Floatation [101] was used to determine the parasitic backgrounds of the heifers. The "Flukefinder" (Visual Difference, Moscow, ID) was used to determine the number of liver fluke eggs per gram during the experiment.

Blood samples were collected twice monthly and analyzed by the Texas Veterinary Medical Diagnostic Laboratory, at Texas A&M University, for a complete ruminant diagnostics panel. Results for the hepatic enzyme (GGT and AST) and serum protein (bilirubin, albumin and globulin) concentrations received from the diagnostic test were used to assess changes induced by *Fasciola hepatica* infection.

Ovarian Steroids

Blood samples were collected twice monthly from day 0 to day 60 via jugular venipuncture, and subsequent samples were collected twice weekly until the completion of the project. After each collection, the blood serum was separated by centrifugation and frozen at -20° C for a subsequent radioimmunoassay (RIA). Blood samples were collected from a pregnant cow (at approximately 8 mo gestation) and from an ovariectomized cow to serve as hormone controls in the RIA.

Concentrations of estradiol 17-β and progesterone were analyzed by RIA after the hormones were extracted from the serum, using the Ultra-sensitive Estradiol RIA DSL-4800 (sensitivity= 1 pg/ml; cross-reactivity of 2.40% and 0.21% for estrone and 17α estradiol; Diagnostic Systems Laboratories, Inc., Webster, Texas) and the

progesterone assay as previously described (sensitivity= 0.2 ng/ml) [102], respectively. Intra- and inter-assay coefficients of variation (as determined by serum from the pregnant control cow) for estradiol were 3.10% and 3.41%, respectively, and for progesterone were 3.02% and 7.91%, respectively.

Estrus Detection

To determine the onset of puberty, we used serum progesterone concentration and the HeatWatch® System (DDX, Inc., Denver, CO). An elevation in progesterone concentration (>1 ng/ml of serum) in at least three consecutive samples was defined as a luteal phase resulting from initiation of estrous cyclicity accompanied by ovulation and formation of a corpus luteum.

The electronic HeatWatch® System was used to aid in the detection of estrus by continuously monitoring the mounting activity (number of mounts and duration) for each heifer. On day 165, transponders were assigned and fixed to the tail head of the heifers and the onset of puberty was determined by the occurrence of at least three mounts within a 4-hr interval, which was followed by an elevation in serum progesterone as described above.

Artificial Insemination

After the onset of the second estrus (interval from pubertal estrus ranged between 17 and 24 days), each heifer was artificially inseminated approximately 12 hr after the initiation of estrus. A single technician used frozen-thawed semen from one Angus bull (29AN1458) to inseminate the heifers.

Statistical Analyses

The Mixed Procedures function of the SAS 8.1 program (SAS Institute Inc., Cary, NC) was used to determine if the rate of change of monitored variables was different between treatment groups. This type of analysis is sometimes referred to as growth curve analysis [103]. Monitored variables included: (1) hepatic enzyme concentrations from day 0 to day 112 post-infection and the 16-wk interval preceding puberty, (2) estradiol and progesterone concentrations from day 0 to the end of the study (day 393) and the 8-wk interval preceding puberty, (3) serum protein analysis from day 0 to day 393, and (4) average BW from day 0 to day 393 and the 16-wk interval preceding puberty. Analysis of variance was used to analyze treatment effects on age and BW at first ovulation. Correlations between age at puberty and the maximum concentration and area under the curve (AUC) for fecal egg counts and hepatic enzymes were analyzed using the General Linear Model. AUC was calculated by taking the sum of all values for a given heifer across time, multiplied by a constant variable (14) for the length of time (days) between sample periods to find the area as described in equation (a) below. Conception rate was analyzed using a Chi-Square analysis.

Equation (a) AUC= 14 x concentration sum

Medical Treatments

The following medications were administered during the project:

1. Liquamycin (LA200) was administered to three control heifers and one infected heifer for pink eye infections (8/2/2002-9/20/2002).

2. Two control heifers developed abscesses from the Liquamycin injections and were administered Nuflor (6 mg/pound), Banamine and Dexamethasone (9/18/2002- 9/30/2002).
3. Ringworm lesions were routinely treated with topical 2% iodine solution.
4. Heifer #2 (control group) aborted her 6-mo-old fetus (6/10/2003) which was diagnosed to result from an infection with *Listeria ivanovii*. Procaine Penicillin G was administered daily at 60 cc subcutaneously for 7 days and then at 30 cc for 7 days. She was isolated from the trial heifers to reduce the risk of transmission to the pregnant heifers. The fetus and one milliliter of the heifer's serum was submitted to the Texas Veterinary Medical Diagnostic Laboratory, Texas A&M University for an abortion screen.

Results

One control heifer (No. 3) displayed progesterone concentrations over one ng/ml for the duration of the project. Upon palpation, it was determined she was approximately 7-mo pregnant and had conceived prior to her arrival at the Texas A&M University research facility. All blood samples and fecal samples collected from this heifer were excluded from the analyses.

Approximately 45 days post-conception, a control heifer (No. 17) experienced embryo loss as determined by reduced progesterone concentrations and a return to estrus. On January 28, 2003, she was bred via artificial insemination and determined to be pregnant by elevated concentrations of progesterone (>1 ng/ml) to day 35. By day 38

post-insemination, progesterone concentrations were below one ng/ml and she returned to estrus 42 days post-insemination, indicating embryonic loss. Data from this heifer were incorporated into the analysis, and she was considered pregnant.

In the control group, one heifer (No. 2) aborted at six months of gestation (6/10/2003). The fetus and one milliliter of the heifer's serum were submitted to the Texas Veterinary Medical Diagnostic Laboratory, Texas A&M University for an abortion screen and were diagnosed as resultant from infection with *Listeria ivanovii.*

Treatment Effects

Body weight, GGT, AST, bilirubin, albumin, globulin, and estradiol and progesterone concentrations were analyzed using the SAS Mixed Procedure to determine significant effects of *Fasciola hepatica* infection. Treatment group did not affect BW or concentration of either estradiol or progesterone in serum during the study (Table 2). BW had a curvilinear effect over time ($p=0.0003$) that was not ($p>0.1$) modified by group (Table 2; Figure 1). Estradiol had a quadratic effect with time ($p<0.0001$) that was not modified by group ($p>0.1$; Table 2; Figure 2). Progesterone had a linear effect over time ($p<0.0001$) that was not modified ($p>0.1$) by group (Table 2; Figure 3). Mean BW and mean serum concentration of estradiol and progesterone by treatment group on days 0 and 393 are presented in Table 3. All heifers and blood samples, regardless of group designation, were handled in a similar manner and analyzed together. After day 112, there were decreases in serum GGT and AST concentrations. Therefore, the growth curve model was used to analyze the changes in serum GGT and AST only

Table 2. Effects (probability level) of treatment group and time on body weight and concentration of estradiol and progesterone from day 0 through day 393.

	Group	Time	Time*Group	Time*Time	Time*Time*Group
BW	NS	< 0.0001	NS	0.0003	NS
Estradiol	NS	NS	NS	< 0.0001	NS
Progesterone	NS	< 0.0001	NS	NS	NS

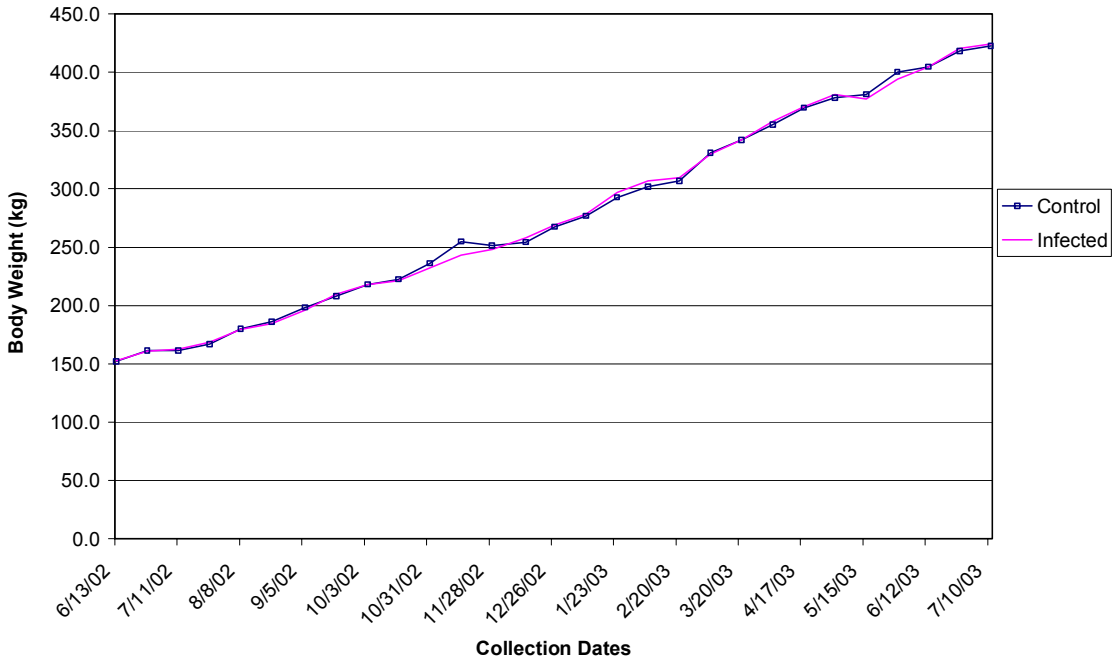

Figure 1. Mean body weight by treatment group from day 0 to day 393.

during the interval from day 0 to 112 when concentrations were either basal or increasing for control and infected heifers, respectively. The analyses of GGT and AST from day 0 to day 112 resulted in significantly higher concentrations in the infected group at the beginning of the study (p=0.0009 and p=0.0002, respectively; Table 4). However, analyses of the enzyme concentrations in serum during the first 6 wk (day 0 to

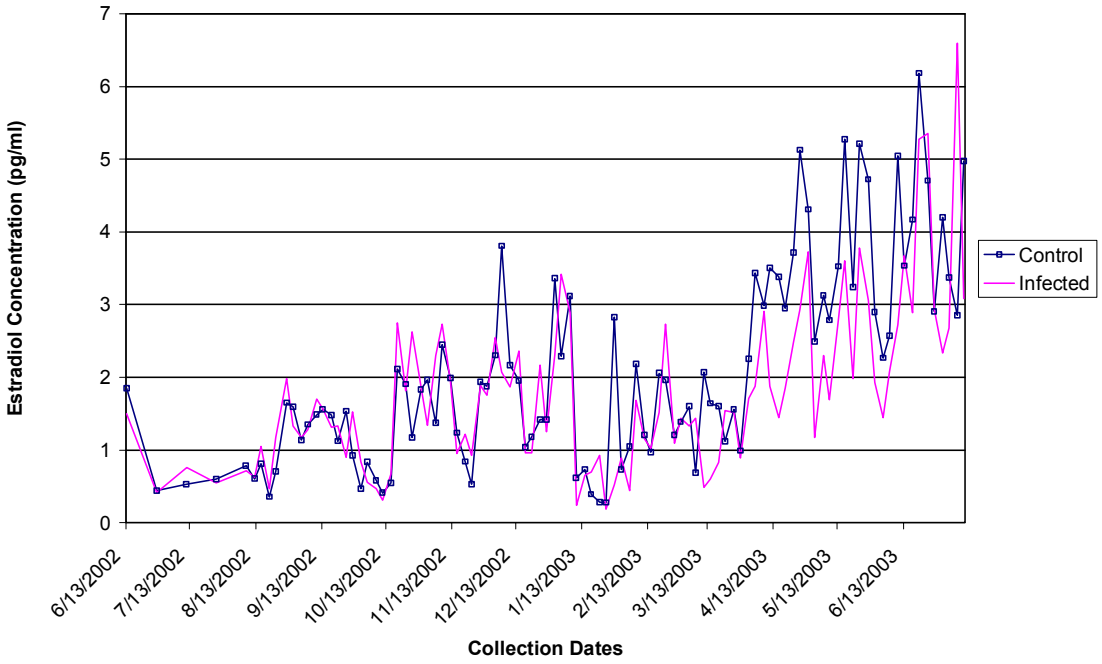

Figure 2. Mean serum concentration of estradiol by treatment group from day 0 to day 393.

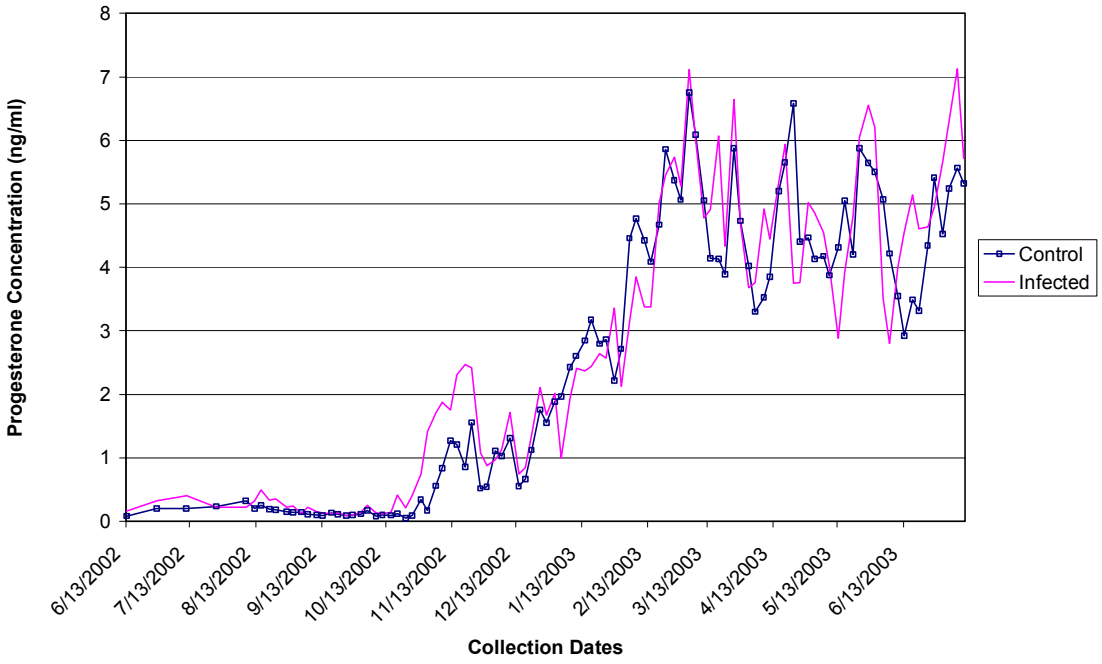

Figure 3. Mean serum concentration of progesterone by treatment group from day 0 to day 393.

Table 3. Mean (± SE) body weight and mean (± SE) serum concentration of estradiol, progesterone, GGT, and AST on days 0 (start) and 393 (end) of the study.

	Day 0		Day 393	
	Control	Infected	Control	Infected
Body Weight (kg)	152 ± 6	153 ± 3	422 ± 12	424 ± 13
Estradiol (pg/ml)	1.85 ± 0.44	1.50 ± 0.46	4.98 ± 1.67	3.08 ± 1.23
Progesterone (ng/ml)	0.08 ± 0.01	0.16 ± 0.03	5.32 ± 1.33	5.71 ± 1.00
GGT (U/l)	16 ± 1.0	16 ± 0.6	18 ± 1.5	18 ± 1.6
AST (U/l)	62 ± 4.3	70 ± 6.4	59 ± 2.3	58 ± 3.9

Table 4. Effects (probability level) of treatment group and time on GGT and AST concentrations in serum of heifers from day 0 through day 112.

	Group	Time	Time*Group	Time*Time	Time*Time* Group
GGT	0.0009	0.0008	0.002	NS	NS
AST	0.0002	0.0006	0.051	0.011	0.086

day 42) indicated that the AST and GGT concentrations were not different between treatment groups at the beginning of the project (data not shown). Based on the graphs in Figures 4 and 5 (illustrating GGT and AST concentrations from day 0 to 393), the increase in GGT and AST occurs after day 42, suggesting that the group analysis may be incorporating an interval rather than the specific time points at the beginning of the dataset. GGT had different intercepts for treatment group (p=0.0009) and a linear effect with time (p=0.0008) that was also different between groups (p=0.002; Table 4). AST intercepts differed between treatment groups (p=0.0002) and a linear effect tended to be different between groups (p=0.051). AST had a curvilinear effect with time (p=0.011) that was not significantly (p=0.086) modified by group (Table 4). Albumin had a

quadratic effect with time ($p<0.0001$) and globulin had a linear effect with time ($p<0.0001$). Neither of the effects was modified by group ($p>0.1$ and $p=0.071$, respectively; Table 5; Figures 6 and 7). Bilirubin was not related to time or to group ($p>0.1$; Table 5; Figure 8).

Age and Weight at First Ovulation (Puberty)

Due to malfunction of the receiver unit of the Heat Watch System, we were unable to record mounting data for the first estrus (which occurred before day 165) from two control and two infected heifers. For this analysis, we defined the onset of puberty by an elevation in serum progesterone concentration for 7 to 10 days (normal luteal function) which followed the pubertal ovulation. Age and weight at puberty were not different ($p>0.1$) between the infected and control groups (Table 6).

Intervals Preceding Puberty

To determine if body weight and GGT, AST, estradiol, and progesterone concentrations differed between groups prior to the onset of puberty, specific intervals were analyzed using the SAS Mixed Procedure. Due to collection times, the interval prior to puberty for body weight, GGT and AST concentrations consisted of eight samples (16 wk) while the interval for estradiol and progesterone included 16 samples (8 wk). There were no differences ($p>0.05$) in body weight, serum enzyme concentrations or serum steroid hormone concentrations between groups in the 8- or 16- wk interval prior to puberty (Tables 7 and 8). Both estradiol and progesterone had linear ($p<0.001$) effects over time but were not modified by group ($p>0.1$; Table 9; Figures 9 and 10).

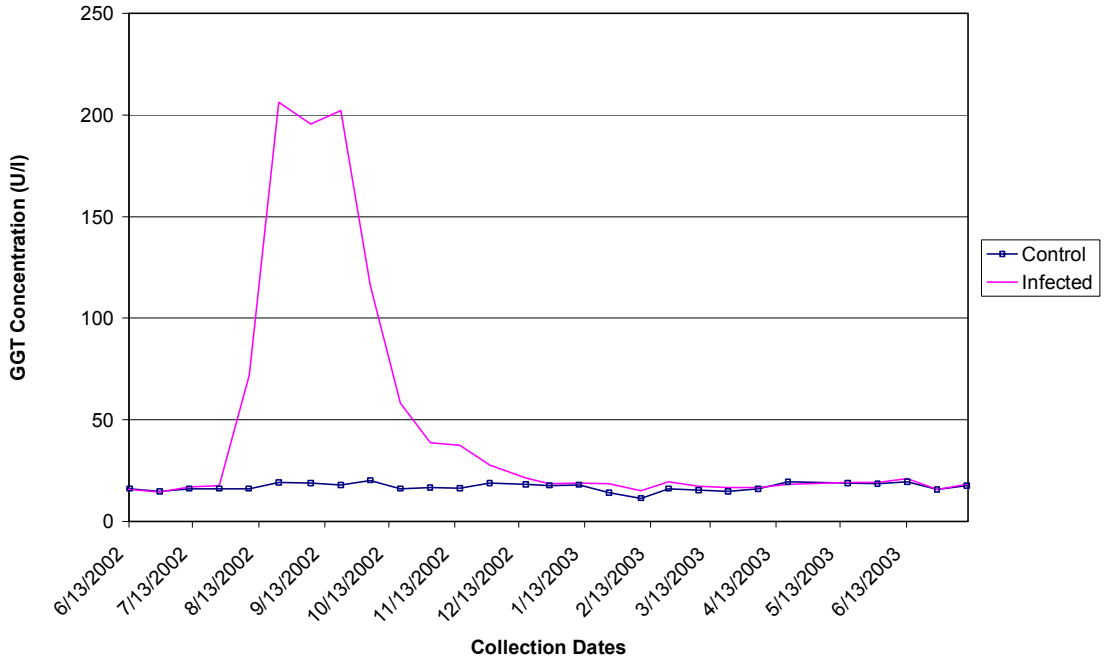

Figure 4. Mean serum concentration of GGT by treatment group from day 0 to day 393.

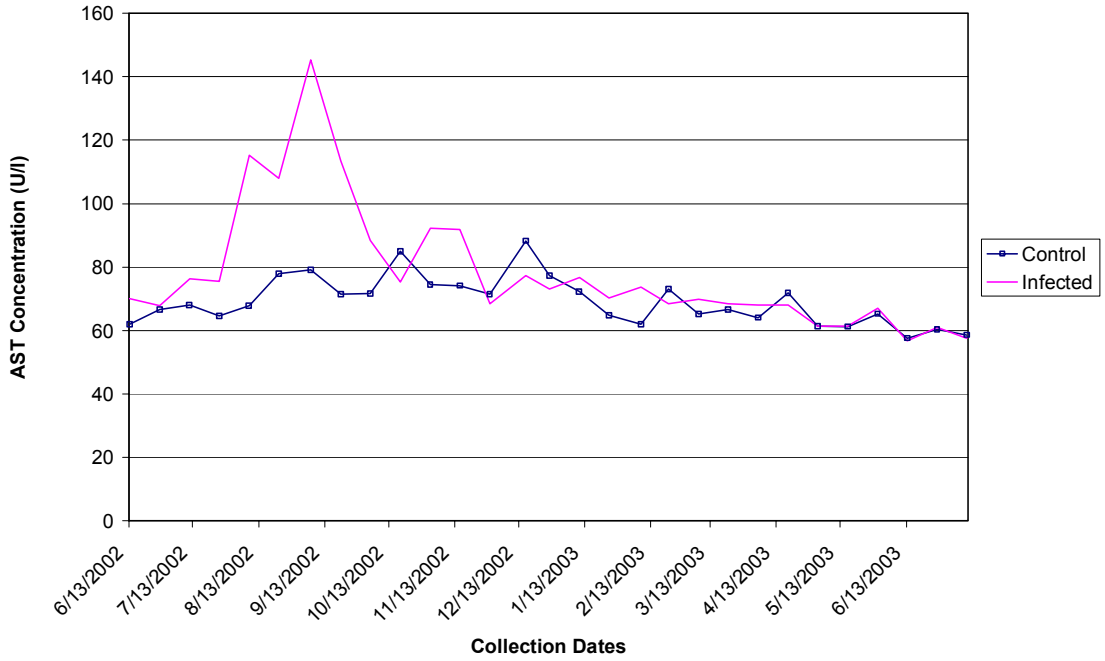

Figure 5. Mean serum concentration of AST by treatment group from day 0 to day 393.

Table 5. Effects (probability level) of treatment group and time on albumin, globulin, and bilirubin concentrations from day 0 through day 339.

	Group	Time	Time*Group	Time*Time	Time*Time* Group
Albumin	NS	NS	NS	<0.0001	NS
Globulin	0.095	<0.0001	0.071	NS	NS
Bilirubin	NS	NS	NS	NS	NS

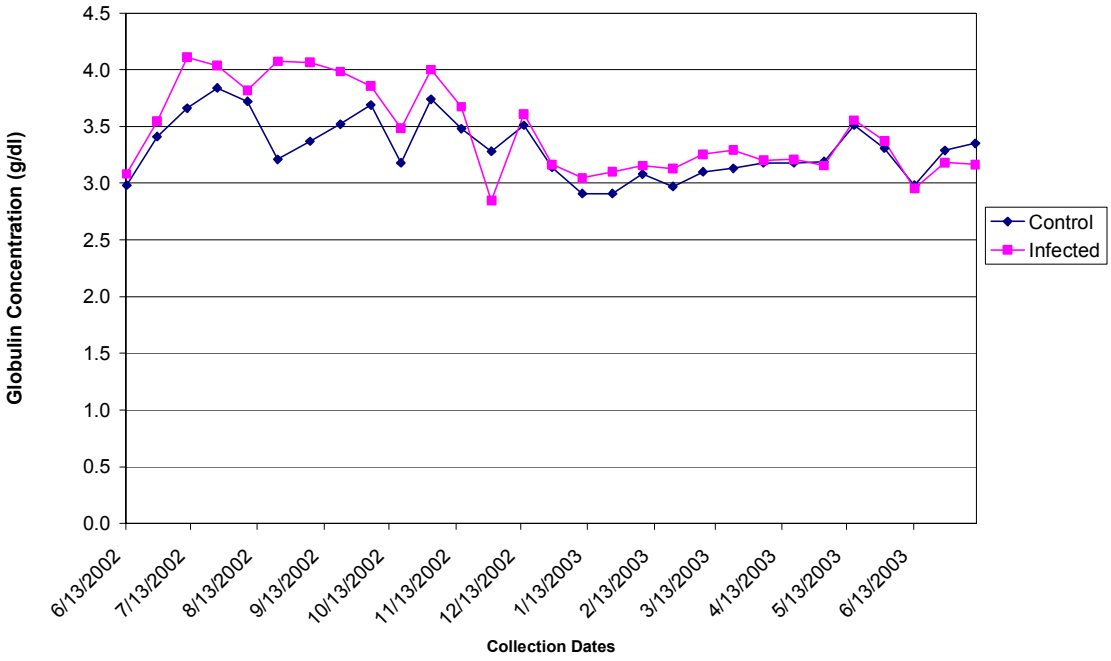

Figure 6. Mean serum concentration of globulin by treatment group from day 0 to day 393.

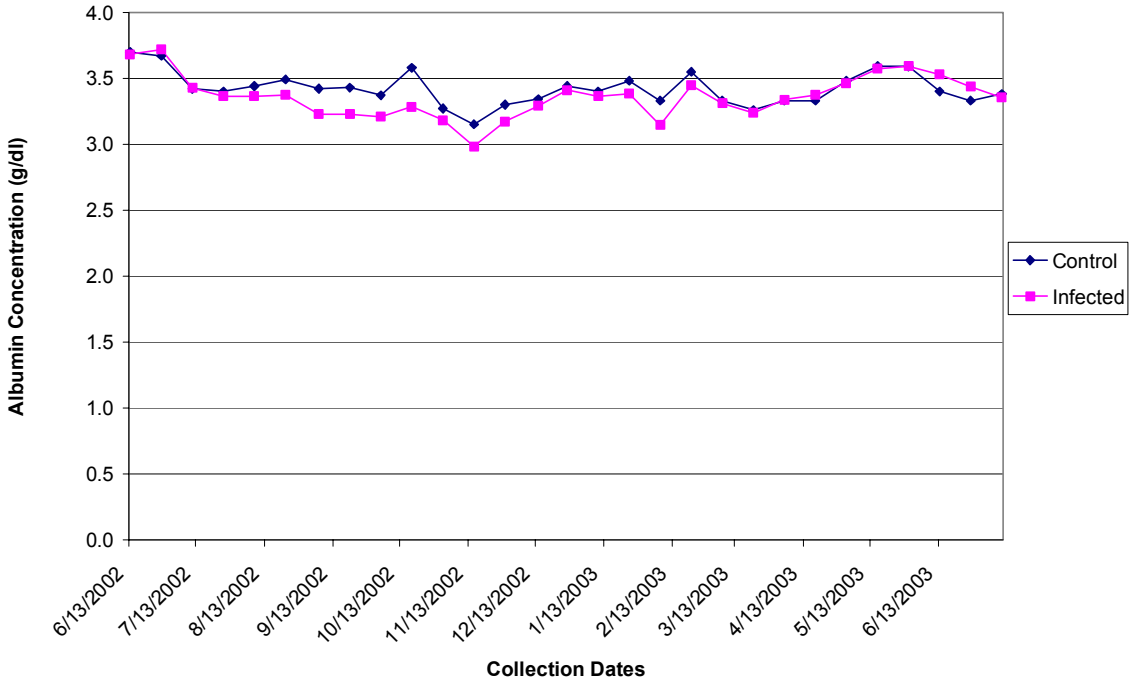

Figure 7. Mean serum concentration of albumin by treatment group from day 0 to day 393.

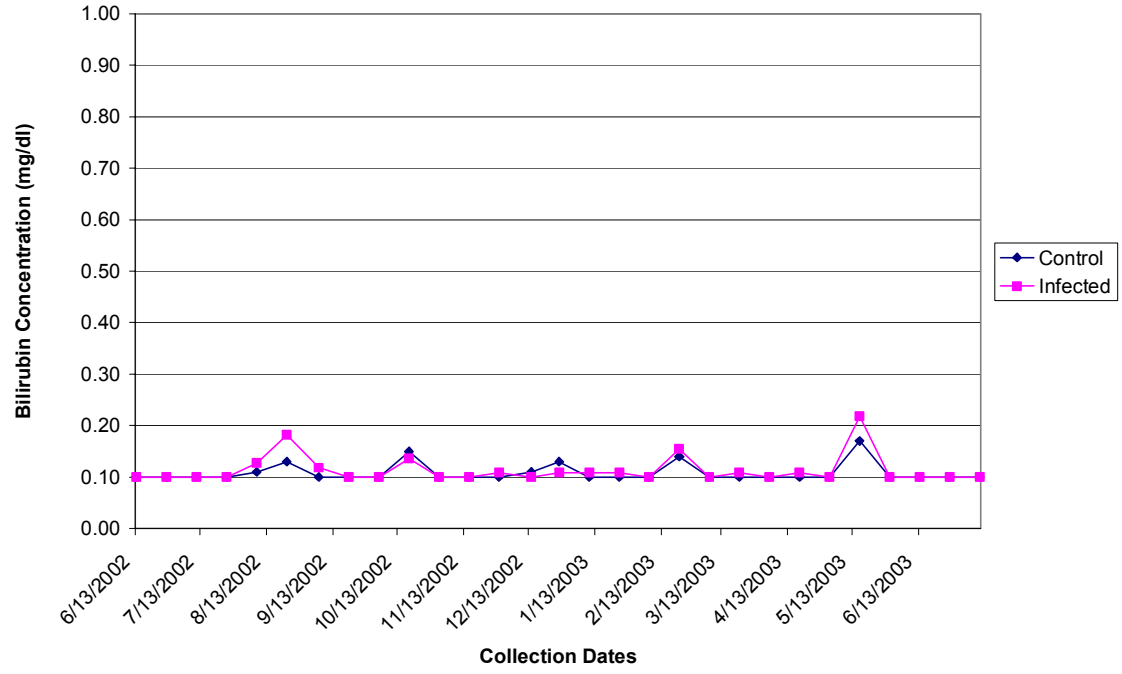

Figure 8. Mean serum concentration of bilirubin by treatment group from day 0 to day 393.

Table 6. Mean (± SE) age and weight at first ovulation (puberty) by treatment group.

	Treatment Group		
	Control	Experimental	P-value
Age At First Ovulation (days)	329 ± 19.48	339 ± 18.57	NS
Weight At First Ovulation (kg)	290.0 ± 12.25	300.0 ± 11.57	NS

Table 7. Mean (± SE) estradiol and progesterone concentration in serum during the 8-wk period preceding puberty by treatment group.

	Control	Infected
Estradiol (pg/ml)	1.73 ± 0.26	1.58 ± 0.15
Progesterone (ng/ml)	0.46 ± 0.19	0.30 ± 0.13

Estradiol had a quadratic effect ($p=0.032$) over time that was not significantly ($p=0.064$) modified by treatment group (Table 10). Weight had a linear effect over time ($p<0.0001$) but was not modified by group ($p>0.1$; Table 10; Figure 11). GGT was different between treatment groups ($p<0.05$) at the start of the 16-wk period prior to puberty but had no further change with time ($p>0.1$; Table 10; Figure 12). AST was different ($p<0.5$) at the start of the 16-wk period prior to puberty and had a quadratic time effect ($p<0.05$). Time effects were not significantly modified by group ($p>0.1$; Table 10; Figure 13).

Table 8. Mean (± SE) body weight and mean (± SE) concentration of GGT and AST during the 16-wk period preceding puberty by treatment group.

	Control	Infected
Body Weight (kg)	240 ± 8	249 ± 8
GGT (U/l)	18 ± 0.3	58 ± 4.5
AST (U/l)	73 ± 1.3	87 ± 3.6

Table 9. Effects (probability level) of treatment group and time on serum ovarian steroid hormone concentrations during the 8-wk period preceding puberty.

	Group	Time	Time*Group	Time*Time	Time*Time* Group
Estradiol	0.083	<0.0001	NS	0.032	NS
Progesterone	NS	0.0009	NS	0.064	NS

Figure 9. Mean serum concentration of estradiol during the 8-wk period preceding puberty by treatment group.

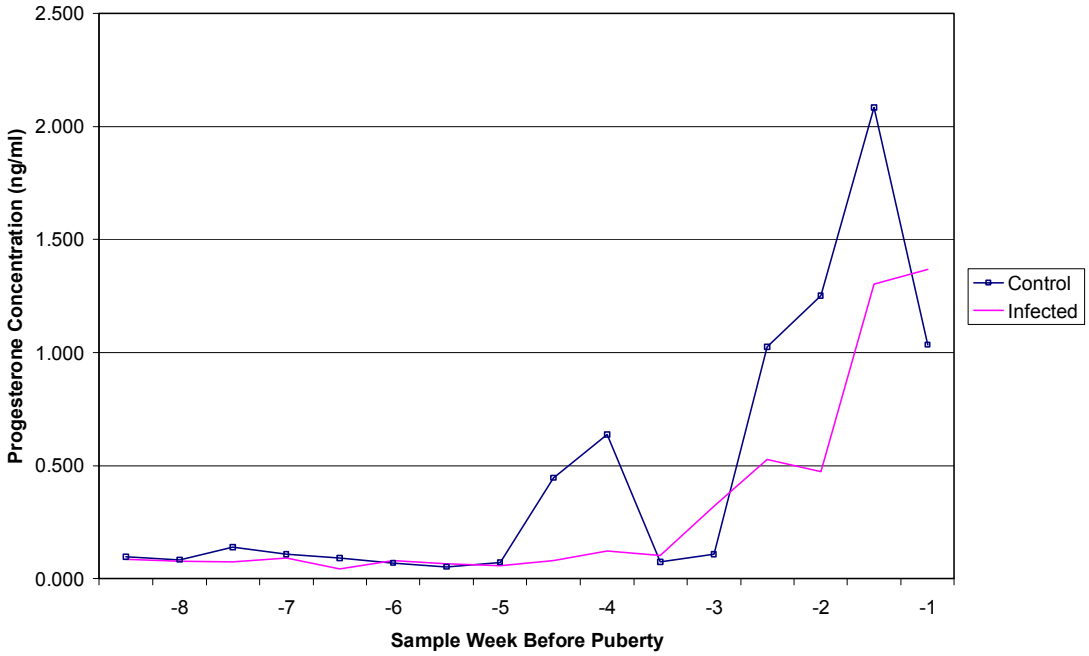

Figure 10. Mean serum concentration of progesterone during the 8-wk period preceding puberty by treatment group.

Table 10. Effects (probability level) of treatment group and time on body weight and serum GGT and AST concentrations during the 16-wk period preceding puberty.

	Group	Time	Time*Group	Time*Time	Time*Time* Group
Weight	NS	<0.0001	NS	0.081	NS
GGT	0.047	NS	NS	NS	NS
AST	0.045	NS	NS	0.03	NS

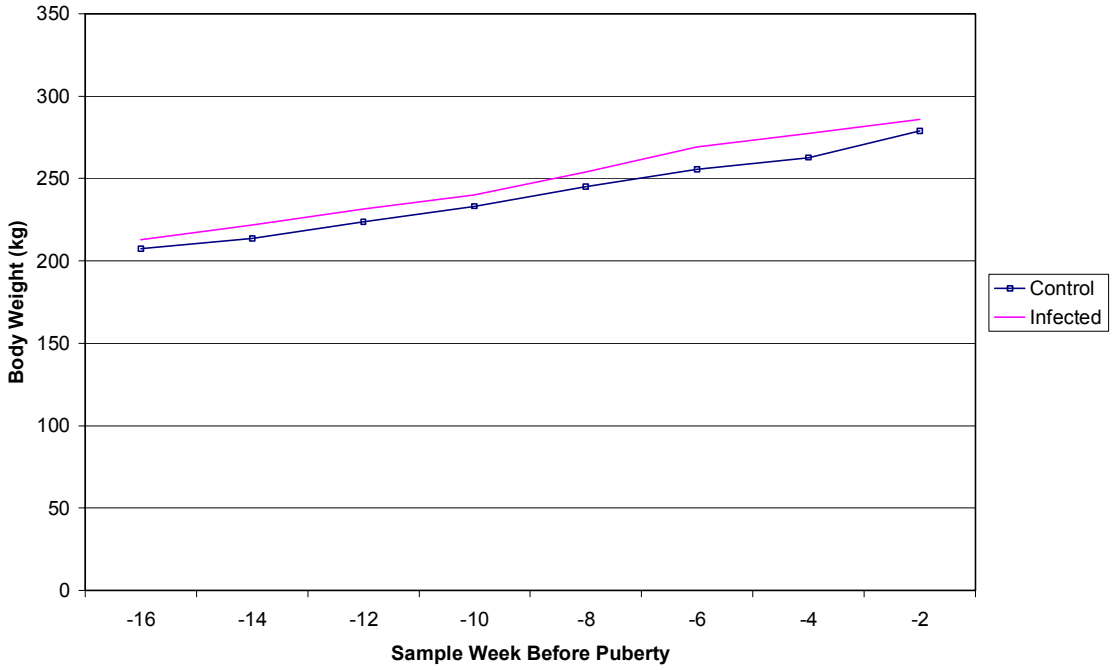

Figure 11. Mean body weight during the 16-wk period preceding puberty by treatment group.

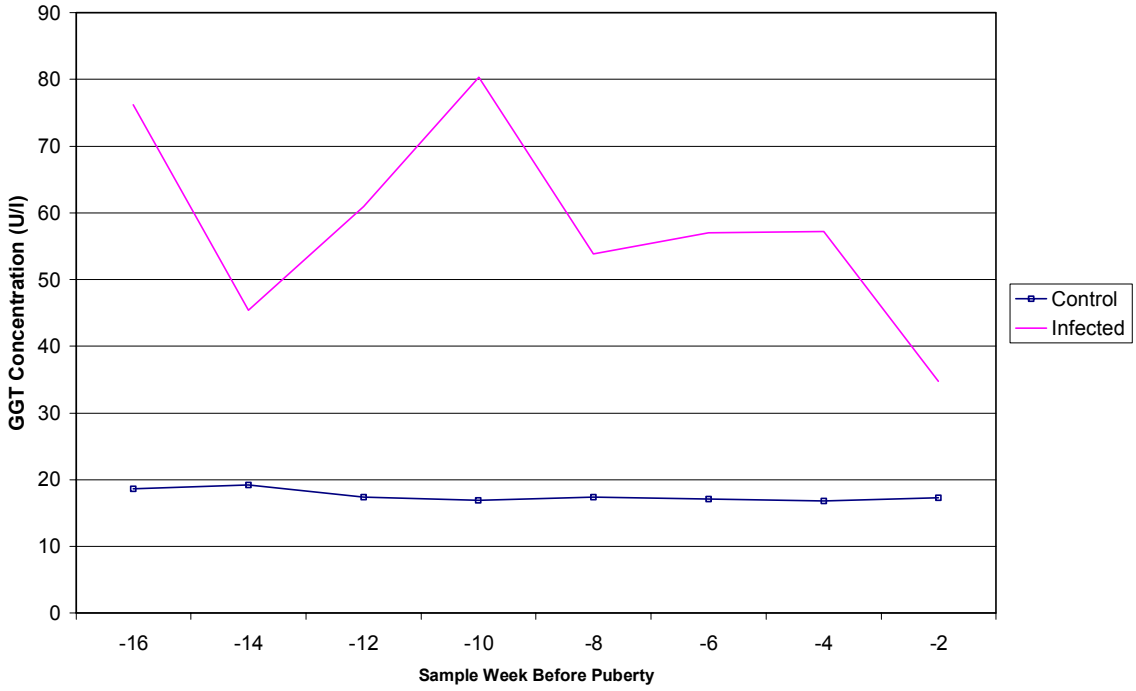

Figure 12. Mean serum concentration of GGT during the 16-wk period preceding puberty by treatment group.

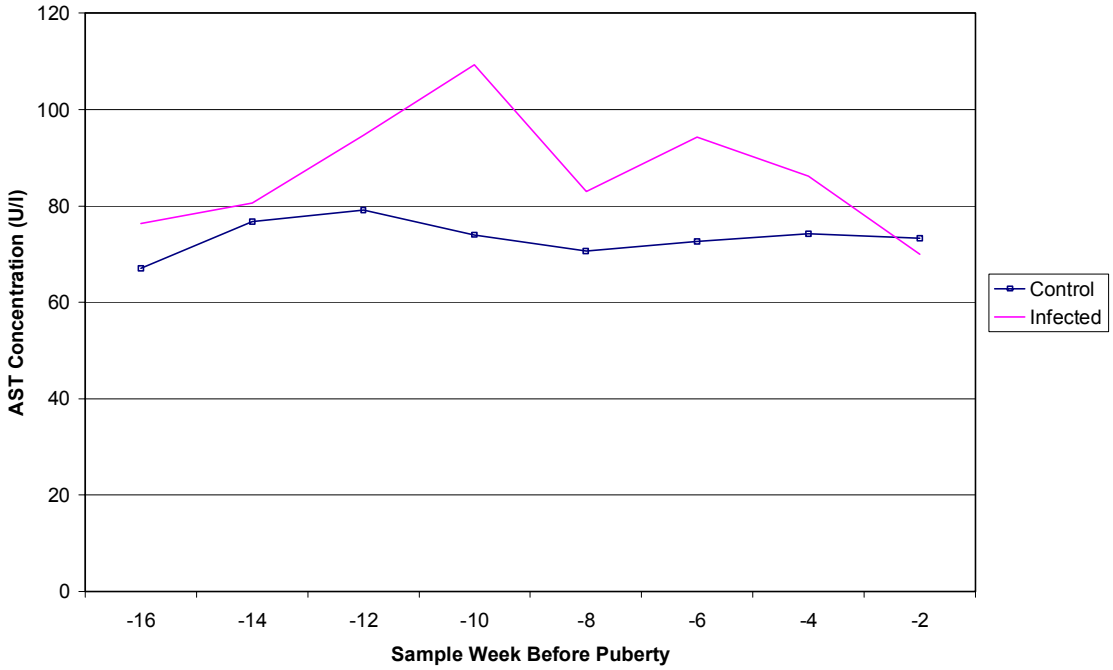

Figure 13. Mean serum concentration of AST during the 16-wk period preceding puberty by treatment group.

Table 11. Correlation coefficients and probability level for age at puberty with maximum values or area under the curve for GGT, AST, or fecal egg counts in the infected group.

		Maximum Value			Area Under the Curve		
Age at Puberty		GGT	AST	Fecal Egg Count	GGT	AST	Fecal Egg Count
	R^2	0.027	0.24	0.067	0.012	0.13	0.091
	p-value	0.63	0.13	NS	0.75	0.28	NS

Hepatic Enzymes and Fecal Egg Counts

There were no significant correlations for age at puberty of infected heifers with maximum value for GGT, AST, or fecal egg counts ($p>0.1$; Table 11). There were no

significant correlations for age at puberty of infected heifers with area under the curve for GGT, AST, or fecal egg counts ($p>0.1$; Table 11). Mean maximum value and area under the curve for GGT, AST, and fecal egg counts for infected heifers are listed in Table 12.

Table 12. Mean (± SE) for the maximum value and area under the curve for GGT, AST, and fecal egg counts in the infected group.

	Mean Maximum Value	Mean Area Under the Curve (Units)
GGT (U/l)	292 ± 20.6	18358 ± 20.8
AST (U/l)	168 ± 18.2	31742 ± 19.6
Fecal Egg Count (eggs/gm)	20 ± 20.2	975 ± 19.9

First Service Conception Rates

A Chi-Square analysis was utilized to determine the effects of *Fasciola hepatica* infection on the conception rates of heifers. There was no significant difference in conception rate between the control and infected heifers (Table 13).

Table 13. Number of heifers that did or did not conceive to a single AI service by treatment group.

	Control	Infected
Pregnant	5	5
Non-pregnant	5	6
Chi-Square (α= 0.05, df= 1)	0.00263	

Discussion

Precocious Puberty and Pre- and Perinatal Mortality

Hypothalamic sensitivity to estrogens in the prepubertal female inhibits the release of gonadotropins by the anterior pituitary; however, during the first few months after birth, the hypothalamus is non-responsive, allowing for follicular growth. Prior to the onset of heighten sensitivity, estrogen concentrations can induce ovulation and result in estrous cyclicity [104]. If exposed to a bull during this period, the female can become pregnant. Wehrman et al. [105] defined precocious puberty as the occurrence of luteal function in prepubertal females prior to 300 days of age. In 120 beef heifers, Wehrman et al. [105] observed precocious puberty in 16.6% of the heifers over two years and bull exposure did not significantly affect the rate of occurrence. Precocious puberty occurred at an average of 194 ± 12.4 days of age, and those heifers had normal luteal function for 65 ± 10.5 d. The inhibitory response of the hypothalamus resumed at this time, and the heifer became anestrous (260 ± 15.3 days of age on average) until the typical age for puberty [105]. In the current study, puberty occurred by 120 d of age in one heifer that conceived by natural service.

Embryonic mortality refers to the death of a conceptus during the embryonic period, the time from conception to the end of differentiation, approximately 45 days [106]. The embryonic mortality rate in Holstein-Friesian heifers observed over a 30-year period was greater for heifers during their first reproductive cycle compared to cows in their second or third (15.0% versus 8.6% returning for service on day 45) [107].

Diskin and Sreenan [108] further classified the incidence of embryonic mortality and observed decreased embryo survival rates 12 (56%), 16 (66%), and 42 (53%) days post-insemination ($p<0.001$). In the current study, we observed a 90% (9/10) embryo survival rate by day 45 (based on serum progesterone profiles) which is higher than the findings of Diskin and Sreenan [108]. Embryo mortality rates in the current study (10%) by day 45 are similar to the findings observed by Erb and Holtz [107].

Listeria infection in gestating ruminants results in placentitis and abortion, and infected animals show symptoms of depression, increased temperature, retained placenta and fetid uterine discharge [109]. These signs were observed in one heifer that contracted listeriosis and aborted at approximately 6 months of gestation. Sergeant et al. [110] stated, "outbreaks of listeriosis are generally associated with periods of cold, wet weather, nutritional stress and sometimes with the feeding of silage," none of which were associated with this project.

Comparison of Studies

This study was designed to quantify the effects of *Fasciola hepatica* infection on the reproductive performance of prepubertal beef heifers and was designed to extend the results of previous work by López-Díaz et al. [8]. Our objective was to determine whether *Fasciola hepatica* delayed puberty and if a delay in puberty was associated with increased estradiol and decreased progesterone concentrations. Puberty was defined more precisely in the current study with the use of an advanced estrus detection system (HeatWatch®) and confirmation of the onset of the pubertal estrus with progesterone concentrations. López-Díaz et al. [8] used heat mount detectors (Kamar®) for

determining the onset of estrus and patches were checked twice a week, a positive response indicated the heifer had reached puberty within the previous 3 to 4 days. The heat detectors were replaced after the first estrus to ensure cyclicity. The use of the HeatWatch® system allowed for continual observance of mounting activity and reduced the risk of false positives in estrus detection by analyzing the number and duration of mounts received.

Comparison of the two assays used to analyze the estradiol concentrations indicated similar specificity and intra-assay coefficients of variation (1.1 and 0.16% cross reactivity for estrone and estradiol-17α, respectively, and less than 4.7% CV) [8]. The RIA used in the current study was one pg/ml more sensitive (1 pg/ml versus 2 pg/ml, respectively) than the solid phase ^{125}I used by López-Díaz et al. [8]; however, the difference does not account for the high estradiol concentrations reported in the previous study. López-Díaz et al. [8] reported average estradiol concentrations of 10 ± 3 pg/ml and 4.4 ± 0.8 pg/ml ($p<0.05$) for infected and control heifers, respectively, from the day of infection to first estrus, though these concentrations are within normal ranges, as reported by previous studies [25, 27]. Mean estradiol concentration during elevations prior to puberty was 34 ± 15 pg/ml for the control heifers over the 65 day interval prior to puberty, higher than the mean concentration reported by Gonzalez-Padilla et al. [27] during the same time interval (18.3 ± 1.9 pg/ml from -64 to -39 days and less than 10 pg/ml from -38 to 0 days before first estrus). Mean estradiol concentration during the 8 week period prior to puberty in the current study for control and infected heifers was less

than 2 pg/ml (1.73 ± 0.26 pg/ml and 1.58 ± 0.15 pg/ml, respectively), lower than reported by Gonzalez-Padilla et al. [27].

Maximum and mean estradiol concentrations during elevations prior to puberty for the infected heifers were 152 ± 45 pg/ml and 109.8 ± 30 pg/ml, respectively [8], well over normal concentrations. After extensive research, we are unable to locate further studies on sex steroid hormone concentrations after infection with *Fasciola hepatica*; however, sex steroid hormone concentrations have been reported during investigations of liver damage due to cirrhosis, though the results are variable. Shaaban et al. [111] reported decreased estradiol concentration in amenorrhic women with advanced liver cirrhosis, though average concentrations did not differ from the control group (61 ± 17 pg/ml and 84 ± 9 pg/ml, respectively). Maximum concentration (137 pg/ml) was similar to that reported by López-Díaz et al. [8]; however, the extent of the range (17 to 137 pg/ml) reduced the mean to less than the normal limits [111]. Protein analysis for bilirubin and globulins, and albumin revealed increases and decreases, respectively, compared to the normal limits in some patients [111]. Similar trends were reported in amenorrhic women with alcoholic and non-alcoholic cirrhosis, non-cirrhotic alcoholics and patients with other liver disease; 65% of the patients had an estradiol concentration below the normal range, yet the averages were similar (0.07 ± 0.07 to 0.10 ± 0.08 nmol/l; normal range- 0.08 to 0.11 nmol/l; p-value not given) [112]. The high elevations reported by López-Díaz et al. [8] are plausible, though the mean concentration reported could be skewed by the low sample size.

The lower concentration of progesterone in the infected heifers before first estrus was hypothesized to be a consequence of the high estradiol concentrations, initiating a negative feedback on the hypothalamus resulting in a failure to ovulate [8]. In the current study, mean progesterone concentration did not differ between control and infected groups before puberty.

Puberty Analysis

Though the study was well designed in comparison to the previous study on pubertal development, the results from this experiment lead us to the conclusion that we may not have induced a high enough level of infection to have altered the normal metabolic functions of the liver. Our findings from the hepatic enzyme (AST and GGT) and fecal analysis are consistent with previous experiments; however, we were unable to induce clinical signs associated with fascioliasis. In the current study, hepatic enzyme analysis for GGT and AST indicated concentrations were significantly higher in infected heifers by day 56 (data not shown) and analysis from day 0 to day 112 resulted in a linear response ($p=0.0023$ and $p=0.051$, respectively) indicating infection of 600 metacercariae induced liver damage. Even though trauma to the liver is evident, normal liver function may not have been altered to an extent in this study which could have delayed the onset of puberty by impairing the metabolic clearance rate of estradiol (described below).

The conclusion that we may not have markedly altered normal liver function is supported by serum protein analysis for bilirubin, globulin, and albumin. Increased concentrations of bilirubin and globulin, and decreased concentrations of albumin are

common signs of chronic fascioliasis [58, 62, 64, 65]; albeit, protein concentrations were not significantly different between groups in this study, indicating we may have only achieved a sub-clinical level of infection. Though not affected by treatment group, linear and curvilinear decreases in globulin and albumin, respectively, were found. In contrast, a previous report indicated these proteins increase and remained unchanged as age increases, respectively; however nutrition was not taken into consideration in the study [113]. A recent study on metabolic profiles in prepubertal dairy heifers reported that an ADG of 0.7 kg (comparable to the ADG in this study) resulted in decreased globulin levels compared to heifers fed a moderate diet (ADG= 0.9 kg) [114], suggesting that dietary level in the current study may have negatively impacted serum globulin concentrations. Similar findings were observed in cattle with seasonal malnutrition; decreases in concentrations of total protein and its components (albumin and gamma-globulins) were noted [115].

A decrease in body weight normally associated with parasitic infection [65] was not evident in the infected heifers. Previous studies by Lacau-Mengido et al. [116], Larson et al. [117], Loyacano et al. [89], and Zajac et al. [1] all reported increased weight gains in beef heifers naturally infected with parasites after ivermectin treatment.

Increases in estradiol concentrations are associated with age [118] and growth of the dominant follicle during the mid- and late-follicular phase [119]. Concentrations are higher for the last non-ovulatory and first ovulatory follicular waves than for the non-ovulatory waves 9 to 12 wks prior to puberty which can be associated with the increase in follicular diameter [120]. Melvin et al. [121] observed an increase in concentration 3

mo to 1 mo prior to puberty ($p<0.03$). Similar to the findings observed by Evans et al. [120] and Melvin et al. [121], a linear and curvilinear increase in estradiol concentration was observed during the 8-wk interval prior to puberty ($p<0.0001$ and $p=0.032$, respectively); however, concentrations were not influenced by infection of *Fasciola hepatica* ($p>0.1$).

During the peripubertal period, transient increases in progesterone are observed due to subfertile ovulations and are typically lower, shorter in duration than a normal luteal phase, and do not follow an observed estrus [28, 31, 32, 33]. The linear increase in progesterone during the 8-wk interval prior to puberty, in our study, can be explained by transient increases in progesterone from ovulations of follicles not resulting in sustained luteal function. Although the heifers experienced ovulation and increased progesterone concentrations, onset of puberty date was not recorded until progesterone concentrations were elevated for three consecutive samples.

Changes to the experimental design that could have altered the outcome are described below:

Management

In the current study, the control and infected heifers were maintained in dry lots (on concrete surfaces) and fed a mixed ration, supplemented with coastal Bermuda grass hay instead of being raised on pastures and fed supplements to support growth, which is routine in traditional livestock management. As stated previously, the average daily gain was 0.68 kilograms which is considered an average to high plane of nutrition. *Fasciola hepatica* infection may exert a negative impact on puberty when nutrition is marginal,

suggested by the analysis completed by Larson et al. [117]. In this previous study, treatment with ivermectin hastened the onset of puberty and improved pregnancy rates in heifers that were maintained on a marginal plane of nutrition (pastured on tallgrass prairie) and naturally infected with parasites [117]. Heifers on pasture may be more susceptible to the potential effects of fascioliasis under the conditions of a restricted nutritional plane compared to heifers developed under optimal nutrition. However, effects of *Fasciola hepatica* infection on puberty would need to be analyzed taking into consideration heifers fed a low nutritional plane may alter the onset of puberty due to decreased body conditioning; hence, the low weight gain of the heifers may be more influential in delaying the onset of puberty than the infection.

Dosage of Metacercariae

The infected group in our study was administered 600 metacercariae intraruminally in a single bolus to induce a subclinical fascioliasis infection based on the increase of hepatic enzyme concentrations in circulation. In comparison to previous experiments, the effect of acute alteration in liver function at lower doses may not simulate the effects of chronic exposure to *Fasciola hepatica* received by cows on pasture. Previous experiments have been conducted at various doses of administered metacercariae in sheep and cattle to determine the impact that infection has on several enzyme concentrations. Sykes et al. [78] experimentally infected 5-month-old sheep with 3, 8, or 14 metacercariae daily, 5 times a week for 22 weeks, or with a single dose of 200 metacercariae. It was reported that GGT and GD (glutamate dehydrogenase, also known as GLDH) concentrations were associated with dosage; however AST concentration was

not related to the dose of metacercariae [78]. Heifer and bull calves (4 to 5 months of age) were experimentally infected with 1000, 100 or 10 metacercariae in three doses (total infection of 3000, 300, 30 metacercariae) and then sacrificed to determine the number of flukes in the liver [71]. It was determined that the number of flukes present in the liver was a more accurate indicator of infection than hepatic enzyme concentrations, reporting the mean number of flukes found at necropsy was 211, 78, and 10 at dose levels of 3000, 300, and 30 metacercariae, respectively [71]. Ross [84] showed massive infection levels of metacercariae resulted in considerable destruction of liver tissue in cattle. At infection levels of greater than 2500 metacercariae (2500, 5000, or 15,000), there was a greater extent and severity of cirrhosis of the liver outside of the bile duct. Livers from cattle infected with 1300 metacercariae had a greater extent of fibrosis in the bile duct than the higher dose levels [84].

Throughout the study, we based the level and severity of *Fasciola hepatica* infection on the concentration of hepatic enzymes released in the circulation, plasma protein concentrations and fecal egg counts. Previous studies have noted that beyond these findings, clinical signs of fascioliasis were not observed to indicate the true severity of the infection (indicated in this study) [72, 75, 79]. In two of these studies, postmortem analysis of the livers indicated enlarged fibrotic and calcified bile ducts and cell destruction [72, 75]. Overall the condition of the liver after severe fascioliasis infection includes plaque formation, fibrosis, and cellular destruction. In our study we estimated the severity of the infection achieved based on serum analyses, but we do not know the true extent of liver trauma since we did not euthanize the heifers or obtain a liver biopsy.

There is evidence to support the hypothesis that natural infections in cattle (i.e. raised on pasture) show more severe signs of fascioliasis than experimental infections due to the development of a chronic infection. Several studies state experimental infection achieved a sub-clinical level of fascioliasis (indicated in this study) [72, 77]; whereas natural infections reached acute, subacute and chronic levels [64]. To reach a chronic level of infection normally observed in cattle [64], metacercariae of *Fasciola hepatica* could be administered in low doses for extended periods of time to induce a sustained infection; for example, 5 metacercariae every other day for an 80-day period [76].

Hepatic Enzyme Analysis

Hepatic enzymes are released as hepatic cells are damaged, hence its diagnostic use as an indicator for liver trauma [72]; however not all hepatic enzymes can be utilized for accurate diagnosis of fascioliasis. Wyckoff and Bradley [71] reported the lack of sensitivity for liver function associated with concentrations of serum albumin, total protein and bilirubin, as well as the lack of liver specificity associated with AST concentrations [75, 77]. Increased concentrations of AST could be related to cellular tissue damage, such as skeletal tissue and cardiac muscle, possibly induced by handling [71, 75, 77]. GGT and GD are more sensitive indicators of *Fasciola hepatica* infection [75, 77, 78] and it has been reported that markedly increased concentrations are related to liver damage by the flukes [72, 77, 78]. The use of another liver specific enzyme, such as GD, might show more definitive levels of infection in conjunction with GGT.

Future Studies: Hormones Affected by *Fasciola hepatica* Infection during Puberty

Estradiol 17β concentrations in prepubertal and pubertal heifers were investigated in this experiment; however, alterations in the metabolic clearance rate (MCR) of estradiol in the liver was not explored. Fleming and Fetterer [7] demonstrated a decreased ability of the liver to metabolize exogenous testosterone in infected postpubertal rams, even though endogenous concentrations were not different between infected and control rams during the prepubertal period, suggesting that compensatory mechanisms were maintaining concentrations in normal ranges. López-Díaz et al. [8] suggested the increase in estradiol concentrations observed in prepubertal heifers infected with *Fasciola hepatica* was a result of decreased MCR. In our experiment we did not observe this increase in endogenous estradiol concentration after infection. Fleming and Fetterer [7] suggested the possibility that exogenous estradiol administered to prepubertal heifers could have the same effect, a decrease in the metabolic clearance rate by the liver, masked by compensatory mechanisms to maintain normal concentrations.

Insulin-like growth factor-1 (IGF-1) is produced by the liver [67] and was found to elicit a dose-related increase of GnRH (LHRH) from the hypothalamus in rats [122], suggesting its role in the timing of puberty. Further study showed increases in circulating concentration of IGF-1 during the late proestrus phase ($p<0.001$) and administrations of exogenous IGF-1 to immature rats induced LH release and advanced the onset of puberty as determined by vaginal opening (34 ± 0.36 days of age vs. 38.9 ± 0.40 days of age; $p<0.001$) [123]. Similar observations were made in sheep; IGF-1

concentrations were greater at 252 days of age in pubertal females (326 ± 16 ng/ml vs. 249 ± 8 ng/ml; $p<0.01$) [124].

Estrogen treatment for growth-hormone deficiency prevents the rise of IGF-1 suggesting that abnormal estrogen metabolism, observed in some patients with chronic liver disease [125], could decrease the concentration of circulating IGF-1 [126]. Findings from Stuver et al. [127] supports this hypothesis after noting reduced concentrations of IGF-1 in humans suffering from hepatocellular carcinoma and metastatic liver cancer, which has been attributed to parenchymal damage. As mentioned previously, elevated concentrations of AST are correlated with damage of the liver parenchyma during the migratory phase of the adult form of *Fasciola hepatica* [71, 75]. These findings suggest that IGF-I concentrations might be altered after *Fasciola hepatica* infection if the parenchyma is extensively damaged.

Growth hormone (GH) regulates growth and protein metabolism by stimulating the release of IGF-I from the liver [5, 67]. Moderate increases in GH have been detected at the onset of puberty [5, 67]; it was noted that GH concentrations were lower at 40 days than at 17 days prior to puberty in heifers [5]. Liver trauma from *Fasciola hepatica* infection could damage the GH and GH-receptor interactions, resulting in increased circulatory concentrations of GH and decreased concentrations of IGF-1. Patients with chronic hepatitis (CH) had GH concentrations 15 times greater than control patients (161 ± 271.9 μU/L vs. 10.9 ± 4.4 μU/L; $p<0.01$). As expected, CH patients had reduced concentrations of IGF-1 compared to control patients (14.9 ± 9.7 nmol/L vs. 37.6 ± 14.9 nmol/L; $p<0.001$) [128], suggesting that this hypothesis is plausible.

Leptin is synthesized and secreted by adipose tissue and evidence indicates that it may play a role in the hypothalamo-pituitary-gonadal axis [129]. It has been shown that leptin concentration increases during puberty in humans [129]; and in prepubertal heifers, serum concentration of leptin increased beginning 16 weeks prior to the onset of puberty (3.8 ± 0.4 ng/ml vs. 6.4 ± 0.4 ng/ml; $p<0.0001$) [130]. If adipose tissue deposition is delayed by *Fasciola hepatica* infections due to decreased body conditioning, a common sign of fascioliasis, then age at puberty could be increased due to depressed serum leptin concentration.

Conclusion

Infection loads of 600 metacercariae of *Fasciola hepatica* did not alter steroid hormone concentrations in prepubertal heifers from day 0 to day 393 post-infection or during the eight week interval prior to the onset of puberty. Heifers in the infected group were on average 10 days older at puberty; however, age and weight at puberty and conception rate after artificial insemination were not significantly different from control heifers. Administration of a single bolus of metacercariae of *Fasciola hepatica* to 4-mo-old heifers induced liver trauma but did not induce clinical signs of fascioliasis. The effects of fascioliasis on altered liver function were associated with an elevation in circulating GGT and AST during a 3-mo-period (between two and five months post-infection); however, typical clinical signs associated with chronic fascioliasis (increased concentrations of plasma bilirubin and globulin and decreased albumin concentration) were not observed. Although low numbers of fluke eggs were detected in the feces of

some heifers for several months following infection, there were no differences in either performance or the physiological traits between the infected and control groups. These results suggest that we may not have achieved a level of infection with 600 metacercariae to substantially alter liver function and steroid hormone concentrations. Further studies are warranted to determine whether chronic exposure to *Fasciola hepatica* (which simulates pasture conditions) or experimental infection in larger or more frequent boluses during the prepubertal period will significantly alter steroid hormone concentrations and delay the onset of puberty in heifers.

CHAPTER IV

MOUNTING ACTIVITY IN PUBERTAL AND GESTATING HEIFERS

Introduction

Radiotelemetry provides continuous monitoring of estrus behavior for determining the appropriate time for artificial insemination. Studies have reported use of radiotelemetry to detect estrus activity (number of mounts, total duration of mounts, and estrus duration) for adult cattle [9, 42]. However, few studies were found that used radiotelemetry to monitor estrus activity in beef heifers and none were found that used radiotelemetry to quantify estrus activity in pubertal heifers. Identifying pubertal estrus is economically beneficial to producers, in that producing a first calf by 24 months of age requires breeding of the heifer on one of its first few estrus events. Thus, there is a need to document the characteristics of estrus behavior in peripubertal heifers utilizing radiotelemetry and compare differences in behavior during the pubertal and second estrus, and to estimate the predictability of these activities on pregnancy outcome.

Estrus activity in gestating females has been reported in sheep [45] and cattle [10, 46]; though with low occurrence rates, misdiagnosis of true pregnancy can lead to serious complications and economic loss. Over a 30-year period, Erb and Morrison [10] observed the mounting activity of gestating dairy heifers and cows and determined the rate of occurrence by visual inspection. Erb and Morrison [10] reported an incidence rate of 5.6% in cows with 55% of the estrus activities occurring 35 days post insemination. Therefore, assessment of estrus activity by radiotelemetry in beef heifers

after artificial insemination can provide useful information concerning the frequency of estrus activity and interestrus intervals that occur during gestation.

Hypotheses: Estrus activity (number of mounts, total duration of mounts and duration of estrus) will be greater during the second estrus in pubertal heifers as determined by HeatWatch® and in heifers conceiving after artificial insemination. The interestrus interval in gestating heifers will approximate the typical estrous cycle of 21 days.

Objectives: Using radiotelemetry, determine estrus activity in pubertal and gestating beef heifers including:

1. Comparison of the first and second estrus activity,
2. Prediction of pregnancy rate determined by the activity from the second estrus,
3. Interestrus interval in pregnant heifers, and
4. Prediction of the intensity of the estrus activity of pregnant heifers determined by the second estrus activity.

Materials and Methods

Estrus Detection

Twenty-two prepubertal (age= 9 mo) Angus-sired heifers were housed as a single group in a 10 x 30 m pen on concrete surfaces. Heifers were fitted with HeatWatch® transponders and continuously monitored for estrous activity. One heifer was excluded from the project and analysis after discovering she had conceived prior to her arrival at the Texas A&M University research facility. Blood samples were collected via jugular

venipuncture twice weekly and analyzed by radioimmunoassay for progesterone concentration [102]. Puberty was defined by a minimum of three mounts in a 4-hr interval according to the HeatWatch® system and confirmed by the occurrence of the first normal luteal phase (serum progesterone concentrations greater than one ng/mL for at least ten days).

Estrus activity recorded included total number of mounts, total duration of mounts, and duration of estrus. Heifers were artificially inseminated with frozen/thawed semen approximately 12 hours after the onset of the second estrus after puberty. Heifers that conceived (confirmed by ultrasound 30 days post-insemination) were monitored during gestation (range from a minimum of 35 days to a maximum of 220 days gestation for individual heifers) for total number of mounts, total mount duration and interestrus interval. Patches that contained the transponder were replaced as needed.

Statistical Analysis

Data obtained from the HeatWatch® system were analyzed using the Mixed Procedures to compare total number of mounts, total duration of mounts, and duration of estrus between the pubertal and second estrus activities. The procedure analyzed the fixed effects while controlling for the repeated measurements by heifer as random. This is sometimes referred to as the "random effects model" [103]. Logistic regression was used to determine if the activities of the second estrus were associated with pregnancy (yes/no). Data from the nine heifers that conceived after artificial insemination were analyzed by the random effects model (described above) to determine whether activities of the second estrus were predictive of the total estrus activities during gestation. Chi-

square analysis was used to determine differences among the percentage of interestrus intervals (<10 days, 10-16 days, 17-24 days, or >24 days) observed during gestation.

Results

Animals

Data from one heifer (No. 3) were excluded from the analysis after it was determined that it had conceived prior to its arrival at the Texas A&M University research facility. Two heifers conceived after artificial insemination but suffered either embryonic loss (heifer No. 17) or abortion due to Listeria infection (heifer No. 2). Data, during the period in which these two heifers maintained a pregnancy, were included in the analysis. Further detail on these three heifers and their conditions is provided in the results section of chapter 3.

Estrus Activity in Pubertal Heifers

Mean duration of estrus was longer ($p=0.0031$) for the second (17.5 ± 3.4 hr) than for the pubertal (12.4 ± 3.4 hr) estrus. Total mount duration and number of mounts did not differ ($P>0.1$) between the pubertal and second estrus (Tables 14 and 15).

Mean number of mounts ($p<0.05$) and mean duration ($p<0.1$) at second estrus were greater in the heifers that conceived after AI. Total mount duration at second estrus was not associated with pregnancy outcome ($p>0.1$; Tables 16 and 17).

Table 14. Mean (± SE) number of mounts, total mount duration and estrus duration for the pubertal and second estrus.

Pubertal Estrus (n=17)			2nd Estrus (n=21)		
Number of Mounts	Total Mount Duration (sec)	Estrus Duration (hr)	Number of Mounts	Total Mount Duration (sec)	Estrus Duration (hr)
51.5 ± 4.1	93.1 ± 14.2	12.42 ± 1.20	54.6 ± 7.6	92.7 ± 12.8	17.54 ± 1.08

Table 15. Significance levels for number of mounts, total mount duration and estrus duration between the pubertal and second estrus.

	P-value
Number of Mounts	NS
Total Mount Duration (sec)	NS
Estrus Duration (hr)	0.0031

Table 16. Mean (± SE) number of mounts, total mount duration and estrus duration at second estrus between heifers that did or did not conceive after artificial insemination.

Pregnant Heifers			Non-Pregnant Heifers		
Number of Mounts	Total Mount Duration (sec)	Estrus Duration (hr)	Number of Mounts	Total Mount Duration (sec)	Estrus Duration (hr)
70.1 ± 10.1	111.7 ± 17.7	20.13 ± 1.22	40.6 ± 7.4	75.3 ± 13.6	15.18 ± 1.79

Table 17. Significance levels for number of mounts, total mount duration and estrus duration at second estrus between heifers that did or did not conceive after artificial insemination.

	P-value
Number of Mounts	0.049
Total Mount Duration (sec)	NS
Estrus Duration (hr)	0.058

Estrus Activity during Gestation

Heifers that conceived after artificial insemination were monitored through varying lengths of gestation (a minimum of 35 days to a maximum of 220 days). A total of 73 estrus events were detected after conception in the nine pregnant heifers. The SAS program was utilized to plot the interestrus intervals, the period of time from the onset of one estrus event to the onset of the next event. The interestrus intervals during gestation were distributed as follows: <10 d= 48%, 10-16 d= 27%, 17-24 d= 10%, and >24 d= 15% (Table 18, Figure 14). Even though almost one-half of the estrus events occurred fewer than 10 days apart, the frequency of estrus events did not differ ($p>0.1$) among interestrus intervals. Characteristics of the estrus when insemination occurred (second estrus) were compared with the total estrus activities during gestation in the nine pregnant heifers. Number of mounts ($p=0.035$) and total duration of mounts ($p=0.022$) at second estrus were predictive of number of mounts during gestation (Table 19). However, number of mounts and total mount duration at second estrus were not associated ($p>0.1$) with either total mount duration of interestrus interval during gestation (Table 19). Duration of second estrus was not associated ($p>0.1$) with any of the characteristics of estrus during gestation (Table 19).

Table 18. Percentage of estrus events occurring during gestation by interestrus interval.

Interval	Number of Events	Percentage of Total (n=73)
<10 days	35	48%
10-16 days	20	27%
17-24 days	7	10%
>24 days	11	15%

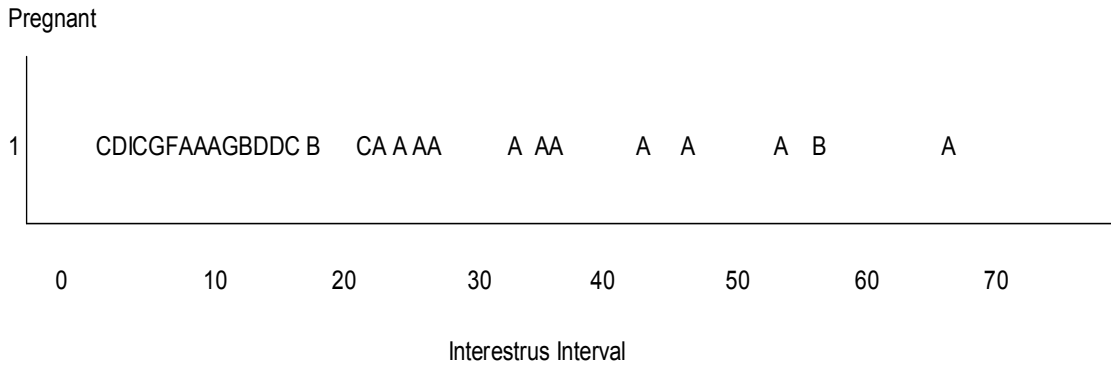

Figure 14. Occurrence of estrus events after conception in nine heifers during gestation (minimum 35 d, maximum 220 d). Interestrus interval was defined as the number of days from the onset of one estrus event to the onset of the next estrus event. A- 1 obs., B- 2 obs., C- 3 obs. … I-9 obs.

Table 19. Significance levels for predicting number of mounts, total duration of mounts, and interestrus interval during gestation based on the number of mounts, total mount duration and estrus duration during the second estrus.

	Estrus Characteristics During Gestation		
Estrus Characteristics During 2nd Estrus	Number of Mounts	Total Mount Duration	Interestrus Interval
Number of Mounts	0.035	NS	NS
Total Mount Duration (sec)	0.022	NS	NS
Estrus Duration (hr)	NS	NS	NS

Discussion

Estrus Behavior as Determined by Radiotelemetry and Factors Affecting Activity

Using radiotelemetry, producers are able to continuously observe mounting activity in dairy and beef herds and utilize this information to enhance their breeding program by allowing for more accurate estrus detection and timing for artificial insemination. Mounting activity during estrus can be influenced by breed, synchronization methods, number of females in estrus, temperature, and location. Landaeta-Hernández et al. [131] reported that synchronization of cows with PGF2α affected estrus duration and number of mounts received when compared with spontaneous estrus (16 ± 1h vs. 9 ± 1 h, $p<0.0001$; 34 ± 4 mounts vs. 8 ± 4 mounts, $p<0.0001$, respectively); however, duration and total mounts were similar when the temperature-humidity index of 75 (THI75) was included in the statistical model.

Activity during spontaneous estrus events for beef heifers in the present study is similar to reports of activity measured by radiotelemetry during a synchronized estrus (with minor differences depending on breed of the heifer and on the synchronization protocol). After synchronization with a norgestomet ear implant, Rae et al. [48] reported mean estrus duration in beef heifers of 6.65 ± 1.16 h, 8.52 ± 1.20 and 11.90 ± 1.22 h for Brahman, Angus and Brahman-Angus crosses, respectively ($p=0.03$). Richardson et al. [132] reported similar estrus duration in beef heifers after synchronization with GnRH + PGF2α (11 ± 0.7 h), P_4 + GnRH + PGF2α (12 ± 0.6 h) and P_4 + PGF2 α (12 ± 0.7 h). The mean estrus duration of 14 ± 0.8 h reported by Stevenson et al. [9] in beef heifers after synchronization with MGA + PGF2α is similar to the estrus duration in the current

study of 12.42 ± 3.42 h and 17.54 ± 3.42 h for the pubertal and second estrus, respectively.

Number of mounts during estrus is more variable among the reports in the literature. Rae et al. [48] reported as low as 19 ± 3.6 mounts in Angus heifers and as high as 37 ± 5.5 mounts in Angus-Brahman crosses ($p=0.02$) and remarked that the number of mounts was correlated with the duration of estrus. Richardson et al. [132] observed between 31 ± 3 and 39 ± 4 total mounts depending on synchronization protocol used (P_4 + GnRH + PGF2α and GnRH + PGF2α). Similar to results of the present study, Stevenson et al. [9] reported that heifers received over 50 mounts during estrus (50.1 ± 6.4 mounts). Total mount duration observed in the current study was similar to that reported by Richardson et al. [132] between 81 ± 9 and 100 ± 11 seconds.

Hurnik and King [133], using a time lapse videorecorder to observe the activity of 12 postpartum dairy cows, reported that mounting activity increased when the number of females in estrus increased (number of cows in estrus, one: 11.2 mounts, two: 36.6 mounts, three: 52.6 mounts, four or more: 49.8 mounts). The small herd size of 21 animals in the current study reduced the incidence of multiple heifers being in estrus at the same time and may have suppressed the number of mounts received by estrual females. A more restrictive setting (four to five postpartum cows in open-housed pens with their calves) noted the difficulties of accurate estrus detection with a time-lapse video system, when only 20% of estrus events were detected by more than ten mounts and 27% were detected by only one mount. Lack of mounting activity by non-estrual

females occurred in 24% of all estrus events indicating the "socially restrictive housing conditions of a small group size" [133].

A temperature-humidity index equal to 75 (THI75) on the day of estrus, tended to affect the duration of spontaneous estrus ($p=0.09$) compared to cows synchronized with PGF2α (9 ± 1 h vs. 16 ± 1 h, respectively) as determined by HeatWatch® [131]. The THI-75 index for five days ($r= -0.46$; $p<0.003$) or one day ($r= -0.51$; $p<0.0008$) before the onset of estrus and on the day of estrus ($r= -0.57$; $p<0.0001$) was significantly correlated with estrus duration, indicating that estrus duration decreased as the THI75 increased on the day of estrus or up to five days prior to estrus. Similar findings were observed for total number of mounts received during estrus; THI75 on the day of estrus influenced total number of mounts ($p<0.005$). The THI75 for five days ($r=-0.49$; $p<0.0008$) or one day ($r= -0.58$; $p<0.0001$) before estrus and on the day of estrus ($r= -0.68$; $p<0.0001$) were correlated with the number of mounts received during estrus [131]. Gwazdauskas et al. [37] previously reported a significant curvilinear relationship between mounting activity and maximum daily temperature; as temperature increased to 25°C, mounting activity increased (approximately 19°C, 2 m/h; 0°C, 7 m/h; 25°C, 9.5 m/h). Although approximately one-half (11/21) of the heifers reached puberty during the winter months (December 1 through February 28), temperature and humidity is a factor in Texas throughout the year and was not taken into consideration in this study.

As mentioned previously, type of housing influenced the number of mounts received during estrus and the interestrus interval as observed by visual inspection (barn-housed: 8.7 ± 0.4 mounts/h, 36.7 ± 2.8 d; drylot: 6.1 ± 0.2 mounts/h, 29.5 ± 3.8 d;

pasture: 5.5 ± 0.2 mounts/h, 29.5 ± 3.8; $p<0.05$) [37]. Similar findings were observed by De Silva et al. [134] with barn-housed dairy cows receiving more mounts per hour than free stalled or pastured cows (11.2 ± 0.9 mph, 6.5 ± 6.8 mph, 5.4 ± 2.9 mph, respectively; $p<0.05$). In preferences testing, the mounting activity by Holstein cows increased nearly three times when estrual teaser females were tied on soil as compared to concrete surfaces, indicating that mounting surface affected activity (soil: 2.4 ± 0.5 average mounts per 30-min test period, concrete: 1.0 ± 0.3 average mounts per 30-min test period; $p<0.05$) [135]. Females in the current study were housed on concrete surfaces, which may have affected estrus behavior by decreasing the desire to mount heifers on these surfaces.

Behavior at Pubertal and Second Estrus

The current study appears to be among the first to describe the estrus behavior at the pubertal and second estrus in beef heifers using radiotelemetry. Only one other study was found characterizing estrus behavior in dairy heifers using radiotelemetry and reported total number of mounts and total mount duration differences before first AI and second AI after synchronization. Richardson et al. [132] used virgin, pubertal Holstein heifers; however, average age before treatment was 13 ± 0.1 mos, suggesting the heifers were cycling before the start of the study. In the current study, estrus duration was longer during the second spontaneous estrus compared to pubertal estrus (17.54 ± 3.42 h and 12.42 ± 3.42 h, respectively; $p=0.0031$); however, total mount duration and total number of mounts did not differ ($p>0.1$). Producers with inadequate estrus detection may not detect heifers in their pubertal estrus due to the shorter duration, resulting in

delayed breeding and postponement in first calving, a concern for producers intending to have first-calf heifers calve by 24 months of age.

The current findings do not resemble mounting activity of females after periods of anestrous (eg. after postpartum anestrous). Hurnik and King [133] noted estrus duration in beef cows during their first, second and third postpartum estrus events were shorter (3.9 h, 4.2 h, and 4.8 h, respectively) when compared to previous reports on beef heifers and cows housed with their calves, suggesting that comparisons between pubertal and first postpartum estrus events are not appropriate.

Heifers that conceived after artificial insemination had significantly higher numbers of mounts during their second estrus ($p<0.05$) and tended to have longer durations of estrus ($p=0.058$) than heifers that did not conceive. These results suggest that intensity of estrus behavior, based on the number of mounts and duration of estrus, can be incorporated into the breeding schedule for pubertal heifers and determining the likelihood of pregnancy.

Estrus Behavior of Gestating Heifers

Few authors have remarked on the occurrence of estrus behavior after conception in livestock species, though the potential implications for the producer can be costly. Erb and Morrison [10] reported 5.6% of 6,751 pregnant dairy cows over a 30-year study displayed estrus behavior after breeding with 5.6% of these events occurring 21 days post breeding. More recently, Thomas and Dobson [46] observed estrus after conception in 5.7% of pregnant dairy and beef cows and remarked they were shorter in duration, but otherwise were indistinguishable from non-pregnant cycling cows.

In a smaller sample size, we observed that the nine pregnant heifers demonstrated estrus activity during gestation with individual heifers showing a minimum of four to a maximum of 16 estrus events (heifers were observed from at least 35 days up to 206 days post-insemination, respectively). Due to the time schedule of the previous study on fascioliasis in prepubertal beef heifers, we were unable to continue monitoring the heifers to parturition, which resulted in the variation in the duration for which they were observed. The use of the Mixed Procedures from SAS for this analysis took into account the differences in observation lengths and was not a factor in the outcome. Interestrus intervals ranging from 17 to 24 days, the typical duration of non-pregnant estrous cycles, occurred in only 10% of the estrus events during gestation, with the majority occurring less than 10 days apart. Total mount duration and the number of mounts received during the second estrus influenced the number of mounts received during estrus events after conception ($p<0.035$).

Estrus activity after conception may be initiated by follicular wave development. In cycling heifers with estrous cycles characterized by two follicular waves, the first anovulatory wave occurred on day 2 ± 1 (ovulation = day 0) and the second wave resulting in the development of an ovulatory dominant follicle, on day 10 ± 4 [136]. Heifers characterized by three follicular waves followed a similar pattern; the first anovulatory wave occurred on day 0, followed by a second anovulatory wave on day 10, and a third ovulatory wave on day 16 post ovulation [136]. Follicular waves in pregnant cows are initiated approximately every ten days as determined by ultrasonography (9.3 ± 0.2 days from wave one to two, 8.5 ± 0.5 days from wave two to three) and did not differ

between the time interval for non-pregnant heifers (10 ± 0.6 days from wave one to two, 10.2 ± 0.6 days from wave two to three) [137].

These findings, along with previous work, further emphasize the importance of proper pregnancy diagnosis in cattle. Cows and heifers showing behavioral signs of estrus 17 to 24 days post conception can be misdiagnosed as non-pregnant and rebred resulting in increased semen expenses and possible paternity questions, or it can lead to culling of the "infertile" females for slaughter. Breeding schedules which incorporate prostaglandin, an increasing trend in today's industry due to the utility of estrus synchronization and economic value, can induce abortion if administered to females misdiagnosed as non-pregnant.

Conclusion

Duration during the second estrus was longer than the pubertal estrus, although number of mounts and total mount duration did not differ between estrus events. Estrus duration tended to be associated with pregnancy outcome, and the number of mounts received during the second estrus was greater in heifers that conceived than in heifers that did not conceive after artificial insemination. During gestation, 48% of the estrus events in heifers occurred less than 10 days apart while an interestrus interval of 17-24 d occurred in only 10% of estrus events indicating that the majority of estrus events after conception do not resemble normal estrous cycles in non-pregnant heifers. The number of mounts and total mount duration during the second estrus were predictive of the number of mounts received after conception.

CHAPTER V

SUMMARY AND CONCLUSIONS

Experiment 1

Experimental infection of prepubertal heifers with 600 metacercariae of *Fasciola hepatica* develops a sub-clinical level of fascioliasis but does not alter normal liver function, resulting in non-appreciable differences in circulating concentrations of sex steroid hormones. The results presented in this thesis, support the following conclusions:

1. Serum concentrations of hepatic enzymes were significantly elevated in infected heifers indicating liver trauma was induced,
2. Normal liver function was not markedly affected as determined by the lack of clinical signs of fascioliasis (i.e. decreased albumin and increased globulin and bilirubin concentrations),
3. Serum concentrations of estradiol and progesterone were not significantly different between groups, suggesting the metabolic clearance function of the liver was not altered, and
4. Age at puberty was not delayed in infected heifers.

Future studies on the effect of *Fasciola hepatica* on pubertal development are warranted due to the contrasting results reported in this study and those published by López-Díaz et al. [8] to confirm that 600 metacercariae does not alter serum steroid hormones resulting in a delay in the onset of puberty. Areas of further research were alluded to in the discussion section of chapter 2 and include:

1. Increased dosage or frequency of *Fasciola hepatica* administered to prepubertal heifers to induce sufficient trauma to the liver, determined by clinical signs of fascioliasis, or to simulate a "natural" infection,
2. Sacrifice of the infected heifers to determine the extent of liver damage induced during the migratory phase of the disease to support the findings of the hepatic enzyme analysis, and
3. Administration of exogenous estradiol to observe possible impacts of infection on the metabolic clearance rate to determine if regulatory mechanisms are maintaining normal concentrations of endogenous estradiol.

The results of this study do not confirm the findings by López-Díaz et al. [8] and raise further questions on the true impact of fascioliasis on pubertal development. Under the conditions of the current experiment, we reject our own hypothesis that *Fasciola hepatica* infection of 4-mo-old beef heifers will alter serum steroid hormone concentrations and delay puberty.

Experiment 2

Analysis of the HeatWatch® data obtained during estrus detection demonstrated similarities in the pubertal and second estrus activity, in terms of total number of mounts and total mount duration. Duration of the second estrus, however, was longer and tended to be associated with pregnancy outcome. Total number of mounts received during the second estrus was greater in heifers conceiving to artificial insemination. After conception, all nine heifers displayed estrus activity; 48% of the events occurred

less than 10 days apart while only 10% of the events occurred between 17 and 24 days. The number of mounts received after conception was related to the number and total mount duration received at the time of artificial insemination (second estrus).

This experiment was conducted concurrently with experiment 1; due to time constraints, analysis of the gestating heifers was minimized, warranting future research in this area. In this study, only nine heifers were monitored during gestation, all of which displayed estrus activity after conception. Research using larger sample sizes will allow for an accurate account of the occurrence rate of activity after conception and will confirm the time distribution of estrus events presented in this report. The use of FSH analysis may demonstrate an association of follicular waves with the occurrence of estrus events post-conception.

Several studies have remarked on the characteristics of estrus behavior using radiotelemetry; however, this is the first known research completed on characterizing spontaneous pubertal and second estrus events in beef heifers and their association with pregnancy outcome. Though few studies have demonstrated interestrus intervals in gestating heifers, this appears to be the first report using radiotelemetry and the first report on the association of activity after conception to the activity at artificial insemination.

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