INFLUENCE OF DIETARY FAT SOURCE ON REPRODUCTIVE PARAMETERS IN MARES

by

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ABSTRACT

Sixteen cycling mares were used to study the effects of different fat supplements (corn, soybean, and fish oil) on reproductive parameters. Mares were supplemented with oil at a rate of 342 mg/kg BW/d for two consecutive estrous cycles, with blood samples collected at each ovulation and 9 days post-ovulation. Cycle length was recorded, and serum triglycerides, non-esterified fatty acids (NEFA), progesterone, and estradiol concentrations were analyzed in serum. Fat supplementation had no effect on cycle length, serum triglycerides, progesterone, and estradiol. Decreasing NEFA concentrations throughout the study were similar to previous published literature. Cyclic changes observed in progesterone and estradiol concentrations were in accordance with expected hormone fluctuations in the estrous cycle of the mare. The results of this study indicate that the oils commonly added to feeds have no effect on the reproductive markers measured.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER ONE: INTRODUCTION

In an effort to increase caloric density in equine diets without increasing feed intake, supplemental fat is often used. The horse has been shown to be able to efficiently digest up to 20% fat in the total diet (Potter et al., 1992). Fat sources commonly utilized are plant and vegetable oils such as soybean oil and corn oil since they are readily available and highly palatable. The reproductive success of an animal depends on its nutritional state. A lack of dietary energy coupled with poor body condition can negatively affect reproduction (Henneke et al., 1984). Dietary fat supplementation has been proven to affect follicular growth, pregnancy rates, and milk composition in many livestock species (Mattos et al., 2002). Ovarian and uterine function is also affected by adding fat to the diet (Beam and Buttler, 1997; Mattos et al., 2000; Santos et al., 2008). Oils derived from plants have been known to possess estrogenic compounds termed phytoestrogens that can have an effect on reproductive processes. The purpose of this study was to determine the reproductive effects of adding fat sources to the diets of normally cycling mares. The fats utilized in this study were fish oil and soybean oil as treatment oils and corn oil serving as a control. The main interest in soybean oil is the presence of high levels of the phytoestrogens, genistein and daidzein, and their potential effects on reproduction. The primary interest in fish oil is the omega-3 polyunsaturated fatty acid (PUFA) involvement in eicosanoid production which is essential in reproductive processes. The following literature review will discuss the normal estrous cycle in the non-pregnant mare, phytoestrogens, the influence phytoestrogens have on

reproduction, phytoestrogenic effects in the horse, fatty acids, influence of dietary fats on reproductive parameters, and observed effects of dietary fat supplementation in the horse.

The Estrous Cycle in the Non-Pregnant Mare

The estrous cycle is under endocrinological control dictated by the hypothalamic-pituitary-gonadal axis. It is important to note that the mare is a seasonal breeder and therefore, the cycle is primarily driven by photoperiod, or day length. Other factors such as nutrition, body condition, age, and temperature play a minor role (Davies Morel, 2008). Photoperiod and other factors act to control the breeding season to allow the most opportune time of delivery of the foal, in the spring. Photoperiod is perceived by the pineal gland in the base of the brain. Melatonin is produced by the pineal gland in low light conditions. When day length is short, melatonin production is high. Melatonin inhibits the release of gonadotropin releasing hormone (GnRH) from the hypothalamus and therefore inhibits the release of the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH). When day length is long, maximal sunlight, the inhibition of melatonin is removed and GnRH production follows along with the release of FSH and LH (Davies Morel, 2008). This allows the development of follicles that secrete estrogen which induce estrus behavior. LH will assist in the final maturation of the follicle and cause ovulation.

When day length is long, endocrinological control of the estrous cycle is dictated by the hypothalamic-pituitary-gonadal axis. GnRH, is released from the hypothalamus.

It travels to the pituitary gland via the hypothalamic-pituitary portal system and stimulates the release of FSH and LH. Roughly 80% of GnRH released is passed through this portal system, while the remaining 20% is passed to the central nervous system to influence behavior (Davies Morel, 2008).

Once released, FSH and LH travel to their target organs, the ovaries. FSH stimulates follicular development and is one of the two hormones that drive gonadal development. LH is the other hormone responsible for the development of the gonads in the mare. FSH is released into the general circulation and its concentration levels suggest a biphasic release pattern. Elevated FSH levels occur at ovulation, day zero, and at days 9 to 12 of an average 21 day estrous cycle in the mare. The FSH peak at ovulation completes final follicular development before ovulation and starts the development of new follicles for the next ovulation (Davies Morel, 2008). Follicles release estrogen which contributes to receptive behavior, when a mare is in estrus. LH causes the final maturation of the dominant follicle and causes ovulation. LH levels rise several days before ovulation and peak just after ovulation. A few days after ovulation LH levels drop to low diestrus levels.

Formation of a corpus luteum (CL) is the result of an ovulated follicle. The luteal tissue within the CL releases progesterone (P4). Progesterone levels rise within 24 to 48 hours post ovulation and reach maximum levels 5 to 6 days after ovulation. The lifespan of the CL within the non-pregnant mare is roughly 15 to 17 days. Progesterone inhibits the release of the gonadotropins, FSH and LH. In the mare, progesterone only inhibits

the release of LH and does not inhibit FSH. A second rise in FSH occurs from days 9 to 12 while progesterone levels are high.

If conception did not occur then prostaglandin F2α (PGF2α) is released from the endometrium of the uterus. This hormone lyses the CL and progesterone production ceases, allowing the release of LH. Prostaglandin F2α levels are highest around days 14 to 16 after ovulation, when lysis of the CL is in progress (Stout and Allen, 2002). These levels drop around day 18 when luteolysis of the CL is complete (Stout and Allen, 2002). Oxytocin drives the release of PGF2α. Oxytocin is synthesized in the endometrium of the mare (Budik et al., 2012). At 14 to 17 days after ovulation, oxytocin receptors on the endometrium rise. This allows oxytocin to bind to these receptors, allowing the endometrium to produce PGF2α which then lyses the CL and returns the mare to estrus (Davies Morel, 2008).

CHAPTER TWO: GENERAL REVIEW OF LITERATURE

Phytoestrogens

Phytoestrogens are estrogenic compounds derived from plants. Phytoestrogens have the ability to induce estrogen-like and antiestrogenic effects in animals and humans (Liu et al., 2006). Found in plants or their seeds, phytoestrogens can be divided into three classes: the isoflavones, coumestans, and the lignans (Murkies et al., 1998). Soybeans have high levels of the isoflavones daidzein and genistein, and smaller amounts of the isoflavone glycitein. Small amounts of the isoflavonoid-derived coumestan, coumestrol is present in soybeans as well (Dixon, 2004; Franke et al., 1999; Wang, 1994).

Phytoestrogens and Reproduction

The similarity between phytoestrogens and 17-β estradiol (E2) allows for the phytoestrogens to bind to estrogen receptors to promote or inhibit estrogenic responses (Ferreira-Dias et al., 2013; Kuiper et al., 1998; Woclawek-Potocka et al., 2005). Phytoestrogens have displayed a higher affinity for estrogen receptor β when compared to estrogen receptor α (Ferreira-Dias et al., 2013; Kuiper et al., 1998). Phytoestrogens have been found to affect not only sexual behavior but also the function and morphology of reproductive organs in multiple species of either sex (Nynca et al., 2013; Kurzer and Xu, 1997). Phytoestrogens may influence the estrous cycle via competitive binding with E2

(Ferreira-Dias et al., 2013). The absorption, metabolism, and biotransformation of phytoestrogens can be influenced by the reproductive status of the animal such as, pregnancy or phase of ovarian cycle (Ferreira-Dias et al., 2013; Woclawek-Potocka et al., 2008). Clover and alfalfa pastures rich in phytoestrogens have caused infertility issues in cattle and sheep by decreasing plasma P4 and E2 levels (Ferreira-Dias et al., 2013; Adams, 1995; Obst and Seamark, 1970; Newsome and Kitts, 1977).

Coumestrol, a coumestan present in soybeans, alfalfa, and clover, exhibits strong estrogenic activity and has a high affinity for estrogen receptors than genistein in rats (Dixon, 2004; Tinwell et al., 2000). The rat estrous cycle is suppressed when coumestrol is fed in female rat diets (Dixon, 2004; Whitten et al., 1995).

Soy based products such as soybean oil, are rich in the phytoestrogens genistein and daidzein (Kwack et al., 2009; Eldridge and Kwolek, 1983). Genistein has been shown to possess weak estrogenic properties (Nynca et al., 2013). In porcine granulosa cells, genistein inhibited basal P4 secretion (Nynca et al., 2013). Genistein becomes antiestrogenic when administered with estradiol (Murkies et al., 1998; Messina, 1994). Administering genistein coupled with estradiol decreases the estradiol uptake in the uterus (Murkies et al., 1998; Messina, 1994). Genistein and daidzein inhibit the secretion of P4 by pig granulosa cells and it has been suggested that they may have an influence on follicular function (Nynca et al., 2013; Basini et al., 2010; Nynca et al., 2009; Tiemann et al., 2007). Nynca and et al. (2013) confirmed prior reports of phytoestrogens having an inhibitory effect on P4 secretion and discovered that genistein increased basal estradiol secretion in pigs. Genistein competes with estradiol for binding to the estrogen receptor

at low concentrations (Murkies et al., 1998; Wang et al., 1996). Genistein supplementation to ovariectomized sows resulted in uterine hypertrophy and proliferation of epithelial cells of the endometrium (Ferreira-Dias et al., 2013; Ford et al., 2005). When genistein and daidzein were administered in the same amount to humans, genistein attained higher plasma levels than daidzein (Dixon, 2004; Setchell et al., 2001). This is caused by a greater bioavailability displayed by genistein and daidzein is more widely spread throughout the body (Dixon, 2004; Setchell et al., 2001).

Daidzein has been shown to exhibit estrogen-like and estrogen-independent effects (Liu et al., 2006; Jia et al., 2003; Guo et al., 2004). In chickens, daidzein increases germ cell proliferation (Liu et al., 2006). Estrogen is involved in germ cell development and differentiation (Liu et al., 2006; Andrews et al., 1997). Daidzein is metabolized by intestinal bacteria to produce equol in the equine gut (Dixon, 2004; Setchell et al., 2002; Wang, 2002; Ferreira-Dias et al., 2013). Since equol conversion is dependent upon the microbial population, it is able to be influenced by fiber and carbohydrate levels in the diet (Dixon, 2004; Lampe et al., 1998). Equol is significantly more estrogenic and may be responsible for the physiological effects of phytoestrogen isoflavonoid ingestion (Dixon, 2004; Setchell et al., 2002). Equol binds to both estrogen receptors α and β (Dixon, 2004). Competition for estrogen receptor binding can induce antiestrogenic activity (Dixon, 2004). Genistein and equol are strong estrogen agonists (Dixon, 2004; Zava and Duwe, 1997). In humans, Genistein and equol displace bound estrogen and testosterone and could possibly affect the clearance rates and availability of androgens and estrogens (Dixon, 2004; Pino et al., 2000).

Phytoestrogens in the Horse

A recent study testing the dietary effects of coumestrol in mares discovered that ingestion of coumestrol over a long term period caused a lack of ovulation despite follicular growth and administration of the ovulation inducing drug human chorionic gonadotropin (hCG) (Ferreira-Dias et al., 2013). Alfalfa and clover are the main sources of coumestrol (Dixon, 2004; Nelsen et al., 2002).Coumestrol displays strong estrogenic activity and is considered one of the more potent phytoestrogens (Ferreira-Dias et al., 2013). This same study also found that coumestrol induced uterine edema and uterine fluid accumulation in the lumen with failure to clear (Ferreira-Dias et al., 2013). After the removal of coumestrol from the diet for 2-3 weeks after completion of the study, the mares returned to normal ovarian cyclicity and were able to clear uterine fluid accumulation. After the ingestion of estrogenic plants, the presence of coumestrol and its metabolite methoxycomestroul increased in the blood. This same study noted high levels of E2 in the blood and low levels of P4 in mares receiving the coumestrol rich diet. Persistent anovulatory follicles corresponding to high levels of plasma coumestrol and its metabolite were also recorded in this study. Coumestrol disrupts follicular growth and maturation leading to a failure in ovulation (Ferreira-Dias et al., 2013; Burns and Douglas, 1981). Decreased ovulation rates and conception rates have occurred in sheep after long term ingestion of coumestrol (Ferreira-Dias et al., 2013; Adams, 1995).

Fatty Acids

Fatty acids are an important component of lipids. They consist of a chain of carbon atoms with hydrogens attached and mainly occur as esters. A carboxylic group is attached to the alpha end and a methyl group is attached on the omega end. In plasma, fatty acids exist in non-esterified form as free fatty acids (Abayasekara and Wathes, 1999). If there are no double bonds present then the fatty acid is classified as a saturated fatty acid. If a double bond is present then it is classified as an unsaturated fatty acid. If two or more double bonds are present then the fatty acid becomes a polyunsaturated fatty acid (PUFA).

Polyunsaturated fatty acids can be divided into omega-3 fatty acids, omega-6 fatty acids, and omega-9 fatty acids. Naming of fatty acids is dependent upon the position of the first double bond in relation to the omega end. Therefore, an omega-3 fatty acid has the double bond positioned between the third and fourth carbon from the omega end. An omega-6 fatty acid would have the double bond between the sixth and the seventh carbon of the omega end of the molecule. Since mammals do not have the ability to synthesize these polyunsaturated fatty acids in their bodies, omega-3 and omega-6 fatty acids are considered to be essential fatty acids. The essential polyunsaturated fatty acids α-linolenic acid and linoleic acid are the precursors for the omega-3 and omega-6 series of fatty acids (Hall et al., 2004). Linoleic acid and α-linolenic acid must be supplied in the diet due to the lack of the enzymatic action necessary to incorporate double bonds to carbon atoms prior to the ninth carbon atom in the fatty acid chain (Hall et al., 2004;

Miles and Calder, 1998). Linoleic acid is an omega-6 polyunsaturated fatty acid found in corn oil, safflower oil, and soybean oil. Metabolism of linoleic acid produces arachidonic acid (Hall et al., 2004). The omega-3 polyunsaturated fatty acid, α-linolenic acid is present in linseed oil, canola oil, and soybean oil. α-linolenic acid is a component of chloroplasts and therefore is present in fresh, leafy forage (Schmidt, 2010). When α-linolenic acid is further metabolized it produces eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which are the main omega-3 fatty acids present in fish oil (Hall et al., 2004; Miles and Calder, 1998; Calder, 1998).

Influence of Dietary Fat Source on Reproductive Parameters

Fat sources commonly utilized in equine diets are plant and vegetable oils such as soybean oil and corn oil since they are readily available and highly palatable. More recently, another fat source utilized in the equine industry is fish oil. This marine-derived fat source is rich in omega 3 fatty acids. Omega 3 fatty acids are polyunsaturated fatty acids (PUFAS) that have gained interest in the industry due to their benefits in both livestock and human diets. Polyunsaturated fatty acids have been found to affect reproduction primarily through their role in eicosanoid formation (Schmidt, 2010; Abayasekara and Wathes, 1999).Omega-3 and omega-6 fatty acids are imperative in growth, reproduction, brain development, and vision (Schmidt, 2010; Gurr et al, 2002). Omega-3 fed rats displayed higher ovulation rates than rats fed omega-6 fatty acids (Trujillo and Broughton, 1995). The authors of this study speculated that the observed

ovulation rates were in response to an observed decrease in PGE2 production paired with an increase in PGE3 production (Trujillo and Broughton, 1995).

The two most common omega 3 fatty acids found in fish oil and flaxseed oil are EPA and DHA (Schmidt, 2010). Supplemental fish oil fed to horses increased EPA and DHA plasma concentrations (King et al., 2008; Hoffman et al., 2011). EPA competitively inhibits cyclooxygenase resulting in the reduction of eicosanoid synthesis (Trujillo and Broughton, 1995; Wolfe, 1982). EPA also reduces eicosanoid synthesis through competition with arachidonic acid (Trujillo and Broughton, 1995; Wolfe, 1982). Diets rich in omega-6 fatty acids increase arachidonic acid availability for eicosanoid synthesis (Trujillo and Broughton, 1995; Kinsella et al, 1990). Competition for desaturation enzymes exist between omega-3 fatty acids and omega-6 fatty acids (Simopoulos, 1991). The inhibition of cyclooxygenase has been shown to inhibit ovulation through a reduction in the prostaglandins PGF2a and PGE2 (Trujillo and Broughton, 1995; Osman and Dullaart, 1976; Espey, 1983; Satoh et al., 1985; Espey et al., 1986).

Arachidonic acid, EPA, and DHA are all incorporated into the phospholipid membranes of cells which can then be mobilized upon chemical or physical activation producing eicosanoids (Hall et al., 2004). Prostaglandins are eicosanoids and are an imperative component in reproduction. The eicosanoid group encompasses prostaglandins, prostacyclins, thromboxanes, leukotrienes, and lipoxins. They act in very low concentrations and usually act locally close to the cells that produce them. Eicosanoids are involved in pain and fever production, blood pressure regulation, blood

clotting, and reproduction (Voet et al., 2008). Linoleic acid is converted to arachidonic acid via enzymatic desaturation and elongation. Phospholipase A2 frees arachidonic acid from cellular membranes. Free arachidonic acid is now able to be metabolized into eicosanoids through three major pathways: the cyclooxygenase (COX) pathway, the lipoxygenase (LOX) pathway, and the cytochrome P450 monooxygenase pathway (Wang and DuBois, 2010). The COX pathway yields proinflammatory eicosanoids of the one and two series variety such as prostaglandins and thromboxanes (Hall et al., 2004; Wang and DuBois, 2010). The LOX pathway produces the four series eicosanoids such as leukotrienes (Hall et al., 2004; Wang and DuBois, 2010). The P450 pathway produces epoxyeicosatrienoic acids, hydroxyl eicosatetraenoic acids (HETES), and hydroperoxyeicosatetraenoic acids via metabolism of arachidonic acid (Wang and DuBois, 2010).

Fat Supplementation in the Horse

In an effort to increase caloric density in equine diets without increasing feed intake, supplemental fat is often used. Fish oil has more recently become a common fat source in equine diets due to the presence of omega-3 fatty acids. A number of studies have been completed to discover the effects of dietary fat supplementation in the horse (Potter et al., 1992).

A study was conducted by Davison et al. (1991) to determine if fat supplementation to pregnant mares in late gestation would have an influence on

reproductive performance and lactation during early lactation. The study began 60 days prior to expected foaling date and ended 60 days after foaling. Foals from the fat supplemented mares displayed higher plasma lipid concentration at birth, day 10, and day 30. Fat supplemented mares had higher percent fat in milk on days 10 and 60 post-foaling. Foals from the fat supplemented mares tended to have higher body weights within the first week of life compared to controls, but no difference in body weights between groups was noted after this point. No difference in reproductive performance was seen between treatment groups; however, a trend was observed for a shorter postpartum interval and fewer cycles to pregnancy in the fat fed mares (Davison et al., 1991).

A study conducted to determine the effects of fish oil and corn oil supplementation on plasma fatty acid profiles in horses by Hall et al. (2004), revealed a 29% increase in plasma omega-6 fatty acids in corn oil supplemented horses after 6 weeks of consumption. These plasma concentrations persisted through the 8 and 12 week mark. No change was noted in plasma omega-3 fatty acid concentrations in these horses. Fish oil supplemented horses displayed a 24% decrease in plasma omega-6 fatty acid concentrations after 6 weeks of consumption with marked increases in plasma omega-3 fatty acid concentrations at 6, 8, and 12 weeks when compared baseline concentrations. Fish oil supplemented horse showed higher concentrations of omega-3 fatty acids at 6, 8, and 12 weeks when compared to the corn oil supplemented horses. An increase in arachidonic acid, EPA, and DHA plasma concentration was noted in fish oil supplemented horses when compared to concentrations in corn oil supplemented horses.

In corn oil supplemented horses, no change in plasma concentrations of arachidonic acid, EPA, and DHA was shown over time for the length of the study. The increased concentrations of arachidonic acid, EPA, and DHA observed in the fish oil supplemented horses were decreasing 4 weeks after cessation of the supplementation, however, arachidonic acid and DHA concentrations were still higher than baseline values. Both groups displayed a decrease in α-linolenic acid when compared to baseline values. These concentrations returned to baseline 4 weeks after cessation of supplementation (Hall et al., 2004).

In a study completed by King and associates (2008), omega-3 fat supplemented mares displayed an increase in circulating EPA and DHA concentrations after 3 days of supplementation. These concentrations progressed to a dose dependent peak level 7 days after onset of supplementation. When mares were supplemented with 40 g of EPA and DHA per day, peak EPA concentration in plasma was more than 13 times more than the control values. Plasma concentration of DHA was roughly 10 times the control value. In this study, the time required to return to basal concentrations after cessation of supplementation was 9 days. In the omega-3 supplemented mares an increase in the omega-3 type fatty acids, docosapentaenoic acid and stearidonic acid, was noted. Arachidonic acid concentration in the plasma also increased in the omega-3 supplemented mares (King et al., 2008).

In a fish oil study, mares receiving EPA and DHA displayed a longer period between foaling and first post-partum ovulation compared to control mares and to mares receiving DHA only (Schmidt, 2010; Poland, 2006). In the EPA and DHA supplemented

mares there was a tendency to hold pre-ovulatory follicles significantly longer than the control group and the DHA only group (Schmidt, 2010; Poland, 2005).

The inclusion of omega-3 fatty acids in equine diets resulted in a decrease in the production of inflammatory markers (Schmidt, 2010; Dinnetz, 2009). A shift from producing pro-inflammatory eicosanoids to anti-inflammatory eicosanoids occurred due to supplementation of omega-3 fatty acids (Schmidt, 2010; Calder, 2002). It is the observed decreases in eicosanoid production after omega-3 supplementation that is of interest due to the vital role eicosanoids play in reproduction.

CHAPTER THREE: OBJECTIVE

The objective of this study was to determine the reproductive effects of fat supplementation in normally cycling mares. Supplemental fish oil and soybean oil served as treatments and corn oil served as the control. We hypothesize that, when compared to corn oil, fish oil supplementation will increase cycle length and result in higher progesterone concentrations on day 9 post-ovulation. We further hypothesize that, when compared to corn oil, soybean oil supplementation will decrease cycle length and result in higher estradiol concentrations at ovulation denoted as day 0.

CHAPTER FOUR: MATERIALS AND METHODS

This study was approved by the Middle Tennessee State University (MTSU) Institutional Animal Care and Use Committee (Protocol #13-004) and was conducted at the MTSU Horse Science Center in Murfreesboro Tennessee. A total of 18 mares were used in this project. Mares were kept indoors for the duration of the study and only allowed turnout into sand and dry lot pens to avoid unknown intakes of fresh pasture. Mares were placed under lights for 16 hours every day, beginning at 7:00 am and ending at 11:00 pm, in an effort to extend reproductive cyclicity. Mares were followed through three ovulations. The first ovulation denoted as the base ovulation (BOV) began the feeding of the diets, the second ovulation was denoted as the end of cycle one (C1), and the study and collections ended at the third ovulation (End OV). The project began in September 2012 and ended in December 2012. Two mares were removed from the study in early November due to inadequate lighting and a managerial error leaving the total of mares used to 16. If mares failed to ovulate after BOV or C1 then final collections occurred 60 days after their recorded BOV.

Diet

Three different oils were used in this study: corn oil, soybean oil, and fish oil. Corn oil served as the control group while soy and fish oil were used in the treatment groups. Individual mares were stratified by body weight and body condition score

(Henneke et al. 1983) and randomly assigned to groups. Each group consisted of 6 mares. Mares were provided a ration to maintain body weight and meet NRC recommendations (NRC, 2007), providing 0.6% BW daily of a commercial concentrate (Purina Strategy) and approximately 1.2% BW of prairie grass hay. The assigned oil was top-dressed to the concentrate at a rate of 342 mg/kg BW/d. Daily ration was divided into two equal feedings. During the duration of this study, mares received no additional foodstuffs. All grain refusals from previous feedings were collected, weighed, and recorded prior to each feeding. Due to numerous refusals of fish oil at initiation, fat-free cherry syrup was added to increase palatability.

Estrus Detection and Cycle Monitoring

Mares were given an intramuscular injection of Prostaglandin F2α to initiate estrus as needed at the beginning of the project. Mares were not given Prostaglandin F2α injections after base ovulation occurred. Mares were checked for sexual receptivity on a daily basis by leading them individually to a stallion and checking for behavioral signs of estrus. They were assigned a behavioral teasing score ranging from 0 to 4 (0=cold; 1=cold, tolerant; 2=indifferent; 3=hot, winking; 4=hot, broke down). Once estrus behavior was evident, noted by a 3 or 4 score, mares were placed in stocks and monitored daily via rectal palpation and ultrasonography until ovulation occurred. Follicular size and reproductive tract tone, edema, and presence of uterine cysts were recorded. Once

ovulation occurred, blood was collected, mares were weighed and assigned a body condition score (Henneke et al., 1983).

Blood Collection

Blood was collected via jugular venipuncture at each ovulation: base ovulation (BOV), cycle 1 ovulation (C1), cycle 2 ovulation (End OV), and between days 9 and 11 after BOV and C1 ovulations. For simplicity, the day 9 to 11 sample will be referred to as D9. A total of 5 blood collections occurred per mare (BOV, BOV D9 C1, C1 D9, and End OV). Two vacutainer tubes, one serum and one plasma, were filled with blood collected from the jugular vein at each collection. Blood collections were allowed time to clot before processing. Blood was processed at 3000 RPM for 15 minutes in a refrigerated centrifuge at 4º C. Once centrifuged, the serum aliquots were collected and placed in labeled microcentrifuge tubes and frozen until analysis could occur.

NEFA Analysis

A NEFA-HR Assay (Wako Chemicals USA, Inc., Richmond, VA), previously validated for use in the horse, was performed to determine the concentration of non-esterified fatty acids (NEFA). Briefly, the HR Series NEFA-HR is an enzymatic calorimetric assay and works off the principle that when NEFA in serum are treated with acyl-CoA synthetase in the presence of adenosine triphosphate and CoA, form the acyl-

CoA and adenosine monophosphate and pyro-phosphate are formed. The acyl-CoA is oxidized to produce hydrogen peroxide which in the presence of peroxidase forms a purple color which can then be measured to determine the amount of NEFA in serum. The standards used were as follows: 0, 20, 40, 60, 80, 100, 120, 150, 200, 400, and 600 mmol/L. First, 10 µL of the blank, standards, and specimens were pipetted in duplicate into each well. Next, 200µL of Color Reagent A Solution was added to each well and the well plate was then incubated at 37ºC for 5 minutes. The absorbance of each well was then measured at 560nm (Abs 1). Next, 100µL of Color Reagent B Solution was then added to each well and incubated for 5 minutes at 37ºC. After incubation, the absorbance of each well was again measured at 560 nm (Abs 2). Final value was obtained by subtracting Abs1* F from Abs2, where F is the correction factor for changes in volume, in this case the correction factor was 0.68. Acceptable covariance used for this assay was 10%.

Triglyceride Analysis

A L-Type TG M Microtiter Procedure (Wako Chemicals USA, Inc., Richmond, VA), previously validated in the horse, was performed to determine the total number of triglycerides in serum. The kit is calorimetric and works off the premise that when a sample is mixed with Color A, free glycerol in the sample is decomposed by the enzymes present in Color A. Color B is then added and the triglycerides in the sample are hydrolyzed to glycerol and free fatty acids in an enzymatic reaction catalyzed by

lipoprotein lipase. Glycerol is converted to glycerol-3-phosphate which is then oxidized to produce hydrogen peroxide. The production of hydrogen peroxide causes HMMPS (N-(3-sulfopropyl)-3-methoxy-5-methylaniline) to produce a blue pigment. The amount of triglycerides contained in each sample is determined by measuring the absorbance of this blue color. Provided standards were diluted as necessary to produce standards with concentrations ranging from 0 mg/L to 530 mg/L. 4µL of each standard and sample were pipetted in duplicate into each well. 90 µL of Reagent 1 was added to all wells and contents were gently mixed. The well plate was incubated for 5 minutes at 37º C. The absorbance was measured at 600nm (Abs 1). 30 µL of reagent 2 was added to each well and the contents were mixed via gentle rotation. The well plate was incubated for 5 minutes at 37º C. The absorbance was again measured at 600nm (Abs 2). Final absorbance was calculated by subtracting Abs 1 from Abs 2. Acceptable covariance used for this assay was 10%.

Progesterone Assay

Serum samples were sent to the Veterinary Endocrinology Laboratory at University of California Davis for analysis. In order to test the progesterone concentrations in plasma during the different stages of the cycle at the time of collections, a progesterone enzymeimmunoassay (EIA), previously validated for the horse, competitive assay was performed. Serum samples served as the specimens. The plates Immulon 1B were coated with anti-progesterone antibody at 1:5000 dilution. 100µl of

each serum sample were pipetted into glass tubes. 2ml of petroleum ether were added to each tube including the internal controls placed at the beginning and every 20 samples. After petroleum ether was added samples were mixed and then placed in a methanol/dry ice bath to freeze the serum. The ether was poured off into separate tubes and allowed to evaporate overnight. Progesterone standards were suspended in 100% ethanol and allowed to evaporate overnight. EIA plates were then washed three times with wash buffer. The HRP conjugate at 1:60000 dilution was added to the standards and samples. Plates were incubated for 2 hours at room temperature and then washed with wash buffer three times. The ABTS substrate was made and all plates were incubated for 45 minutes at room temperature until the conjugate wells were at an optical density of 0.8 to 0.9. 100 µl of stop solution was added and plates were read at 490 nm.

Estradiol Assay

A Siemens estradiol double antibody radioimmunoassay (RIA), previously validated for the horse was used to determine estradiol concentrations in the serum at each collection. Serum samples were used as specimens and processed at the Veterinary Endocrinology Laboratory at the University of California Davis. This was a two day procedure. On day one, 250 µl of serum samples were pipetted in duplicate into glass tubes. 250 µl of gelding serum served as a negative control. Stallion serum served as low (50µl), middle (100µl), and high (200µl) positive controls. Estradiol standard curve was pipetted in duplicate, in ethanol, from 2.5pg to 200pg per tube. 3ml of diethyl ether was

added to extract each serum sample and then mixed at full speed for 30 seconds. Tubes were reserved for total counts, non-specific binding, and maximum binding. The serum was frozen and ether was poured off and allowed to evaporate overnight. On day two, 200µl of buffer was added to each tube, except total count tubes, and then vortexed for 30 seconds for re-suspension of evaporated samples. 100µl of reconstituted antibody was added to each sample except total counts and non-specific binding tubes, then mixed for 5 seconds, covered and incubated for two hours at room temperature. 100 µl of 1251 Estradiol tracer was added to each tube and mixed for 5 seconds before incubation commenced for one hour at room temperature. 1 ml of cold precipitation solution was added to each tube except total count tubes and then incubated for 10 minutes at room temperature. Total count tubes were then set aside for counting at a later time. Tubes were spun for 15 minutes at 3800 rpm in a pre-chilled centrifuge. The liquid supernatant was aspirated from the antibody pellet. Each sample was counted for 30 seconds on the gamma counter. Concentration was calculated from a log-logit curve, with total count tubes taken as 100%.

Statistical Analysis

Data were summarized as least squares means and standard errors. A mixed model with repeated measures was used to test the effects of diet and day on cycle length, serum non-esterified fatty acids and triglycerides, progesterone and estradiol using SAS (Ver 9.2, SAS Inst., Inc., Cary, NC). Model effects included diet, day and their

interaction, diet*day, with horse as the subject and day as the repeated effect. Results were considered statistically significant at $P < 0.05$, with a tendency towards significance at $0.05 < P < 0.10$.

CHAPTER FIVE: RESULTS AND DISCUSSION

All horses readily consumed corn and soybean oil supplementation. At the time of project initiation, refusals (n=2) in horses supplemented with fish oil occurred and in an attempt to increase palatability, fat-free cherry syrup was added. Although refusals in fish oil horses still occurred on occasion, the amount refused lowered. All horses remained healthy for the duration of the study with the exception of one fish oil supplemented mare who experienced colic on day 14 post-ovulation of her second cycle; however, it is not believed that this attributed to fat supplementation nor had any effect on final results.

Cycle Length

No difference (P=0.17) in cycle length between diets was observed. No difference (P=0.17) between cycle lengths across all diets was present. No difference (P=0.33) was present in the interaction of diet on cycle length. The average length of cycle across all diets for cycle one was 22.3 ±3.0 days. The average length of cycle two was 30.4 ±2.9 days. The average cycle length for corn supplemented horses was 24.6 ±5.0 days for cycle one and 29.7 ±4.7 days for cycle two. The average cycle length for fish oil supplemented horses for cycle one was 18.4 days ±5.6 and 38.2 days ±5.1 for cycle two. Soybean oil supplemented horses showed an average cycle length of 23.8 days ±5.1 for cycle one and 23.20 days (±5.14) for cycle two. Schmidt (2010) also

reported no change in cycle length in fish oil supplemented mares fed at a rate of 280g of a powdered fish oil supplement containing EPA and DHA. The average cycle length in the mare is 21 days. The subtle increase in cycle length during this study, although not significant, could be attributed to season. In the current study, 6 mares failed to ovulate by 60 days of supplementation. In the soybean oil group 1 mare failed to ovulate, in the corn oil group 2 mares failed to ovulate, and in the fish oil group 3 mares failed to ovulate. Since this study was conducted from the months of September to December, these ovulation failures may be attributed to season as mares may have been in the early stages of transition. During these months, mares are naturally entering a transitional period when day and night are equal in length. During this transitional period mares often display erratic estrus behavior without corresponding ovulation. Even if ovulation does occur during this transitional period, CL function is not maintained and mares enter a state of reproductive quiescence.

NEFA

Serum NEFA analysis revealed no difference (P=0.73) between all diets across all days (Figure 3). There also was no difference (P=0.20) in diet*day interactions (Figure 5). There was a trend (P=0.073) between days across all diet (Figure 4). The observed trends were higher NEFA concentrations on BOV when compared to C1D9 and EndOV. These observed decreases in NEFA concentrations are deemed normal as the horse progresses through fat supplementation. O'Connor and associates (2007) reported

similar decreases in NEFA values in fat supplemented horses receiving the same amount of oil, 324 mg/kg of body weight, as in the present study and therefore these results were expected. Elevated NEFA concentrations indicate that dietary energy intake is deficient, therefore, excess energy attributes to decreasing NEFA concentrations.

Triglycerides

No difference (P=0.72) in serum triglycerides between diets across all days was observed (Figure 6). Likewise, no difference (P=0.13) between days across all diets was present (Figure 7). No difference (P=0.61) was detected in diet* day interactions (Figure 8). O'Connor et al. (2007) reported lower serum triglyceride concentrations in fish oil supplemented horses when compared to corn oil supplemented horses on day 63 of supplementation. The changes in triglyceride concentration observed by O'Connor et al. (2007) have been speculated to be attributed to a down regulation in the enzymes necessary for triglyceride synthesis as a result of omega-3 supplementation; however the exact mechanism is unknown (Marsh et al., 1987; Surette et al., 1992). In further contrast to our results, O'Connor et al. (2007) observed a decrease in triglyceride concentrations in soybean oil supplemented horses. This decrease was attributed to an increase in lipoprotein lipase activity and possibly fatty acid oxidation. Increasing lipoprotein lipase activity increases the rate of triglyceride clearance from the bloodstream. Unfortunately, lipoprotein lipase activity was not measured and therefore it is unknown if the observed decreases in triglyceride concentration witnessed by O'Connor et al. (2007) is attributed

to lipoprotein lipase activity or a reduction in triglyceride synthesis due to a lack of necessary enzymes (O'Connor et al., 2007). The results obtained in our study were similar to triglyceride concentrations observed by O'Connor et al. (2007); however, our study had a high amount of variability which may attribute to the lack of differences in the results.

Estradiol

In serum estradiol analysis, no difference (P=0.54) between diets across all days was observed (Figure 9). No difference (P=0.38) was observed in diet*day interactions (Figure 11). A trend (P=0.094) was shown between days across all diets (Figure 10). The trends observed included higher estradiol concentrations at time of ovulation C1 when compared to estradiol concentrations at BOVD9 (P=0.063) and higher estradiol concentrations at EndOV when compared to concentrations on BOVD9 (P=0.008). These rises in estradiol serum concentrations on these days are normal as they coincide with a normal estrous cycle in the mare and never reached abnormal values. Estradiol concentrations begin to rise 24 to 48 hours before ovulation and reach peak values just prior to ovulation.

Progesterone

In serum progesterone concentrations there was no difference (P=0.17) between diets across all days (Figure 12). No difference (P=0.18) between diet*day interactions was observed (Figure 14). A difference (P<0.0001) was observed between days across all diets (Figure 13). Progesterone concentrations were low at time of ovulation (BOV, C1, and EndOV) and higher on day 9 during cycle 1 and 2. These progesterone concentrations are expected as they follow the normal estrous cycle progesterone concentrations in the mare. After ovulation, progesterone concentrations begin to rise with the formation of the corpus luteum (CL). By 5 days after ovulation, the CL is fully functional and begins to secrete progesterone reaching maximal concentrations which are maintained until luteolysis occurs around day 16. A difference observed that was not expected occurred between BOVD9 and C1D9 (P=0.020). BOVD9 progesterone concentrations were higher than C1D9, 9.98 and 6.35ng/ml respectively; however the concentrations reached are within normal progesterone concentrations. The difference observed in this study may be due to season, as mares were supplemented during the months of September, October, November, and December of 2012 when mares are normally in transition. Schmidt (2010) reported that omega-3 supplementation had a limited effect on hormone concentrations in the mare with a trend for fish oil supplemented mare's progesterone concentrations to be higher 3 days post-ovulation when compared to control mares. A significant difference in fish oil mare's progesterone concentrations on day 5 post-ovulation when compared to control mares was also

reported (Schmidt, 2010). While the increases in progesterone in fish oil supplemented mares were reported, the values were within the normal range for the time period. Schmidt (2010) claimed the observed increases may have been a result of more efficient CL that had the ability for increased cholesterol uptake by theca cells to produce more progesterone. The theca cells are able to convert cholesterol, an androgen precursor, to progesterone (Davies Morel, 2008). Varying supplementation concentrations, physiological status of the animal, and the fat source used may attribute to progesterone concentrations. A study conducted by Furtney et al. (2009) reported similar increases in progesterone concentrations in omega-3 supplemented mares across estrous cycles when compared to mares supplemented with animal fat. It has been speculated that the resulting increases in progesterone after fat supplementation may be due to a decreased clearance rate of progesterone in circulation, not an increased rate of synthesis. In our study, samples were collected on day 9 to evaluate progesterone concentrations at this time. Future studies may want to consider sampling more frequently throughout the estrous cycle of the mare to determine progesterone concentrations.

Conclusion

The results of this study indicate that feeding oil at a rate of 324mg/kg of body weight for two consecutive estrous cycles has no implication on the estrous cycle of the mare. Fat source supplementation had no effect on cycle length, serum triglycerides, serum NEFAs, serum estradiol concentrations, and serum progesterone concentrations.

No difference in cycle length was seen. The trend in NEFA values coincided with previous reports of NEFA decreases as fat supplementation occurred. While trends and differences were detected in estradiol and progesterone concentrations, they were deemed normal as they followed the normal hormone concentrations during the estrous cycle of the mare and never reached abnormal values. Oils commonly added to feeds are not detrimental to reproductive markers measured in this study. Therefore, effects on the estrous cycle should not be of concern when selecting a fat source for supplementation in mares.

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APPENDICES

APPENDIX A

Cycle Length Figures

Figure 1: Effect of Diet Across All Days: Cycle Length

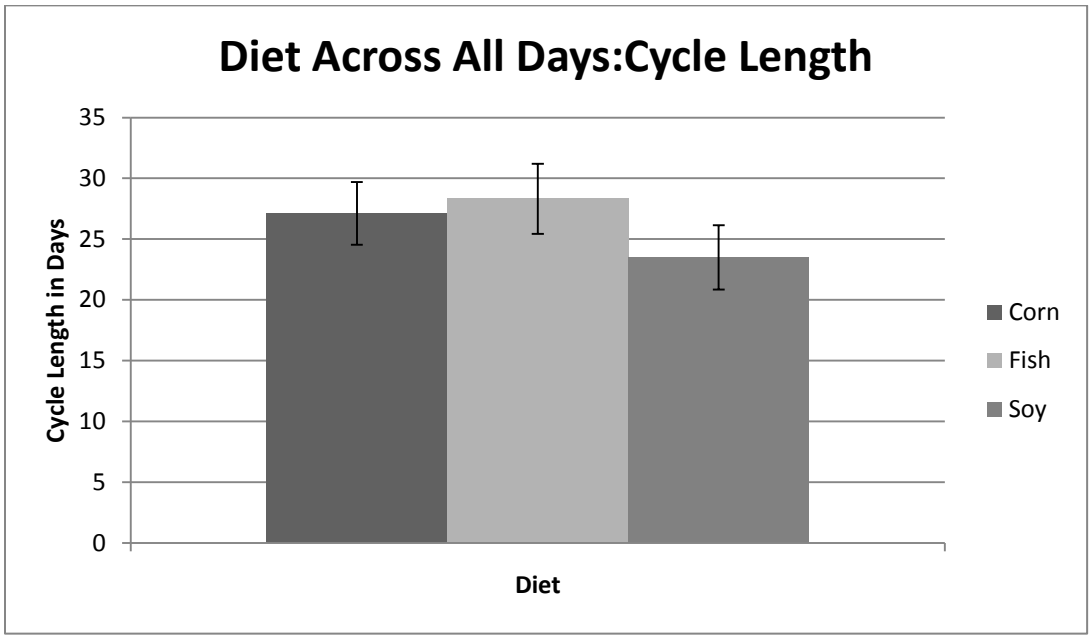

Figure 1. Effect of diet across all collection days on cycle length in 16 mares receiving a supplemental corn oil, fish oil, or soybean oil fat source for 2 consecutive estrous cycles. (P=0.49).

Figure 2: Effect of All Diets: Cycle Length

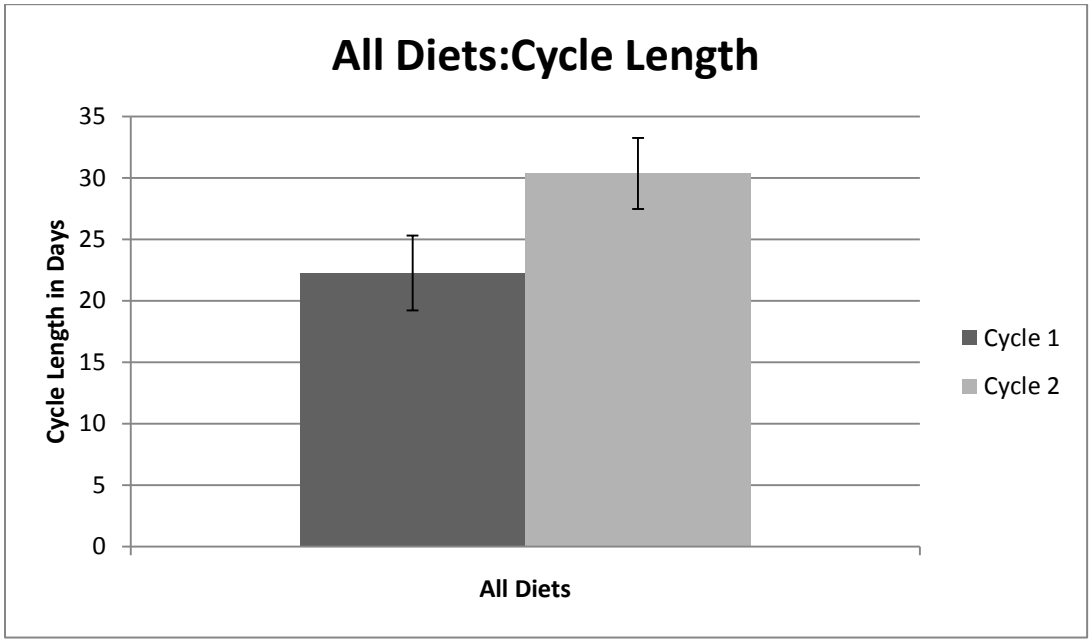

Figure 2. Effect of all diets on cycle length in 16 mares receiving a supplemental corn oil, fish oil, or soybean oil fat source for 2 consecutive estrous cycles. (P=0.17).

APPENDIX B

NEFA Figures

Figure 3: Effect of Diet Across All Days: NEFA

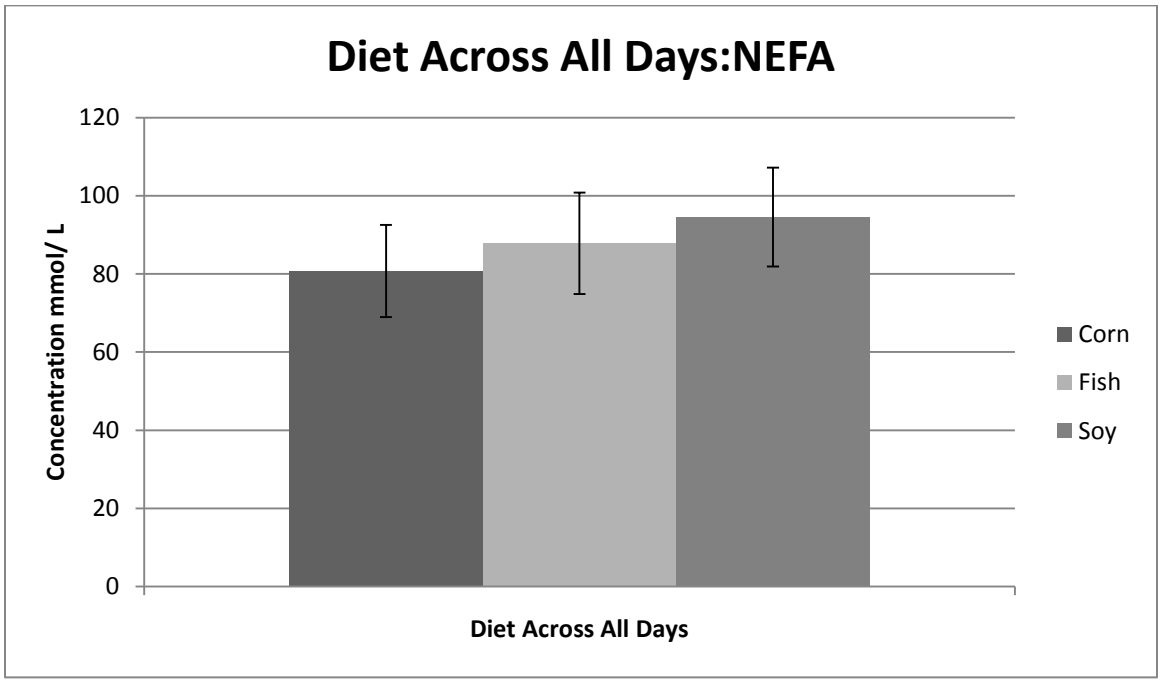

Figure 3. Effect of diet on serum NEFA concentrations across all collection days in 16 mares receiving a supplemental corn oil, fish oil, or soybean oil fat source for 2 consecutive estrous cycles. ($P<0.73$).

Figure 4. Effect of All Diets: NEFA

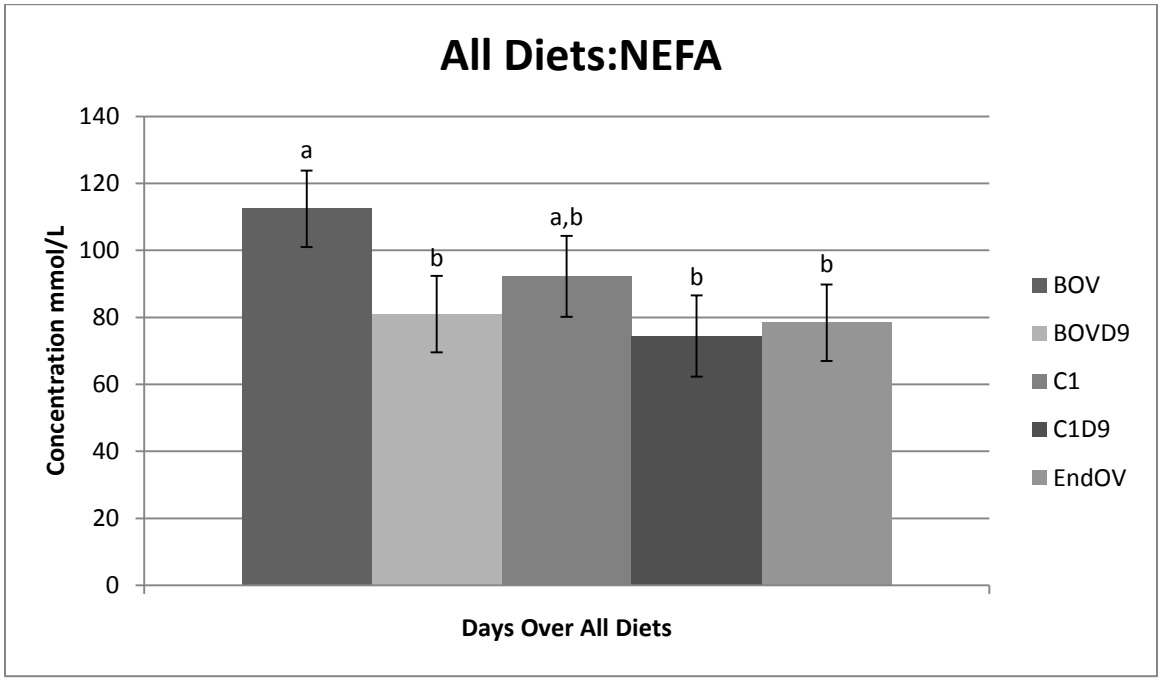

Figure 4. Effect of all diets on serum NEFA concentrations across each collection day in 16 mares receiving a supplemental corn oil, fish oil, or soybean oil fat source for 2 consecutive estrous cycles. Collection days are: Base ovulation (BOV), Base ovulation day 9 (BOVD9), First Ovulation after treatment ensued (C1), First ovulation after treatment ensued day 9 (C1D9), and Final ovulation (EndOV). Days lacking common superscript tend toward significance ($P<0.073$).

Figure 5. Effect of Diet on Day: NEFA

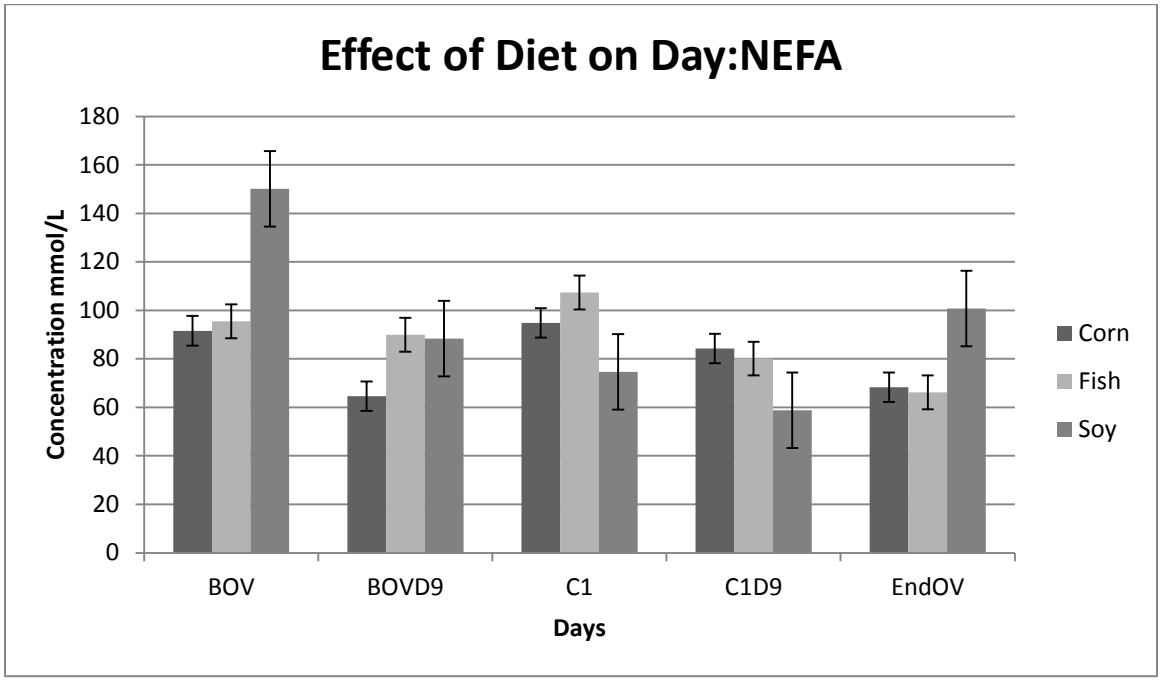

Figure 5. Effect of diet on serum NEFA concentrations on each collection day in 16 mares receiving a supplemental corn oil, fish oil, or soybean oil fat source for 2 consecutive estrous cycles. Collection days are: Base ovulation (BOV), Base ovulation day 9 (BOVD9), First Ovulation after treatment ensued (C1), First ovulation after treatment ensued day 9 (C1D9), and Final ovulation (EndOV). ($P<0.20$).

APPENDIX C

TG Figures

Figure 6. Effect of Diet Across All Days: TG

Figure 6. Effect of diet on serum triglyceride (TG) concentration across all collection days in 16 mares receiving a supplemental corn oil, fish oil, or soybean oil fat source for 2 consecutive estrous cycles. ($P<0.72$).

Figure 7: Effect of All Diets: TG

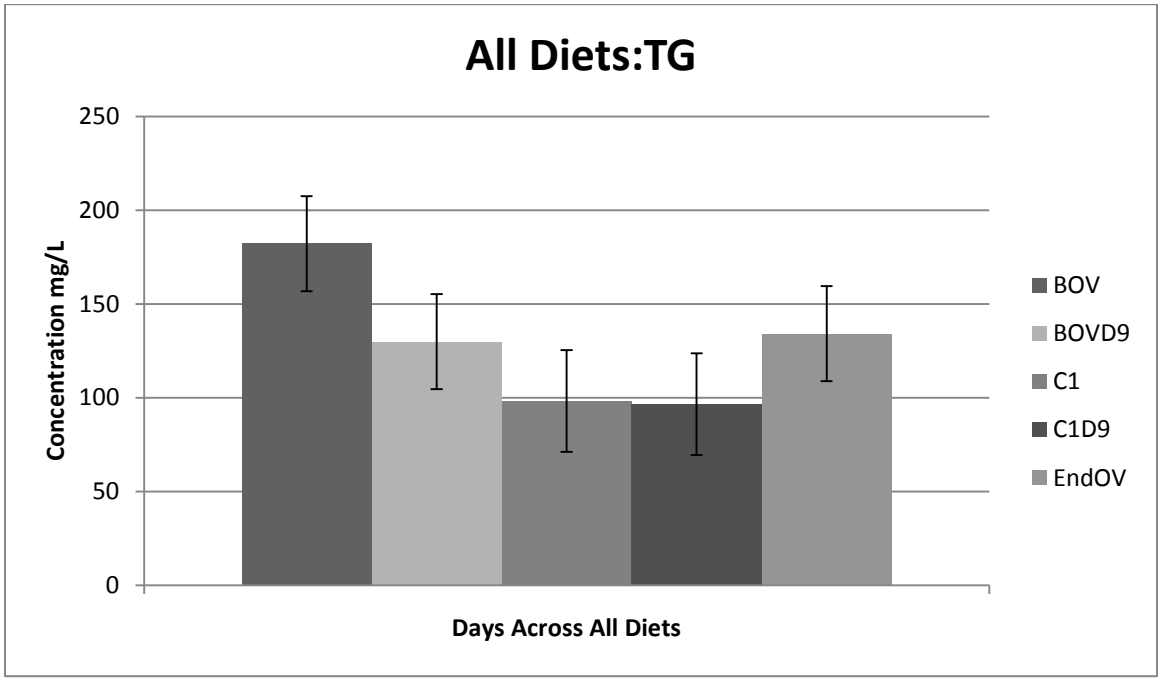

Figure 7. Effect of all diets on serum TG concentrations on collection days in 16 mares receiving a supplemental corn oil, fish oil, or soybean oil fat source for 2 consecutive estrous cycles. Collection days are: Base ovulation (BOV), Base ovulation day 9 (BOVD9), First Ovulation after treatment ensued (C1), First ovulation after treatment ensued day 9 (C1D9), and Final ovulation (EndOV). ($P<0.13$).

Figure 8: Effect of Diet on Day: TG

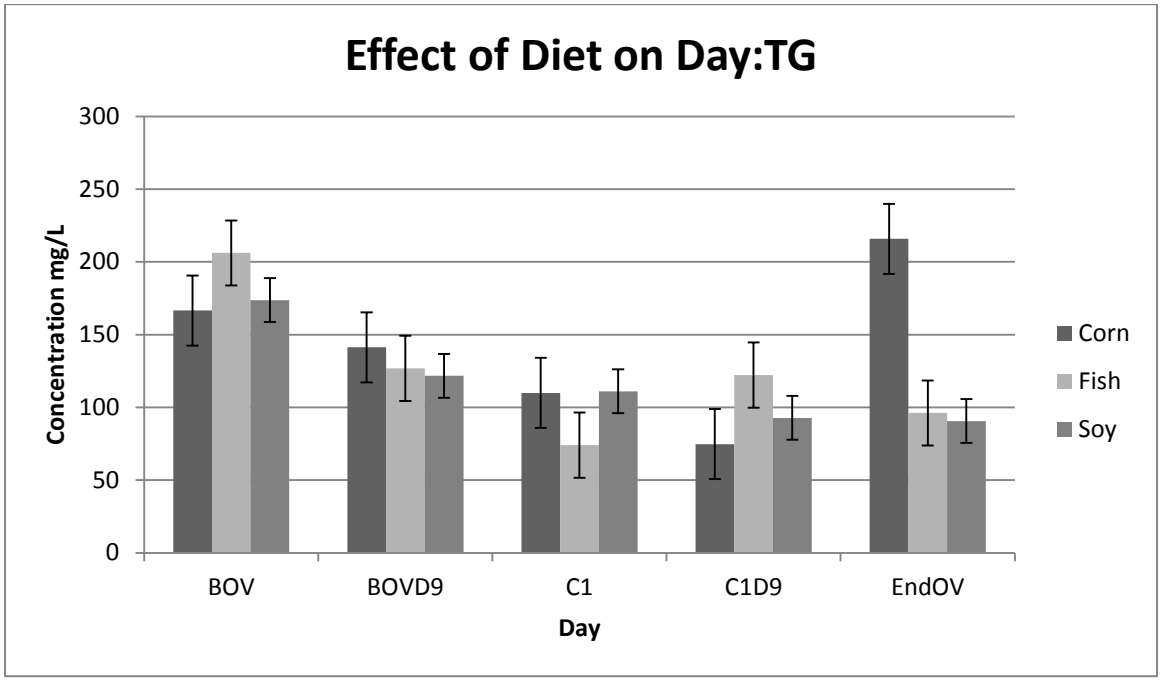

Figure 8. Effect of diet on serum TG concentration on each collection day in 16 mares receiving a supplemental corn oil, fish oil, or soybean oil fat source for 2 consecutive estrous cycles. Collection days are: Base ovulation (BOV), Base ovulation day 9 (BOVD9), First Ovulation after treatment ensued (C1), First ovulation after treatment ensued day 9 (C1D9), and Final ovulation (EndOV). ($P<0.61$).

APPENDIX D

Estradiol Figures

Figure 9. Effect of Diet Across All Days: Estradiol

Figure 9. Effect of diet on serum estradiol concentrations across all collection days in 16 mares receiving a supplemental corn oil, fish oil, or soybean oil fat source for 2 consecutive estrous cycles. ($P<0.54$).

Figure 10: Effect of All Diets: Estradiol

Figure 10. Effect of all diets on serum estradiol concentrations on each collection day in 16 mares receiving a supplemental corn oil, fish oil, or soybean oil fat source for 2 consecutive estrous cycles. Days lacking common superscript tend toward significance ($P<0.09$). Collection days are: Base ovulation (BOV), Base ovulation day 9 (BOVD9), First Ovulation after treatment ensued (C1), First ovulation after treatment ensued day 9 (C1D9), and Final ovulation (EndOV).

Figure 11. Effect of Diet on Day: Estradiol

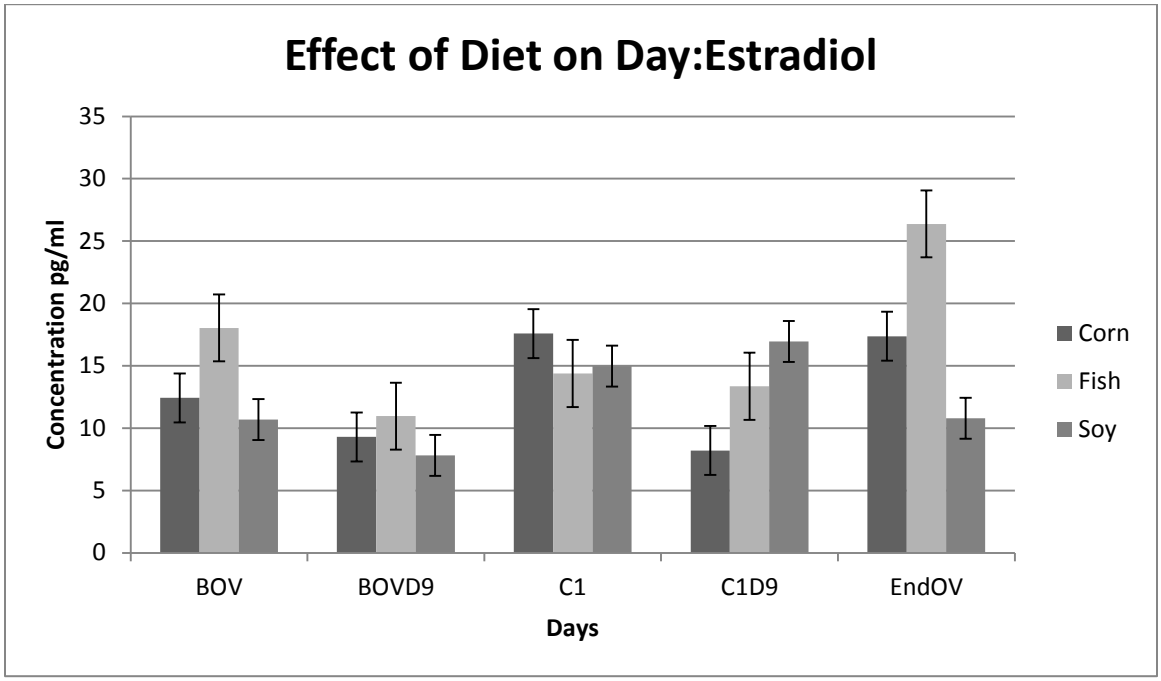

Figure 11. Effect of each diet on serum estradiol concentrations on each collection day in 16 mares receiving a supplemental corn oil, fish oil, or soybean oil fat source for 2 consecutive estrous cycles. Collection days are: Base ovulation (BOV), Base ovulation day 9 (BOVD9), First Ovulation after treatment ensued (C1), First ovulation after treatment ensued day 9 (C1D9), and Final ovulation (EndOV). ($P<0.38$).

APPENDIX E

Progesterone Figures

Figure 12. Effect of Diet Across All Days: Progesterone

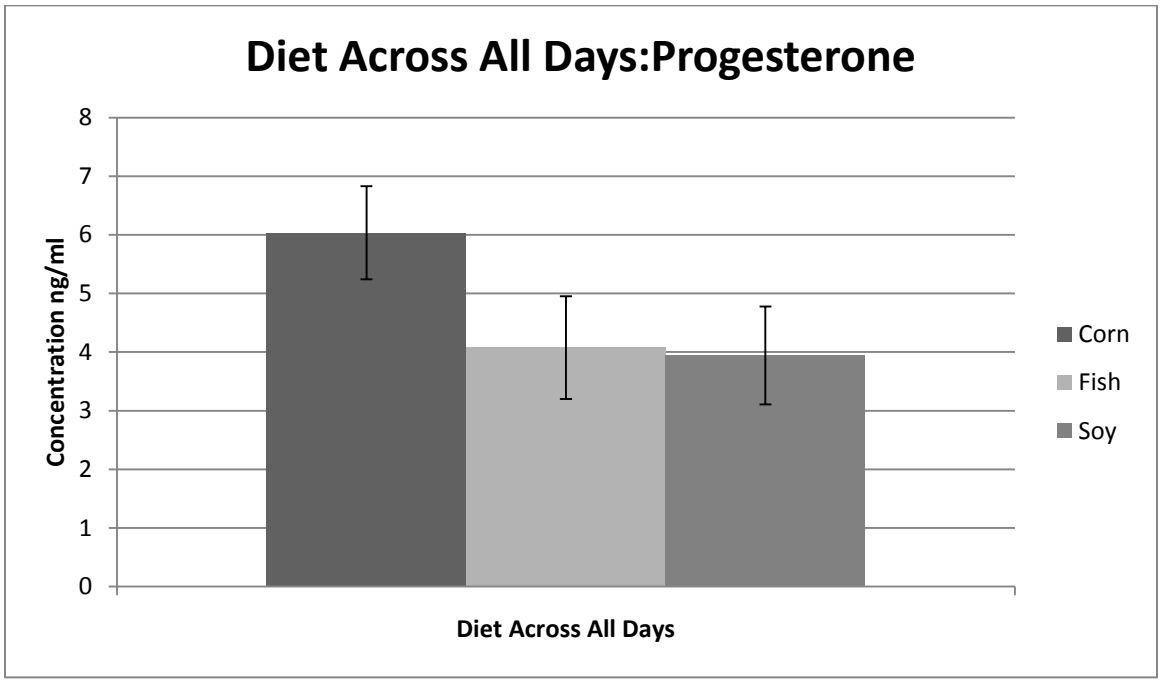

Figure 12. Effect of diet across all collection days on serum progesterone (P4) concentrations in 16 mares receiving a supplemental corn oil, fish oil, or soybean oil fat source for 2 consecutive estrous cycles. ($P<0.17$).

Figure 13. Effect of All Diets: Progesterone

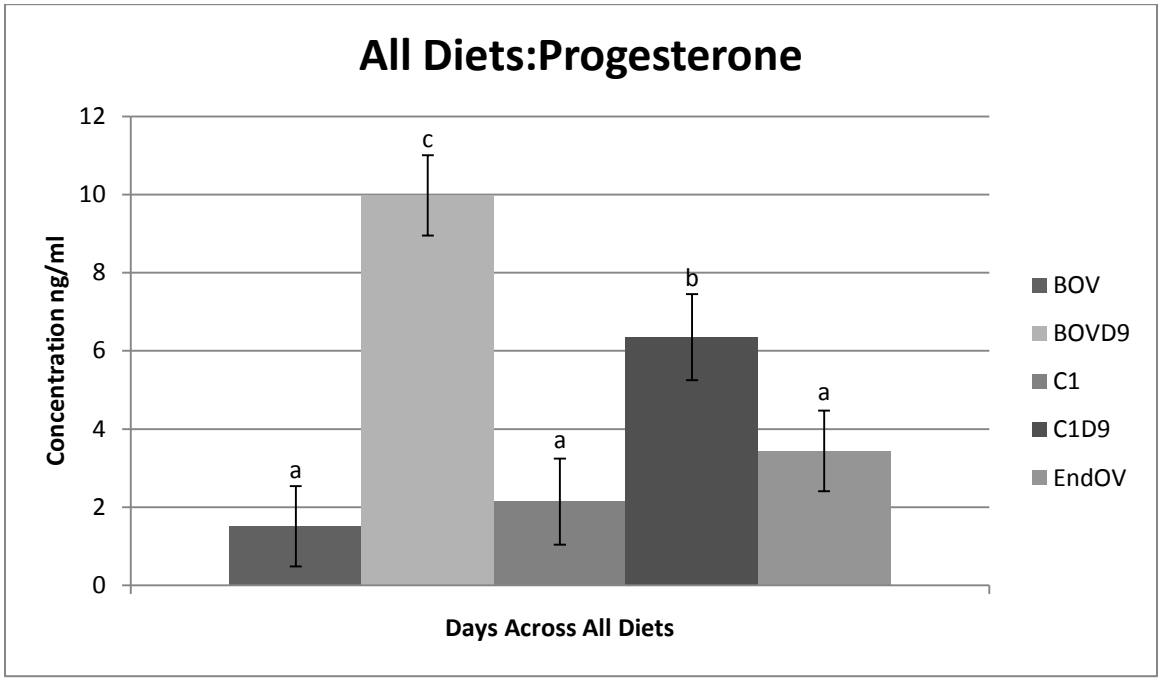

Figure 13. Effect of all diets on serum P4 concentrations on each collection day in 16 mares receiving a supplemental corn oil, fish oil, or soybean oil fat source for 2 consecutive estrous cycles. Collection days are: Base ovulation (BOV), Base ovulation day 9 (BOVD9), First Ovulation after treatment ensued (C1), First ovulation after treatment ensued day 9 (C1D9), and Final ovulation (EndOV). Days lacking common superscript differ ($P<0.0001$).

Figure 14. Effect of Diet on Day: Progesterone

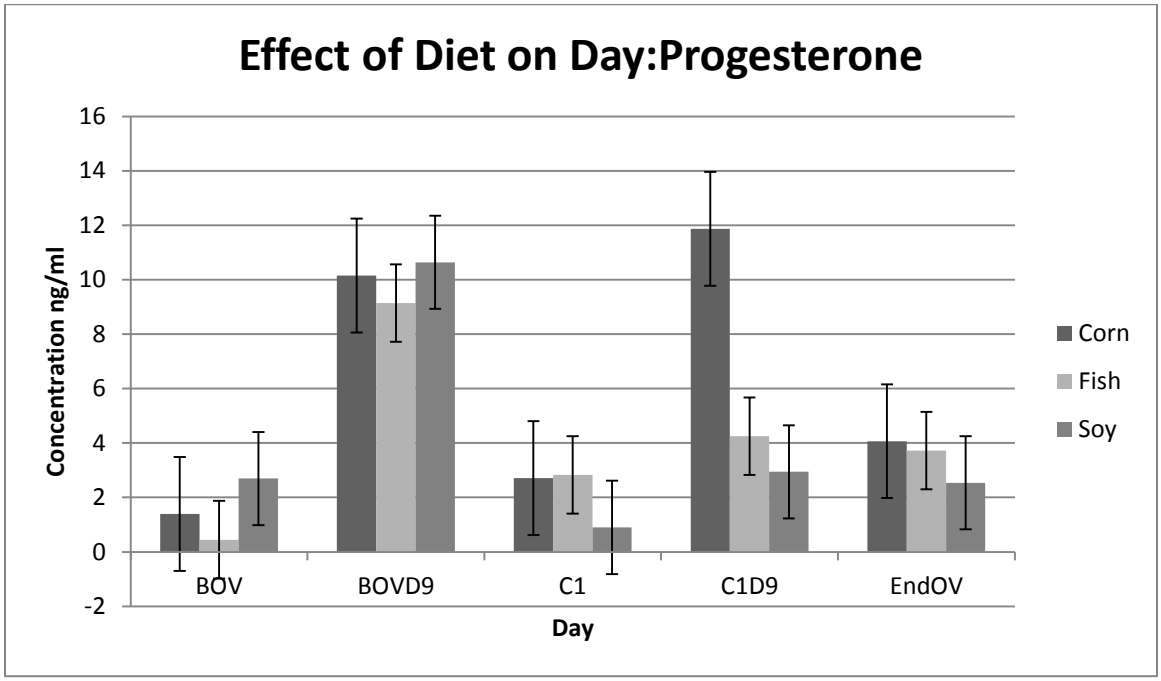

Figure 14. Effect of each diet on serum P4 concentrations on each collection day in 16 mares receiving a supplemental corn oil, fish oil, or soybean oil fat source for 2 consecutive estrous cycles. Collection days are: Base ovulation (BOV), Base ovulation day 9 (BOVD9), First Ovulation after treatment ensued (C1), First ovulation after treatment ensued day 9 (C1D9), and Final ovulation (EndOV). ($P<0.18$).

September 17, 2012

Investigator(s) Name: Holly Spooner, Rhonda Hoffman, John Haffner, Jenna Bertoli, Caitlin Crain
Investigator(s) Email: Holly.spooner@mtsu.edu; Rhonda.hoffman@mtsu.edu; John.haffner@mtsu.edu; jcb6v@mtmail.mtsu.edu; cmc8n@mtmail.mtsu.edu

Protocol Title: "Influence of dietary fat sources on reproductive parameters in mares"
Protocol Number: 13-004

Dear Investigator,

The MTSU Institutional Animal Use and Care Committee has reviewed your research proposal identified above and has approved your research under the PHS definition of animal. Approval is granted for three (3) years. **Please note you will need to file a Progress Report annually regarding the status of your study.**

According to MTSU Policy, an investigator is defined as anyone who has contact with animals for research purposes. Anyone meeting this definition needs to be listed on the protocol and needs to provide a certificate of training to the Office of Compliance. If you add investigators to an approved project, please forward an updated list of investigators and their certificates of training to the Office of Compliance before they begin to work on the project.

Any change to the protocol must be submitted to the IACUC before implementing those changes. Any unanticipated harms to subjects or adverse events must be reported to the Office of Compliance at (615) 494-8918.

You will need to submit an end-of-project report to the Office of Compliance upon completion of your research. Complete research means that you have finished collecting data and you are ready to submit your thesis and/or publish your findings. Should you not finish your research within the three (3) year period, you must submit a Progress Report and request a continuation prior to the expiration date. Please allow time for review and requested revisions. Your study expires **September 17, 2015**.

Also, all research materials must be retained by the PI or faculty advisor (if the PI is a student) for at least three (3) years after study completion. Should you have any questions or need additional information, please do not hesitate to contact me.

Sincerely,

Emily Born

Emily Born
Compliance Officer
615-494-8918
emily.born@mtsu.edu