EFFECTS OF SUPPLEMENTING MARE DIETS WITH MARINE-DERIVED N-3 FATTY
ACIDS ON SERUM, FOLLICULAR FLUID AND FOLLICULAR DYNAMICS DURING
THE ESTROUS CYCLE

by

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Abstract

The objective of this study was to evaluate the reproductive effects of supplementing normally cycling mares with marine-derived omega-3 (n-3) fatty acids during the estrous cycle. Fifteen mares were assigned to a control diet (CONT, n=7) or a fish oil supplemented diet (FO, n=8) containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The FO mares received 18.48 g EPA/10.08 g DHA/mare/d. At the start of the trial, mares were synchronized using a progesterone and estradiol protocol. Following synchronization, mares were monitored with transrectal ultrasonography throughout the second estrous cycle. Ovarian activity, ovulation, and presence of a corpus luteum were noted. Mares were ultrasounded throughout the third estrous cycle until a 35 mm follicle was detected. Upon detection of the 35 mm follicle, hCG was administered. Within 16 hr, transvaginal ultrasound-guided follicular aspiration (TUGA) was performed on the preovulatory follicle, signifying the end of the trial. Follicular fluid was analyzed for fatty acid and hormone concentrations. Serum fatty acids were measured every 2 wk and serum hormone concentrations were analyzed during the second estrous cycle at 5 d to 1 d prior to ovulation, at ovulation, and 3 and 5 d post-ovulation. Samples were also collected prior to hCG administration and on aspiration day for hormone analysis. Serum estradiol-17β, progesterone, luteinizing hormone (LH), and insulin-like growth factor 1 (IGF-1) were measured.

Fish oil supplementation increased ($P < 0.01$) arachidonic acid (ARA), EPA, docosapentaenoic acid (DPA), and DHA in mare serum and increased ($P<0.01$) EPA, DPA, and DHA in follicular fluid. No overall treatment effect was found on serum hormone concentrations during the second estrous cycle, but a decrease ($P<0.05$) in IGF-1 was noted in the FO group on aspiration day. Concentrations of IGF-1 were also lower ($P<0.05$) in follicular fluid in the FO group compared to controls. No other follicular fluid differences were observed. Supplementation resulted in a smaller diameter follicle ($P<0.05$, 38.0±0.47 mm) on aspiration day than the CONT group (39.5±0.5 mm). Dietary n-3 fatty acids modify mare serum and follicular fluid fatty acid profiles, with supplementation of EPA and DHA decreasing serum and follicular IGF-1 concentrations.

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Preface

The inclusion of fat in the horse diet to maximize energy without negatively impacting the animal's glycemic index or digestive tract has been a common practice for many years. As monogastric herbivores, horses are designed to primarily consume forage which is high in fiber but low in DE. However, unlike high amounts of starch and cereal grains, the horse has a relatively high tolerance for fat in the diet and it is considered a "safe" energy alternative to the more traditionally used cereal grains (Bush et al., 2001; Lewis, 2005). In fact, horses can utilize up to 20% added fat in the total diet (Lewis, 2005). The most commonly used forms of fat supplementation for horses are plant and vegetable oils, such as corn oil and soy oil, due to their high palatability and availability. Plant and vegetable oils provide about 3 times more digestible energy than an equal weight of cereal grain, and 3.5 to 6 times more digestible energy than an equal volume of cereal grain (Lewis, 2005). Fat added diets have been proven to be especially beneficial to those horses that have a heavy work load or exercise requirement (Hambleton et al., 1980; Harkins et al., 1992; Eaton et al., 1995). Dietary fat supplementation also affects follicular growth, pregnancy rates, milk composition (Mattos et al., 2002), and ovarian and uterine function in several livestock species (Beam and Butler, 1997; Mattos et al., 2000; Santos et al., 2008). Although not as extensively documented, a positive effect of added fats on reproduction in the equine species has been documented (Kubiak et al., 1987; Davidson et al., 1991; Ordakowski-Burk et al., 2005). Fat supplementation of mares during late gestation and early lactation increased milk fat percentage, blood lipid concentration of their foals, and rate of gain in foals during their first week of life (Davidson et al., 1991). Additionally, this study noted a trend for a shorter postpartum interval and fewer cycles to pregnancy in fat-supplemented mares.

A particular group of fatty acids has been recently highlighted in research and the consumer market as having multiple health benefits for both humans and livestock species. Polyunsaturated fatty acids are defined as fatty acids with multiple double bonds, and can be categorized into omega-6 (n-6) and omega-3 (n-3) families. Omega-6 fatty acids are found in plentiful amounts in common animal feedstuffs such as corn and corn oil. Once digested, n-6 fatty acids produce series 1 and 2 eicosanoids which are inflammatory in nature. Omega-3 fatty acids are not commonly found in high amounts in a typical livestock diet, but can be supplemented with products like flaxseed oil and fish oil. Marine-derived supplements, like fish

oil, are rich in two of the most commonly supplemented n-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Once digested, n-3 fatty acids produce series 3 eicosanoids which are anti-inflammatory in nature. Supplementing n-3 fatty acids can benefit many systems in the body, including the cardiovascular, skeletal, nervous, immune, and reproductive systems (Simopoulos, 1991; Pike and Barlow, 2000; Simopoulos, 2002; Dunnett, 2005; Robinson and Stone, 2006).

Omega-3 fatty acids have received increasing attention in the equine industry, primarily due to their anti-inflammatory properties (Calder, 2002; Munsterman et al., 2005; Skjolaas-Wilson et al., 2005). In stallion reproduction, n-3 fatty acids have been investigated in relation to improving spermatogenesis and cooled semen properties (Brinsko et al., 2005; Harris et al., 2005). In the mare, research has been focused on supplementation of the foal through mare n-3 dietary supplementation (Kruglik et al., 2005; Skjolaas-Wilson et al., 2005; Poland, 2006). During the postpartum estrous period, mares receiving EPA and DHA in the form of fish oil had an increase in time from foaling to ovulation in the EPA and DHA supplemented group when compared to the control group and a group receiving DHA alone (Poland, 2006). The EPA and DHA supplemented mares also held a preovulatory follicle significantly longer prior to ovulation than the other two groups (Poland, 2006).

The current study was designed to further explore Poland's findings regarding an n-3 effect on mare cyclicity. The objectives of this study were to determine the effects of feeding a marine-derived n-3 fatty acid supplement on plasma fatty acid profiles as well as several reproductive factors during the estrus periods of healthy, cycling mares. To this author's knowledge, there have been no such studies evaluating the reproductive effects of n-3 fatty acid supplementation in non-pregnant mares during the natural breeding season.

CHAPTER 1 - GENERAL REVIEW OF LITERATURE

Introduction

Nutrition and reproduction have always been integrally linked in that the reproductive success of an animal depends on its nutritional status. Throughout the years this link has been explored in research, often by altering diets in various ways and observing the resulting changes in reproductive parameters. One of the most significant dietary changes that can be made to influence the reproductive system is the addition of fat to the diet. Several studies on reproductive activity of fat-supplemented cattle demonstrated an increase in diameter and number of follicles present on the ovary, as well as a shorter period to the first postpartum ovulation (Hightshoe et al., 1991; Lucy et al., 1992; Thomas and Williams, 1996). In addition, fat supplementation has also been shown to affect milk composition and fertility in several farm animal species (Ashes et al., 1992; Encinias et al., 2004; Mattos et al., 2000). Originally, it was believed that the improvement in reproduction due to the addition of fat was solely a result of an increase in energy availability to the animal. However, it soon became apparent that individual fatty acids themselves can play a role in influencing reproductive parameters.

Fatty acids are defined as molecules consisting of a hydrocarbon chain with a methyl group at the omega end and a carboxylic group at the alpha end. Fatty acids can be characterized by their structure in many ways. Fatty acids with the hydrocarbon chain having the maximum number of carbon to hydrogen (C-H) bonds and no double bonds present are characterized as saturated fatty acids. Those fatty acids that do not have the maximum number of C-H bonds but have double bonds are considered unsaturated. Furthermore, those unsaturated fatty acids that have more than one double bond present within the hydrocarbon chain are considered polyunsaturated fatty acids (PUFAs).

Polyunsaturated fatty acids can be separated again into omega-3 fatty acids (n-3), omega-6 fatty acids (n-6), or omega-9 fatty acids (n-9). Omega-3 and omega-6 fatty acids are considered to be essential fatty acids because mammals do not have the ability to synthesize them in vivo. Omega-3 fatty acids have a double bond present between the third and fourth carbon from the omega end of the molecule, while omega-6 fatty acids have a double bond present between the sixth and seventh carbon from the omega end of the molecule.

Polyunsaturated fatty acids are a special group of fatty acids because, not only are they essential in the diet, but they are also considered "healthy fats". Omega-3 fatty acids and omega-6 fatty acids must be ingested by mammals due to the inability of mammalian desaturase enzymes to act on fatty acids at a position greater than the ninth carbon from the alpha end. Because of their inability to synthesize these fatty acids de novo, n-3 and n-6 fatty acids are considered essential fatty acids and are vital for many functions including growth, reproduction, vision, and brain development (Gurr et al., 2002). They are also integral elements of phospholipids and cell membranes (Kinsella, 1991). Additionally, PUFAs also have the ability to affect steroidogenesis and some transcription factors controlling gene expression (Wathes et al., 2007).

Typically, PUFAs are naturally consumed by the horse and other livestock in two varieties, alpha-linolenic acid (ALA) and linoleic acid (LA). Linoleic acid (C18:2 n-6) is by far the most prevalent polyunsaturated fatty acid consumed by the modern horse and is provided by many vegetable oils as well as cereal grains. Alpha-linolenic acid (C18:3 n-3) is an n-3 fatty acid plentiful in chloroplasts of plants and can be found in flaxseed or linseed oil, as well as fresh, leafy forage.

In western societies, n-6 fatty acids are more predominant in the diet than n-3s, resulting in a much higher n-6 to n-3 ratio than the 5:1 ratio recommended for humans (Newton, 2001). Equine diets are similar in that feedstuffs high in n-6 fatty acids, such as many cereal grains and vegetable oils, are more commonly fed. The practice of feeding preserved forage versus fresh pasture has also decreased the amount of n-3s naturally consumed. Excess n-6 fatty acids present in the diet has been documented in several species to lead to issues such as cardiovascular problems and inflammation, so feedstuffs high in n-3 fatty acids, such as fish oil and flaxseed, have recently been considered as a way to lower the n-6 to n-3 ratio and achieve a more ideal fatty acid balance (Robinson and Stone, 2006).

Once consumed and digested, LA and ALA travel to the liver and undergo enzymatic desaturation and elongation via n-6 and n-3 fatty acid pathways, respectively (Gurr et al., 2002). Both pathways utilize the same enzymes, resulting in competition between the two pathways, which affects the amount of intermediates and products produced (Figure 1.1, adapted from Bezard, et al., 1994). Delta-6 desaturation enzyme is the first to manipulate the precursor fatty acids, with LA being converted into gamma-linoleic acid (GLA) and ALA transformed into

stearidonic acid. This first step is rate-limiting in both pathways, although delta-6 desaturation enzyme is preferential to the n-3 pathway substrate, ALA. This preference can shift production to favor n-3 pathway products if enough precursor is present (Bezard et al., 1994).

Next, elongation takes place, converting GLA and stearidonic acid into dihomo-gamma-linolenic acid (DGLA) and eicosatetraenoic acid (ETA), respectively. Delta-5 desaturase then proceeds to transform DGLA to arachidonic acid (ARA) in the n-6 pathway and ETA to eicosapentaenoic acid (EPA) in the n-3 pathway. Eicosapentaenoic acid can also undergo another desaturation and elongation reaction to produce docosahexaenoic acid (DHA) via the intermediate docosapentaenoic acid (DPA). The EPA to DHA conversion is reversible, which allows DHA to be converted back to EPA if needed. Once produced from the n-6 or n-3 pathway (or possibly obtained from the diet in the case of ARA), DGLA, ARA, and EPA can enter other cascades resulting in the production of eicosanoids, including prostaglandins, thromboxanes, prostacyclines, and leukotrienes (Bezard et al., 1994).

Omega-3 Pathway   **Omega-6 pathway**
α-linolenic acid   Linoleic acid
(18:3)   Δ6 desaturation   (18:2)
Steridonic acid   γ-linolenic acid
(18:4)   Elongation   (18:3)
Eicosatetraenoic   Dihomo-γ-linolenic
acid (20:4)   Δ5 desaturation   acid (20:3)
Eicosapentaenoic   Arachidonic acid
acid (20:5)   (20:4)
                Elongation
                Retroconversion
Docosapentaenoic   Docosatetraenoic
acid (22:5)   Δ4 desaturation   acid (22:4)
                Retroconversion
Docosahexaenoic   Docosapentaenoic
acid (22:6)   acid (22:5)

Figure 1.1. Synthesis of n-3 and n-6 fatty acids via enzymatic pathways.

A more efficient way of supplementing with n-3 fatty acids is to provide the supplementation in the form of DHA and EPA in the diet. This allows for more rapid absorption into the plasma and tissues due to the omission of the rate limiting step in both pathways in which precursors ALA and LA are converted to GLA and stearidonic acid (Baur, 1994). Eicosapentaenoic acid and DHA are naturally found in marine based sources, especially fatty fish such as salmon and herring, because their diet of phytoplankton is rich in DHA and EPA (Popp-Snijders et al., 1986). Therefore, fish oil is a supplement that is high in DHA and EPA.

Dietary supplementation of n-3 fatty acids results in a gradual inclusion of n-3s into tissues and membranes while simultaneously displacing n-6 fatty acids present (Trujillo and Broughton, 1995; Wathes et al., 2007). This incorporation of n-3s is in direct proportion to the amount consumed by the animal. For example, feeding a diet rich in n-3 fatty acids to rats for three wk resulted in a 50% replacement of n-6 with n-3 fatty acids in rat uterine phospholipids (Trujillo and Broughton, 1995). Recent research has indicated that incorporation of n-3 fatty acids into equine blood plasma and tissues occurs at a faster rate than in humans, with peak concentrations being achieved as quickly as 7 d post-supplementation (Arterburn et al., 2006; King et al., 2008). This indicates that horses are capable of consuming and integrating n-3 fatty acids into their system quickly and effectively. However, the need for supplementation in the horse is still being elucidated and consequently, the ideal level of supplementation in horses has not yet been determined.

Supplementing omega-3 fatty acids into the diet can have many positive health benefits and affect various systems throughout the body, including cardiovascular, skeletal, immune, and reproductive systems. Supplementation in humans has led to increased vascular compliance as well as anti-thrombotic and anti-atherosclerotic effects (Robinson and Stone, 2006). A human study in 1998 also documented omega-3 fatty acids' ability to suppress cytokine mediated aspects of inflammation (Grimble, 1998). Omega-3 supplementation has also shown promise in treating renal disease, skin irritation, autoimmune disease, and possibly cancer in domestic animals (Baur, 1994). Prenatally, n-3 fatty acids, especially DHA, also play an essential role in development of the fetus. Brain development and visual acuity of human babies have been documented to be affected by maternal supplementation during pregnancy (Eilander et al., 2007).

Omega-3 supplementation has increased trot stride length in horses when compared to a corn-oil fed control group (Woodward et al., 2007). Exercising horses also displayed a

decreased heart rate when supplemented with n-3 fatty acids (O'Connor et al., 2004). Researchers have shown that inclusion of omega-3 supplements in the diets of horses resulted in a decrease in TNFα production, which is a major inducer of inflammation (McCann et al., 2000; Dinnetz, 2009). A reduced cortisol secretion in n-3 supplemented horses in response to transport stress has also been observed (King et al., 2009). Based on these findings, n-3 supplements may be able to decrease stress, increase stamina and serve as a possible alternative therapy for various inflammatory conditions, such as arthritis and developmental orthopedic disease, in the horse as well as other livestock species. However, the exciting potential of n-3 supplementation shown in human studies cannot be extrapolated into the equine without further research.

Follicular Development and Hormonal Changes in the Cycling Mare

The mare is classified as a seasonally polyestrus, mono-ovulatory species and typically ovulates a single preovulatory follicle during regular intervals throughout its designated breeding period. As a seasonal breeder, the mare is anovulatory during the winter and fall months and becomes reproductively active during the spring and summer months (Ginther, 1992a). Increasing day length causes the mare's pineal gland to produce less melatonin, thus stimulating endocrine activity and initiation of estrous cycles. The mare's estrous cycle is 21 days long and consists of four stages: proestrus, estrus, metestrus, and diestrus (Palmer, 1978). The proestrus and estrus stages compose the follicular phase, while metestrus and diestrus are components of the luteal phase.

The duration of estrus is an average of 5 to 7 days during which the mare is physically and behaviorally receptive to breeding. Various hormonal changes take place at this time to finish the growth and maturation of the preovulatory follicle as well as prepare the follicle for ovulation. The oocyte is then ovulated from the ovulation fossa approximately 24 to 48 hours prior to the end of estrus, where it then travels through the oviduct to either be fertilized or degenerate (Ginther, 1992b).

Endocrine Activity of the Estrous Cycle

The hypothalamo-pituitary-gonadal (HPG) axis is responsible for hormonal regulation of the reproductive cycle. This axis consists of 3 major organs; the hypothalamus, the pituitary, and the gonads. In a normal estrous cycle, the hypothalamus secretes gonadotropin releasing hormone (GnRH) from neurons in the ventromedial and arcuate nuclei (Senger, 2003).

Gonadotropin releasing hormone travels to the anterior pituitary to cause release of gonadotropins from gonadotrope cells. Gonadotropins then travel to the ovaries where they affect the tissues and create a response. Throughout the HPG axis, positive and negative feedback mechanisms are in place to control endocrine activity during the estrous cycle. The major hormones involved in the estrous cycle of the mare are described in detail below.

Luteinizing Hormone

Luteinizing hormone (LH) is a gonadotropin that is produced and released by the gonadotropes of the anterior pituitary due to stimulation by GnRH. During the anovulatory season, low basal concentrations can be found in the circulation due to progesterone's inhibitory effect on LH (Hafez, 1980). However, during the breeding season an increasing pulsatile release of GnRH from the hypothalamus allows for increased production and release of LH. Unlike other livestock species, no ovulatory surge of LH occurs in the mare. Instead, a progressive increase in LH concentrations occurs over many days (Irvine and Alexander, 1994). Luteinizing hormone travels through the circulation to the ovaries where it binds to available receptors to increase estradiol secretion and assist with maturation of follicles. Levels of LH continue to increase throughout estrus and reach a maximum concentration one to two days after ovulation (Palmer, 1978). This prolonged high level of LH is responsible for initiating the ovulatory process.

Follicle Stimulating Hormone

Follicle stimulating hormone (FSH) is a gonadotropin that, in conjunction with LH, is responsible for follicular growth and development through the proestrus and estrus period of the estrous cycle. Follicle stimulating hormone is regulated by a dual mechanism that independently controls its basal secretion and pulsatile secretion (Padmanabhan et al., 1997). Pulsatile FSH secretion occurs during proestrus and estrus phases of the estrous cycle. The first peak in FSH secretion occurs when the largest follicle is approximately 13 mm in diameter, stimulating the growth of the follicles in the follicular wave (Donadeu and Ginther, 2001). Concentrations of FSH then decline and are suppressed by inhibin produced by the growing follicles until another surge of FSH occurs at the time of ovulation (Hafez, 1980). Follicle stimulating hormone travels through the circulation to the ovaries where it binds to available receptors and stimulates estradiol production and development of follicles.

Estradiol

Estradiol is the major steroid hormone that is the driving force behind estrus behavior and physiological changes in the reproductive tract associated with estrus. Estradiol is produced in the granulosa cells of the follicle under the influence of LH and FSH, and travels through the circulation to its receptor sites throughout the body. Estradiol also exerts positive feedback on GnRH production and the hypothalamus, continually stimulating the release of LH from the anterior pituitary. Although GnRH is also responsible for the synthesis and release of FSH, the growing follicles prevent the increase of FSH concentrations at this time. As they grow, follicles produce inhibin which negatively feeds back on FSH receptors in the pituitary (Senger, 2003). High levels of estradiol in later stages of estrus ensure that LH is primarily responsible for maturation in the later stages of estrus, while FSH serves its role in earlier follicular development when estradiol concentrations are lower.

Estradiol has several receptors throughout the reproductive tract and high estradiol concentrations during estrus result in physiological changes such as hyperemia, relaxation of the cervix, and increases in uterine gland secretions. High concentrations are also directly associated with estrus behavior such as winking of the vulva, receptiveness to the stallion, and frequent urination. Estradiol begins to increase the day prior to follicular deviation and concentrations peak just prior to ovulation (Ginther et al., 2001). During diestrus, follicle development is subdued, but not eliminated, due to basal secretions of FSH (Padmanabhan et al., 1997). Lower FSH concentrations and high progesterone levels prevent the follicles from developing to tertiary follicles and resultant estradiol production is low.

Progesterone

Progesterone is the dominant steroid hormone during the diestrus phase. After ovulation, granulosa and theca cells of the follicle undergo luteinization, form a corpus luteum (CL), and secrete progesterone. Progesterone reaches a maximum about 6 d after ovulation, with concentrations in circulation reaching 10 ng/ml (Senger, 2003). Progesterone has a negative feedback on GnRH production, resulting in minimal LH being secreted. Basal concentrations of FSH are still being secreted, but high progesterone levels do not allow for follicles to mature enough to provide threshold estradiol levels needed for positive feedback on the hypothalamus and anterior pituitary (Senger, 2003).

While under the influence of progesterone, contractions of the myometrium are lessened, uterine secretions to promote conceptus survivability are initiated, and the cervix is rigid and closed. The mare is not receptive to mating and does not show estrus behavior. At the end of diestrus, the CL undergoes luteolysis and progesterone production decreases, resulting in removal of the negative feedback loop to the hypothalamus. As a result, GnRH pulsatile production begins to increase and the follicular phase begins (Ginther, 1992b). Typical progesterone serum concentrations during this period are less than 1 ng/ml or often undetectable (Ginther, 1992b; Amer et al., 2008).

Prostaglandins

Prostaglandins have many functions throughout the body. However, during estrus prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin-E_2 (PGE_2) are the most essential prostaglandins for a normal ovulatory process. Originating from arachidonic acid, PGE_2 and $PGF_{2\alpha}$ production begins to rise in follicular fluid prior to ovulation, promoting inflammation and increased blood flow to the ovary, and aiding in follicular rupture (Espey, 1980; Watson and Hinrichs, 1988; Murdoch and Gottsch, 2003). Prostaglandin-$F_{2\alpha}$ also plays a role in luteolysis of the corpus luteum (CL) at the end of diestrus by altering blood flow to the CL as well as blocking LH's ability to bind to its receptor on the luteal cells. Prostaglandins will be discussed in more detail in a later section.

Insulin-Like Growth Factor 1

Insulin-like growth factor 1 (IGF-1) is a hormone secreted primarily from the liver, involved in the somatotrophic axis, and is also thought to influence follicular growth and ovulation (Daftary and Gore, 2005). Although examining IGF-1's role in equine reproductive activity is still relatively new, support for IGF-1 involvement has been documented by several researchers. It has recently been found to be produced in equine granulosa cells, and insulin can inhibit its production (Davidson et al., 2002). Insulin-like growth factor 1 has been noted to positively affect LH and FSH activity on ovarian tissues in several species, although the mechanism has not yet been elucidated (Spicer and Echternkamp, 1995). Mitosis of granulosa cells during follicular development, androgen production from LH-induced theca cells, and estradiol production are all enhanced by IGF-1. Insulin-like growth factor 1 is also thought to play a role in follicular deviation and dominance by allowing the future dominant follicle to

develop despite limited FSH availability (Ginther et al., 2001; Ginther et al., 2008). Previous research has documented that free IGF-1 concentrations in large follicles increased in mares during the follicular phase and was simultaneous with an increasing estradiol:progesterone ratio (Spicer et al., 2005). Additionally, a recent study revealed that the IGF-1 system plays a crucial role in follicular deviation without affecting steroid or gonadotropin hormone concentrations (Ginther et al., 2008). The growing evidence suggests that IGF-1 and insulin-like growth factor binding proteins (IGFBPs) play a role in many aspects of follicular development and ovulation.

Follicular Development in the Mare

Follicle development begins with the emergence of follicles, which are usually represented as follicles 6 mm in diameter in mares (Ginther, 2000). After emergence, the follicles enter a common-growth phase in which all follicles grow at the same rate and develop granulose cell layers with FSH receptors and theca cell layers with LH receptors. The accumulation of receptors allows follicles to become sensitive to gonadotropin hormone release from the anterior pituitary and undergo further maturation. These follicles go on to develop an antrum and begin to display steroidogenic activity as tertiary follicles. The theca cells, under stimulation of LH, convert circulating cholesterol into androgens, androstenedione, and testosterone through a second messenger system and enzymatic stimulation. Testosterone is then transported by a carrier protein to the granulosa cells where, under stimulation of FSH, a second messenger system activates aromatase to convert testosterone into estradiol. Due to increasing concentrations, estradiol begins a positive feedback loop to the neurons of the hypothalamus and allows further development of tertiary follicles through LH stimulation, including granulosa cells accumulating LH receptors.

After the common growth phase, which lasts approximately 6 days in the mare, the deviation process is initiated when the most developed tertiary follicle reaches a mean of 13 mm in diameter and begins to secrete inhibin (Ginther, 2000). Inhibin serves to negatively feedback on FSH production at the anterior pituitary, resulting in lower FSH concentrations. The decreasing FSH causes all smaller follicles to slow growth and eventually regress, but the largest follicle is able to continue development in spite of low FSH concentrations due to its increased gonadotropin sensitivity (Donadeu and Ginther, 2002). This deviation in size and development occurs when the largest follicle reaches 21 to 23 mm in diameter, effectively separating the

dominant follicle from the subordinant follicles. The dominant follicle, under increasing estradiol and LH concentrations, undergoes further maturation and becomes a preovulatory follicle that is capable of releasing a viable oocyte that can be fertilized and result in a pregnancy. Preovulatory follicles continue to increase in size in the final 6 days prior to ovulation (Hafez, 1980). The preovulatory follicle can vary from 30 mm to 70 mm in diameter, with an average diameter of 40 to 45 mm at ovulation (Blanchard et al., 1998).

Composition of Follicular Fluid

Follicular fluid is composed of serum components as well as locally secreted factors, such as hormones, which play a part in the metabolic activity of follicular cells (Gerard et al., 2002) Follicular fluid is present in the antrum of a tertiary follicle and its content varies with the stage of estrous and the size of the follicle. Gerard et al. (1999) noted that growth of an equine dominant follicle early in estrus is associated with a significant increase in intrafollicular estradiol and progesterone concentrations as well as a decrease in some IGFBPs. During the final maturation of the preovulatory follicle just prior to ovulation, a slight decrease in intrafollicular estradiol concentrations occur along with a continued increase in progesterone concentrations. Furthermore, the presence of an unknown 200,000 molecular weight protein was also detected (Gerard et al., 1999). In a follow-up study using proton nuclear magnetic resonance, equine follicular fluid was found to be comprised of glycoconjugates, lipoproteins, glucose metabolites, amino acids, creatine, and polyamines (Gerard et al., 2002). Follicle maturation was indicated by a decrease in glycoconjucates, trimethylamines, acetate, and estradiol with a subsequent increase in CH_3 groups of lipoproteins and progesterone (Gerard et al., 2002). Increased intrafollicular IGF-1 and changes in IGFBP concentrations have also been shown to be associated with increased estradiol, progesterone, and androstenedione concentrations in developing mare follicles (Bridges et al., 2002; Davidson et al., 2002; Spicer et al., 2005). When compared to other mammals, the mare possesses unique differences in conditions of ovulation and oocyte maturation. For example, the mare has a low in vitro maturation rate when compared other domestic species, which can often exceed 90% (Goudet et al., 1997a). Therefore, it is speculated that equine follicular fluid has species-specific factors that are yet to be determined (Gerard et al., 1999).

The Ovulatory Process in the Mare

Ovulation can be characterized by three main processes: a shift from estradiol to progesterone production, increased follicular pressure, and breakdown of the connective tissue of the follicular wall. The transition from estradiol to progesterone production occurs immediately prior to ovulation. In response to threshold levels of LH, follicular theca interna cells begin to produce progesterone instead of testosterone. This production leads to a slight decrease in estradiol production as well as stimulates the theca interna cells to synthesis collagenase enzymes which will aid in the breakdown of the follicular wall (Senger, 2003).

The preovulatory rise in follicular prostaglandin concentrations in response to increasing LH is necessary for ovulation to occur (Satoh et al., 1985). Increases in PGE_2 result in hyperemia and edema of ovarian tissue, while increasing levels of histamine at the site increase vascular permeability and promote swelling. The increase in blood flow aids the preovulatory follicle in obtaining the hormonal and metabolic factors needed for final maturation. Additionally, a rise in $PGF_{2\alpha}$ leads to smooth muscle contraction of the ovary which, along with PGE_2 and histamine, increases follicular pressure inside the preovulatory follicle (Senger, 2003).

Breakdown of the follicular wall through enzymatic degradation is also aided by prostaglandins. While stimulating ovarian contractions, $PGF_{2\alpha}$ also causes lysosomes in granulosa cells to rupture and release enzymatic components to aid in wall degradation. However, PGE_2 plays a larger role in connective tissue breakdown by activating epithelial cells to produce urokinase-type plasminogen activator (uPA). Plasminogen from serum is then converted to plasmin by the uPA enzyme. Plasmin proceeds to activate collagenases, which break down collagen in the follicular wall, and free tumor necrosis factor-α (TNFα), which results in apoptosis of epithelial cells. Collectively, these events degrade the follicle wall (Murdoch and Gottsch, 2003).

After tissue breakdown and follicular pressure reach a threshold level, ovulation takes place and the ovum is released by way of the ovulation fossa. The evacuated follicle becomes irregularly shaped, follicular cells mix, and local hemorrhaging occurs due to ruptured blood vessels (Senger, 2003). At this stage, the newly ovulated follicle is known as a corpus hemorrhagicum. The CL is formed when granulosa and theca cells are luteinized into large and small luteal cells and begin to produce large amounts of progesterone, initiating the luteal phase of the estrous cycle.

Effect of PUFAs on Reproduction

Polyunsaturated fatty acids affect reproduction primarily through their role in the formation of eicosanoids, especially prostaglandins (Abayasekara and Wathes, 1999a). The most biologically active group of prostaglandins are series 2 prostaglandins which originate from ARA through the cyclooxygenase pathway (see Figure 1.2). Within this pathway, the cyclooxygenase-2 (COX-2) enzyme converts ARA to prostaglandin H_2, which is the universal precursor for all series 2 prostaglandins. The most common and reproductively relevant series 2 prostaglandins are $PGF_{2\alpha}$ and PGE_2, both of which are pro-inflammatory and essential in the ovulatory process. Series 1 prostaglandins, such as PGE_1 and $PGF_{1\alpha}$, are synthesized from DGLA and also have a pro-inflammatory action (Heravi Moussavi et al., 2007).

Supplementation of n-3s results in a shift of production from pro-inflammatory eicosanoids associated with n-6 fatty acids DGLA and ARA, to anti-inflammatory eicosanoids associated with EPA (Calder, 2002). Series 3 prostaglandins, such as PGE_3 and $PGF_{3\alpha}$, are synthesized from EPA and counteract the inflammatory process. Eicosapentaenoic acid also acts as a potent inhibitor of the COX-2 enzyme, effectively reducing the amount of pro-inflammatory prostaglandins being produced, and competes for the same receptor site as ARA on target cells (Calder, 2002). In a 1998 study, rat hepatoma cells were subjected to EPA derivatives in vitro, resulting in a decreased expression of the COX-2 gene, thus inhibiting series 2 prostaglandin production (Larsen et al., 1998). Similar changes were also found in a 2002 study that reported dietary supplementation of n-3 in the form of sardine oil resulted in decreased production of $PGF_{1\alpha}$ and PGE_2 in mice (Broughton and Wade, 2002). Competition between EPA and ARA for mutual enzymes results in a shift of production to the more available substrate.
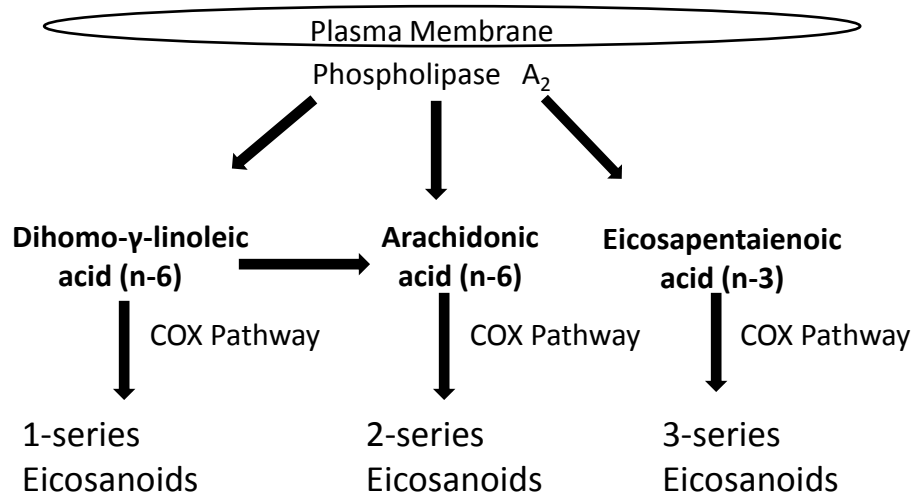
Figure 1.2. Omega-3 and omega-6 substrates for eicosanoid production via the cyclooxygenase (COX) pathway.

PUFA Supplementation and Female Reproduction

When supplemented in the diet, n-3 concentrations in circulation, plasma membranes, and tissues are increased and production of anti-inflammatory prostaglandins is favored, which has the potential to seriously impact female reproduction. Many studies have reported effects of dietary n-3 supplementation on prostaglandin production and subsequent changes in reproductive function. Rats fed n-3s had a higher ovulation rate than those fed with n-6s, which the authors speculated was due to the observed decrease in production of PGE_2 coupled with an increase in production of PGE_3 (Trujillo and Broughton, 1995). A decrease in $PGF_{2\alpha}$ metabolite was also found in response to estradiol and oxytocin injections given 15 d after synchronization in dairy cattle supplemented with fish meal versus dairy cattle not supplemented (Mattos et al., 2002). In a follow-up study, Mattos et al. (2003) exposed bovine endometrial cells to EPA and DHA in vitro, which resulted in inhibition of $PGF_{2\alpha}$. Feeding lactating dairy cows fish meal containing EPA and DHA at 5.4% dietary dry matter also inhibited uterine $PGF_{2\alpha}$ secretion (Thatcher et al., 1997). However, a comparable study disputes Thatcher's and Mattos' results, finding no effect

on $PGF_{2\alpha}$ or COX-2 production after supplementation of dairy cattle with n-3 fatty acids (Heravi Moussavi et al., 2007).

Research has also documented that n-6 supplementation yields lower prostaglandin production. Endometrial samples from n-6 supplemented cows revealed lower $PGF_{2\alpha}$ and PGE_2 levels than n-3 supplemented or non supplemented cows (Cheng et al., 2001). In a follow up study, ewe endometrial cells cultured with n-6 fatty acids and linoleic acid resulted in a lowered production of $PGF_{2\alpha}$ (Cheng et al., 2004). The conflicting literature suggests that many factors may be involved in prostaglandin production's response to fatty acid supplementation and further research is needed to elucidate those factors.

Polyunsaturated fatty acids can affect a wide range of other reproductive parameters across species, including gestation length, lactation, fertility, and development. Recently, direct supplementation of EPA and DHA has gained quite a lot of attention commercially due to their inclusion in many human products. These products mainly target infants and expectant mothers, due to EPA and DHA's reported benefits on early neonatal development and cognition (Kinsella, 1991; Eilander et al., 2007). Birch and others documented significant improvements in visual acuity and visual stereo-acuity of infants fed formula supplemented with PUFAs (Birch et al., 2002; Birch et al., 2005). Pregnant women who consume diets high in n-3 PUFAs, such as Faroese women and Greenland Eskimos, have longer gestation lengths and increased birth weights (Olsen et al., 1991). This occurrence was also noted in 2001, as sows supplemented with salmon oil immediately post service to weaning had a longer gestation length and a lower preweaning mortality when compared to sows being fed a standard commercial diet (Rooke et al., 2001). It has been suggested that the increased pregnancy rates noted with n-3 supplemented diets may be a result of decreased $PGF_{2\alpha}$ secretion and a decreased sensitivity of the CL to $PGF_{2\alpha}$ (Mattos et al., 2000). A 2005 study in ewes supported this claim, as feeding a diet high in n-3 fatty acids resulted in fewer peaks and a lower overall concentration of a $PGF_{2\alpha}$ metabolite measured in plasma on d 14 of the estrous cycle, which may result in delayed regression of the CL (Naddafy et al., 2005). In another study, beef cows supplemented with rumen-protected PUFAs displayed greater pregnancy rates and greater mean serum progesterone concentrations than non-supplemented controls (Lopes et al., 2009). Evidence also points to an improvement in oocyte quality with supplementation of PUFAs. In 2001, varying PUFA content in bovine oocytes was shown to enhance quality and maturation ability (Kim et al., 2001).

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Supplementation of ewes with calcium soaps of fish oil resulted in an increased number, quality, and chilling resistance of oocytes (Zeron et al., 2002).

Alterations in follicular dynamics due to supplementation of EPA and DHA are controversial with conflicting results reported in the literature. In some cattle studies, supplementation of PUFAs resulted in an increase in follicular number as well as the size of the dominant follicle (Beam and Butler, 1997; Robinson et al., 2002a; Bilby et al., 2006) whereas others found no such distinction (Petit et al., 2002; Petit et al., 2004). Gilts fed a dietary n-3 supplement showed no difference in ovulation rate or litter size when compared with controls (Estienne et al., 2006), but gilts fed a protected n-3 fatty acid source 30 d or more prior to breeding produced 1 more piglet than controls at farrowing (Spencer et al., 2004). Sows fed the same protected n-3 fatty acid source 35 d or more prior to breeding had more live embryos at 35 d of gestation compared to control sows (Webel et al., 2004). These discrepancies may be the result of species differences, source and amount of PUFAs being supplemented, feeding duration, or a combination of these factors.

PUFAs and Male Reproduction

Unsaturated fatty acids are necessary components in the spermatozoa in human, livestock, and avian species due to their role in providing fluidity to the sperm plasma membrane. However, too high of inclusion of PUFAs in spermatozoa increases the risk of oxidation and peroxidative damage (Wathes et al., 2007). In male reproduction, research findings on PUFAs' effect on sperm quality and fertility have been mixed and limited. A study in turkeys discovered that reproductive capacity, defined as fertility, embryo viability, and hatching rate, was increased with n-3 supplementation. Benefits were further increased with turkey age, increasing hatching rates by 2 points at 48 to 58 weeks in supplemented males (Blesbois et al., 2004). Supplementing boars with 3% fish oil in the diet resulted in an increased number of sperm in the ejaculate but did not alter freezability in one study (Maldjian et al., 2005), while another study revealed no improvement in nonreturn rates to artificial insemination with semen from boars supplemented with cod liver oil compared to semen from non-supplemented control boars (Paulenz et al., 1995). A recent study revealed that boars supplemented with dietary n-3 fatty acids increased ejaculation duration and sperm per ejaculate when compared with corn-supplemented controls (Estienne et al., 2008). More research in this

area is needed to confirm the value of supplementation on male reproductive health and spermatogenesis.

PUFAs and Equine Reproduction

Omega-3 fatty acid research has received increasing attention in the equine industry, specifically due to their anti-inflammatory properties. In the mare, reproductive research involving n-3 supplementation has been limited. Lactating mares fed a marine-based source of n-3s showed increased levels of EPA and DHA in milk (Kruglik et al., 2005). Further research from the same laboratory documented the effects of fish oil supplementation on mares supplemented 60 d prior to the expected foaling date until the second postpartum estrus (Poland et al., 2006). Poland et al. (2006) found that supplemented mares had the ability to transfer dietary n-3 fatty acids to their foals in utero as well as through milk after the foal is born. This study also indicated that EPA supplementation may play a role in altering follicular dynamics by delaying ovulation post-partum. A 2009 study evaluated marine-derived n-3 supplementation's effects on reproductive factors of quarter horse and miniature mares. No difference among treatments or horse size was noted in number of estrous cycles, but the n-3 supplemented mares showed a tendency for greater serum progesterone concentrations across estrous cycles when compared to mares fed animal fat (Furtney et al., 2009).

In stallions, studies have been centered on PUFA supplementation and improvements in sperm quality and fertility, with special attention being given to cryopreservation and cooling techniques. A University of Arizona study paired 6 stallions by semen characteristics and allocated them to either a control diet or an n-3 supplemented diet (Harris et al., 2005). Researchers found that in n-3 supplemented stallions, lipid plasma concentrations increased by d 19 of supplementation and remained elevated throughout d 90 of the feeding trial. Furthermore, supplemented stallions increased daily spermatozoa output whereas control stallions remained constant. However, no differences where found between groups in motility characteristics or cryopreservation tests (Harris et al., 2005). Another study supplemented 4 stallions with 250 g/d of a DHA-enriched nutriceutical for 14 wk and compared semen quality to their 4 contemporaries fed a control diet (Brinsko et al., 2005). Researchers determined that supplemented stallions exhibited increases in overall sperm motility, velocity, and projectory after 48 hr of cooled storage. It was also noted that a subset of stallions with lower than average

progressive sperm motility (less than 40%) after 24 hr of cooled storage showed improvements in mean progressive motility after 24 hr when fed the DHA supplement. This finding suggests that stallions with poor motility or sperm production may benefit the most from supplementation (Brinsko et al., 2005). In contrast, a Texas A&M University study reported that spermatozoa characteristics from miniature stallions were not affected by dietary supplementation of 150g/d fish oil or 60 g/d flaxseed and algae-based supplement for 84 d (Grady et al., 2009).

Transvaginal Ultrasound Guided Aspiration

Transvaginal ultrasound-guided follicle aspiration (TUGA) is a relatively safe and non-invasive procedure commonly performed to collect follicular fluid and/or retrieve oocytes for oocyte transfer. The procedure involves the manipulation of the ovary through rectal palpation. A needle is then guided through the vaginal wall and into the follicle using a transducer inserted vaginally. Factors that have been show to impact the success of the TUGA procedure include hormone treatment, follicle size, aspiration frequency, breed and reproductive state, and needle size (Squires and Cook, 1996). Although size of needle, ultrasound equipment, and usage of antibiotics and sedatives can vary within the procedure, the basic protocol holds constant for using TUGA in mares. Before initiating the procedure, the mare is often given a rectal relaxer, such as propantheline bromide, and is sedated with intravenous drugs such as xylazine and butorphanol tartrate (Cook et al., 1992; Carnevale and Ginther, 1993). The perineal region is then scrubbed with a disinfectant and an ultrasound probe is lubricated and inserted into the mare's vagina with the transducer facing dorsally. Through transrectal manipulation, the ovary is placed and stabilized against the vaginal wall over the face of the transducer. Due to the large vessels and arteries present in the broad ligament and pelvic region, care must be taken to position the ovary anterior to the broad ligament before maneuvering it to a central and caudal position in the mare for aspiration. The mare's ovary is held within the hand that is palpating rectally and positioned between the bevel of the needle and the abdominal blood vessels (Carnevale, 2008). A needle is then inserted through the vaginal wall and directly into the antrum of the follicle. Follicular fluid is then aspirated using a vacuum pump (Gerard et al., 2004) or a syringe. The time from puncture to follicle evacuation averages 3 min (Carnevale and Ginther, 1993).

Since the 1980s, TUGA has been used for aspiration of follicles and collection of oocytes in both humans and cattle (Pieterse et al., 1988; Cook et al., 1992). The first report of TUGA being performed in horses was in 1992 using a 6-MHz transducer and a single-lumen needle. In this study, four aspirations were performed on preovulatory follicles 20 to 30 hr after hCG was given (Bruck et al., 1992). In another study, three different aspiration techniques were examined to determine the viability of using TUGA for routine oocyte collection in both diestrus and estrus phases (Cook et al., 1992). The first technique used a 12 gauge single lumen needle with repetitive filling and evacuation of the follicle through flushing. The second technique was identical to the first except a wire loop device was attached to the needle and rotated to remove cumulus cells. Technique three involved a 12 gauge double-lumen needle which was used to continuously rinse the follicle for 2 to 4 min via a pressurized IV fluid system. Researchers found technique three to be the most successful at oocyte recovery (84%) from preovulatory mares during estrus and validated TUGA as a safe and effective technique for oocyte recovery (Cook et al., 1992).

Although the incidence of complications is low, some problems and potential hazards are to be noted with TUGA. A small amount of blood is often present in the follicular fluid collected due to the disruption of the vascular follicular wall, but a large amount could indicate internal hemorrhaging or other complications (Carnevale, 2008). In one isolated documented case, severe internal hemorrhage occurred after performing the TUGA procedure on a mare (Vanderwall and Woods, 2002). Other less severe complications that have been recorded include severe ileus, treatable peritonitis, and mild cases of colic (Carnevale, 2008).

No significant effects on fertility have been reported when mares were inseminated after several repeated TUGA procedures, as loss of follicular fluid does not appear to affect establishment of pregnancy (Mari et al., 2005; Vanderwall et al., 2006). Cook (1995) conducted a study to evaluate the effect of repeated TUGA during four consecutive cycles in the mare. It was noted that fewer experimental mares (53%) had a conventional duration of estrus after repetitive aspirations compared with non-aspirated controls (80%). Concentrations of plasma FSH were increased in the experimental group after follicles were aspirated during diestrus when compared with non-aspirated controls. No differences were found between the two groups regarding FSH, LH, or progesterone concentrations during estrus aspirations. No adverse side effects or complications occurred and the author concluded that TUGA was a low-risk repeatable

method for in vivo equine oocyte collection with little effect on estrus cycle characteristics (Cook, 1995). In contrast to Cook's results, another study examining the effect aspiration of three different sized follicles has on plasma LH concentrations in the mare found that increased LH concentrations were present after aspiration of follicles in each size category. Concentrations of FSH were also significantly higher in aspirated mares compared to controls (Hinrichs et al., 1991).

CHAPTER 2 - EFFECTS OF SUPPLEMENTING MARE DIETS WITH MARINE-DERIVED N-3 FATTY ACIDS ON SERUM, FOLLICULAR FLUID AND FOLLICULAR DYNAMICS DURING THE ESTROUS CYCLE

Introduction

The inclusion of fat in the horse diet to maximize energy without negatively impacting the animal's glycemic index or digestive tract has been a common practice for many years. As monogastric herbivores, horses are designed to primarily consume forage which is high in fiber but low in DE. However, unlike high amounts of starch and cereal grains, the horse has a relatively high tolerance for fat in the diet and it is considered a "safe" energy alternative to the more traditionally used cereal grains (Bush et al., 2001; Lewis, 2005). In fact, horses can utilize up to 20% added fat in the total diet (Lewis, 2005). The most commonly used forms of fat supplementation for horses are plant and vegetable oils, such as corn oil and soy oil, due to their high palatability and availability. Plant and vegetable oils provide about 3 times more digestible energy than an equal weight of cereal grain, and 3.5 to 6 times more digestible energy than an equal volume of cereal grain (Lewis, 2005). Fat added diets have been proven to be especially beneficial to those horses that have a heavy work load or exercise requirement (Hambleton et al., 1980; Harkins et al., 1992; Eaton et al., 1995). Dietary fat supplementation also affects follicular growth, pregnancy rates, milk composition (Mattos et al., 2002), and ovarian and uterine function in several livestock species (Beam and Butler, 1997; Mattos et al., 2000; Santos et al., 2008). Although not as extensively documented, a positive effect of added fats on reproduction in the equine species has been documented (Kubiak et al., 1987; Davidson et al., 1991; Ordakowski-Burk et al., 2005). Fat supplementation of mares during late gestation and early lactation increased milk fat percentage, blood lipid concentration of their foals, and rate of gain in foals during their first week of life (Davidson et al., 1991). Additionally, this study noted a trend for a shorter postpartum interval and fewer cycles to pregnancy in fat-supplemented mares.

A particular group of fatty acids has been recently highlighted in research and the consumer market as having multiple health benefits for both humans and livestock species. Polyunsaturated fatty acids are defined as fatty acids with multiple double bonds, and can be categorized into omega-6 (n-6) and omega-3 (n-3) families. Omega-6 fatty acids are found in plentiful amounts in common animal feedstuffs such as corn and corn oil. Once digested, n-6

fatty acids produce series 1 and 2 eicosanoids which are inflammatory in nature. Omega-3 fatty acids are not commonly found in high amounts in a typical livestock diet, but can be supplemented with products like flaxseed oil and fish oil. Marine-derived supplements, like fish oil, are rich in two of the most commonly supplemented n-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Once digested, n-3 fatty acids produce series 3 eicosanoids which are anti-inflammatory in nature. Supplementing n-3 fatty acids can benefit many systems in the body, including the cardiovascular, skeletal, nervous, immune, and reproductive systems (Simopoulos, 1991; Pike and Barlow, 2000; Simopoulos, 2002; Dunnett, 2005; Robinson and Stone, 2006).

Omega-3 fatty acids have received increasing attention in the equine industry, primarily due to their anti-inflammatory properties (Calder, 2002; Munsterman et al., 2005; Skjolaas-Wilson et al., 2005). In stallion reproduction, n-3 fatty acids have been investigated in relation to improving spermatogenesis and cooled semen properties (Brinsko et al., 2005; Harris et al., 2005). In the mare, research has been focused on supplementation of the foal through mare n-3 dietary supplementation (Kruglik et al., 2005; Skjolaas-Wilson et al., 2005; Poland, 2006). During the postpartum estrous period, mares receiving EPA and DHA in the form of fish oil had an increase in time from foaling to ovulation in the EPA and DHA supplemented group when compared to the control group and a group receiving DHA alone (Poland, 2006). The EPA and DHA supplemented mares also held a preovulatory follicle significantly longer prior to ovulation than the other two groups (Poland, 2006).

The current study was designed to further explore Poland's findings regarding an n-3 effect on mare cyclicity. The objectives of this study were to determine the effects of feeding a marine-derived n-3 fatty acid supplement on plasma fatty acid profiles as well as several reproductive factors during the estrus periods of healthy, cycling mares. To this author's knowledge, there have been no such studies evaluating the reproductive effects of n-3 fatty acid supplementation in non-pregnant mares during the natural breeding season.

Materials and Methods

Management of Animals

This study was reviewed and approved by the Institutional Animal Care and Use Committee at Kansas State University. Fourteen Quarter Horse-type mares and one Thoroughbred-type mare between 4 and 16 yr of age were used in this study. Fourteen mares were leased from private owners for the duration of the study and one mare was owned by Kansas State University (KSU). All mares were non-pregnant and cycling throughout the duration of the study. Initially, 19 mares were placed in the study, however 4 mares were removed from the study after failing to establish a normal estrous cycling pattern within the first 30 d. Upon arrival, mares were vaccinated and dewormed according to KSU Horse Teaching and Research Unit herd health management policies. Mares were group housed at the KSU Horse Unit in Manhattan, KS in dry lots and were allowed access to fresh water ad libitum throughout the study. Brome hay was group fed based on 1.5% of each mare's BW and spread throughout 4 bunks for equal access once daily. Mares were also individually fed 1.81 to 2.5 kg of a 12% crude protein sweet feed with their treatment supplement once daily. The amount of grain fed to each mare was adjusted to maintain a body condition score of 5 or above. All feedstuffs and supplements were analyzed to ensure that the two treatment diets met or exceeded the nutritional requirements for horses at maintenance (Tables 2.1, 2.2, 2.4) as per the Horse NRC guidelines (National Research Council, 2007). Additionally, feedstuffs and supplements were analyzed for fatty acid content prior to initiation of the study (Tables 2.3, A.1 and A.2). Treatment diets were initiated on d 0 and continued until follicular fluid was collected from a preovulatory follicle (35 to 45 mm) on the third and final estrous cycle of the study. Grain and supplements were weighed, mixed, and fed daily at 0800 hr. Mares consumed their grain ration in individual 12 x 12 pens and were given adequate time to finish their ration. Any leftover feed was weighed and recorded. Mares were group fed brome hay at 1700 hr daily. The study began on June 8, 2009 and the final mare concluded the trial on August 18, 2009.

Table 2.1 Nutrient profile (on a dry matter basis) of brome hay and concentrate fed to mares during the breeding season

Brome Hay[ac]	
Dry Matter (%)	91.97
Crude Protein (%)	7.56
Acid Detergent Fiber (%)	36.73
Neutral Detergent Fiber (%)	60.32
Digestable Energy (Mcal/kg)	2.34
Nonstructural CHO (per 100 g)	22.35
Fat (%)	2.65
Ash (%)	7.12
Calcium (%)	0.29
Phosphorus (%)	0.18
Potassium (%)	1.35
Concentrate[bc]	
Digestible Energy (Mcal/kg)	2.63
Protein (min %)	12.00
Fat (min %)	3.00
Fiber (max %)	25.00
Calcium (min %)	1.20
Phosphorus (min %)	0.30
Salt (min %)	1.00
Potassium (min %)	0.60
Copper (min ppm)	5.00
Zinc (min ppm)	75.00
Selenium (min ppm)	0.01
Vitamin A (min IU/lb)	2600.00

[a]Proximate Analysis by SDK Laboratories, Hutchinson, KS

[b]Guaranteed analysis provided by Nutrena® Animal Feeds

[c]Values listed are LS means

Table 2.2 Nutrient profiles of fish oil supplement and corn oil fed to mares during the breeding season

Nutrient	Supplements	
	Fish oil[a]	Corn oil
DM (%)	96.19	100
Digestible Energy (Mcal/kg)	3.82	9.19
Total Fat (%)	48.7	
Crude Protein (%)	1	
Crude Fiber (%)	7.19	
Ash(%)	40.25	

[a]Values for fish oil supplement provided by JBS United, Sheridan, IN

Table 2.3 Fatty acid profile of feedstuffs, fish oil supplement, and corn oil fed to mares during the breeding season

	Feedstuffs			Supplements	
	Concentrate[a]	Brome Hay[a]		Corn Oil[a]	Fish oil[b]
Total n-6:					
LA	44.76	25.23		55.71	2.43
ARA	0	0.21		0	0.98
Total n-3:					
ALA	2.9	20.28		1.17	1.08
EPA	0	0		0	13.54
DPA	0	0		0	2.43
DHA	0	0		0	7.29
n-6:n-3	15.43:1	1.25:1		47.62:1	0.14:1

[a]Percent Total Fat calculated from total fatty acids measured by gas chromotography analysis

[b]Percent Total Fat as reported by JBS United, Sheridan, IN

Treatment groups

Mares were divided into two treatment groups based on age, initial BW, and initial body condition score. The treatments consisted of a control diet (CONT, n=7) and a diet supplemented with fish oil (FO, n=8). The CONT group received the concentrate to which 120 g corn oil was added, while the FO group was given the concentrate supplemented with 280 g of Gromega 365™, a powdered fish oil supplement containing EPA and DHA. The fish oil supplement was added to the concentrate and water was added to aid in the mixing and binding of the powder to the concentrate. The FO supplement provided 18.48 g EPA/d and 10.08 g DHA/d for each horse. Gromega 365™ was provided by JBS United, Inc. of Sheridan, IN and is currently marketed for use in the swine industry. Corn oil was used to provide a similar amount of energy to the CONT group as the fish oil supplement provided to the FO group. Corn oil also is extremely low in n-3 fatty acids (Table 2.3), minimizing any confounding effects due to supplementation (Reese, 2003).

Table 2.4 Estimated nutrients provided in total diets for control (CONT) and fish oil supplemented mares (FO) during the breeding season

Nutrient[a]	Req[b]	CONT	FO
DE (Mcal/d)	16.65	23-25	23-25
CP (g/d)	630	783-866	786-869
Ca (g/d)	20	44-52	60-68
P (g/d)	14	18-20	18-20
Ca:P	1.4	2.4-2.6	3.3-3.4
K (g/d)	25	111-115	111-115

[a]Values based on 1.81 - 2.5 kg grain fed/d/mare and 7.48 kg brome hay fed/d/mare

[b]Requirement based on 500 kg horse at maintenance

Synchronization Protocol

Upon initiation of treatment diets on d 0, the mares' estrous cycles were synchronized by a standard progesterone and estradiol protocol (McKinnon and Voss, 1993) to aid in ease of tracking follicular dynamics via ultrasonography. During synchronization, mares were also allowed to acclimatize to the treatment diets. Mares were injected IM in the neck with 1 mL containing 150 mg progesterone and 10 mg estradiol-17β daily from d 0 to d 9 of the trial. On d 9 and 10, 5 mg of Lutalyse® was administered IM. Mares were then ultrasounded to confirm ovulation and the presence of a CL, denoting the end of the first estrous cycle. During the synchronization process, three mares had mild injection site reactions. These reactions were treated with heat packs for 2 to 5 d and alternate locations were used for subsequent injections.

Ultrasonography

Five days after a CL was confirmed following synchronization, mares were monitored every other day through transrectal ultrasonography using a Sonovet 600 ultrasound equipped with a 5 MHz probe. Once a 25 mm diameter follicle was detected, mares were ultrasounded daily through d 5 post ovulation. Ovarian activity, follicular diameter, conformation of ovulation, and presence of a CL were noted.

The same ultrasonography protocol was repeated for the third estrous cycle until a 35 mm follicle was detected. On the day a 35 mm follicle was identified, hCG was administered and the transvaginal ultrasound-guided aspiration (TUGA) procedure was performed the following day, denoting the conclusion of data collection. More detail on the TUGA procedure and hCG administration is provided in the sections that follow. As mentioned previously, four mares were removed from the study after transrectal ultrasonography data revealed the mares failed to establish patterns consistent with normal estrous cyclicity.

Follicular Fluid Collection

Follicular fluid was collected from each mare during the third estrous cycle via a TUGA procedure. When a 35 mm follicle was first detected, 2500 IU hCG (Chorulon®, Intervet/Schering-Plough Animal Health) was administered intravenously that evening at 1800 hr. Administering hCG to mares when a preovulatory follicle of 35 mm or greater is present results in ovulation 24 to 48 hr later in 73% of treated mares (Duchamp et al., 1987). Therefore, giving each mare hCG helped ensure that the preovulatory follicles were maturing at

approximately the same rate and fluctuations in individual mares were minimized. The TUGA procedure began the next day at 1000 hr after a final diameter of the preovulatory follicle was recorded.

TUGA procedure

Before initiation of the procedure, each mare was ultrasounded to determine final diameter of the preovulatory follicle. The mare's tail was then tied up and the vulva and buttocks thoroughly washed. A 0.01 mg/kg dose of Detomidine was administered IV to induce mild sedation. After 1 min, 0.3 mg/kg of Buscupan was given IV over a 30 sec interval. Lidocaine gel was then manually administered to the vaginal wall 2 to 5 min prior to needle puncture. The ovary was rectally manipulated into position against the vaginal wall and an Aloka 5 MHz convex Equine/Bovine transducer was inserted vaginally. Once the preovulatory follicle was located and maneuvered into position, a sterilized lumen needle attached to plastic tubing and a 20 cc syringe was inserted into the probe. The needle was then punctured through the vaginal wall and into the follicle. Simultaneously, slight suction was applied to the needle via the syringe to direct follicular fluid down the tubing. At least 20 mL of follicular fluid was collected per mare and transferred to sterile 50 mL centrifuge tubes. The entire procedure spanned approximately 20 min. Samples were immediately put on ice and later transported to the laboratory for preparation and storage. All TUGA equipment was sanitized with Dulbeccos Phosphate Buffered Saline (Gibcos®, Invitrogen Corp.) prior to and following each procedure and the needle was autoclaved. After the procedure, the mare was allowed to recover from sedation before returning to the pen. For the following 72 hr, the mare was monitored closely for any signs of depression, loss of appetite, or pain. Rectal temperature was also recorded the evening of the procedure and twice daily for the following two days. Each mare's TUGA procedure signaled the end of data collection for that mare.

After transfer to the laboratory, samples were centrifuged in a Beckman J6B centrifuge at 1000 x g (2000 rpm) for 10 min. The cell pellet portion of the follicular fluid was separated into a 1.5 mL microtube and centrifuged again for 10 sec at a steadily increasing rpm to further separate the cells and fluid. Follicular fluid was stored at -70°C until fatty acid and hormone concentrations were analyzed.

Blood Collection

Blood samples were all collected at approximately the same time in the morning, either in the individual pens during morning feeding or in the stocks prior to ultrasound, to minimize differences in fatty acid levels due to circadian rhythms (Orme et al., 1994). For fatty acid analysis, blood samples were collected from mares via jugular venipuncture using 10 mL vacuum tubes without additive. Samples were collected at d 0 of the trial and every two wk until the termination of the study. After collection, tubes were allowed to sit at room temperature for approximately 20 min until clotting and separation occurred. Tubes were then centrifuged at 2000 rpm for 20 min. Serum was stored in 1.5 mL microtubes at -18° C until fatty acid concentrations were analyzed.

Blood samples for hormone analyses were collected from mares during their second estrous cycle at 5, 4, 3, 2, and 1 d prior to ovulation as well as on d 1, 3, and 5 post-ovulation. Samples were also taken during the third estrous cycle prior to hCG administration and the day of follicular fluid collection. Blood was collected via jugular venipuncture using 10 mL and 7 mL vacuum tubes without additive. After collection, tubes were allowed to sit at room temperature for approximately 20 min until separation occurred. Tubes were then centrifuged at 2000 rpm for 20 min. Serum was stored in 5 mL collection tubes at -18° C until hormone concentrations were analyzed.

Fatty Acid Analysis

Serum and follicular fluid fatty acid concentrations were determined using gas chromatography. Although many fatty acids were measured, only the main fatty acids of interest, including linoleic acid (LA), alpha-linolenic acid (ALA), arachidonic acid (ARA), EPA, docosapentaenoic acid (DPA), and DHA, were used for statistical analysis and noted in the results section. The standard operating procedure used to measure long chain fatty acids in serum and follicular fluid is described in detail in the following paragraph.

Serum (500 µl) was dispensed into 10 mL screw cap tubes and allowed to lyophilize overnight. One mL of benzene containing internal standard (400 µg/ml methyl-C13) was then added and samples were vortexed to break up the pellet. Next, 4 mL of Boron Triflouride-

Methanol reagent was added, tubes were gased with nitrogen for approximately 10 sec, and samples were vortexed. Tubes were incubated at 60° C for 60 min, cooled to room temperature, and 4 mL of water and 1 mL of hexane were added. Finally, tubes were centrifuged for 5 min at 2000 rpm/1000 x g and upper layer methyl esters were transferred to a screw top gas chromatography vial for analysis using a Hewlett-Packard Model 5890 gas chromatographer.

Hormone and IGF-1 Analysis of Serum and Follicular Fluid

Estradiol-17β

Serum samples collected during the second and third estrous cycles were analyzed for estradiol-17β using a Becton Dickinson-4800 Ultra-Sensitive Estradiol Radioimmunoassay (RIA) kit from Diagnostic Systems Laboratories, Inc., which was previously validated for equine. The extraction and assay were performed according to the manufacturer's instructions with 400 µL aliquots of serum and samples were counted in a PerkinElmer Cobra II 5005 gamma counter. The intra-assay CV was 6.32% and the inter-assay CV was 6.54%.

Follicular fluid samples for each mare were sent to the Louisiana State University equine physiology lab in Baton Rouge, Louisiana for analysis of estradiol-17β concentrations. A Double Antibody Estradiol RIA kit (DSL-4400) manufactured by Diagnostic Systems Laboratory was used to measure concentration of estradiol in each follicular fluid sample. Prior to the assay, samples were diluted 1:100,000 and run directly at 200 uL per tube. The assay was performed according to the manufacturer's instructions. The intra-assay CV was reported as 7.00%.

Progesterone

Serum samples collected during the second and third estrous cycles were analyzed for progesterone using a DSL-3900 ACTIVE® Progesterone Coated-Tube RIA kit, which was previously validated for equine. The assay was performed according to the manufacturer's instructions with 25 µL aliquots of serum and samples were counted in a PerkinElmer Cobra II 5005 gamma counter. The intra-assay CV was 6.97% and the inter-assay CV was 8.16%.

Progesterone content in follicular fluid samples was measured using a Coat-A-Count® Progesterone RIA kit, which was previously validated for equine. The assay was performed

according to the manufacturer's instructions with 100 µL aliquots of serum and samples were counted in a PerkinElmer Cobra II 5005 gamma counter. The intra-assay CV was 1.0%.

Luteinizing Hormone

Serum samples collected 4 d prior to ovulation, on ovulation, and d 3 post ovulation during the second estrous cycle, during the third estrous cycle, and aliquots of follicular fluid were sent to the Louisiana State University equine physiology lab in Baton Rouge, Louisiana for analysis of luteinizing hormone (LH) concentrations. The radioimmunoassay used to measure LH in these samples has been previously validated and documented in detail (Thompson D.L. Jr et al., 1983) and can measure up to 200 uL of serum. The intra-assay CV was reported as 6.00%.

Insulin-Like Growth Factor-1

Serum samples collected from the day before and the day of the TUGA procedure and aliquots of follicular fluid were analyzed for insulin-like growth factor-1 (IGF-1) content using the DSL-5600 ACTIVE® IGF-1 Coated-Tube IRMA kit, which was previously validated for equine. The extraction and assay were performed according to the manufacturer's instructions with 50 µL aliquots of extracted serum or follicular fluid and samples were counted in a PerkinElmer Cobra II 5005 gamma counter. For serum samples, the intra-assay CV was 4.06%. For follicular fluid, the intra-assay CV was 1.5%.

Prostaglandin $F_{2\alpha}$

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was measured in follicular fluid samples collected during aspiration using a $PGF_{2\alpha}$ Enzyme-Linked Immunosorbent Assay (ELISA) kit manufactured by Neogen Corporation, which was previously validated for equine. The assay was performed according to the manufacturer's instructions with 50 µL aliquots of follicular fluid and samples were counted in a PerkinElmer Victor II microplate reader at 450 nm. The intra-assay CV was 8.9%.

Prostaglandin E_2

Prostaglandin E_2 (PGE_2) was measured in follicular fluid samples using a Correlate-EIA^{TM} PGE_2 Enzyme Immunoassay (EIA) kit manufactured by Assay Designs, which was previously validated for equine. The assay was performed according to the manufacturer's

instructions with 100 µL aliquots of follicular fluid and samples were counted in a PerkinElmer Victor II microplate reader at 405 nm. The intra-assay CV was 14.2%.

Statistical Analysis

A randomized complete block design was utilized for this study, with mares being allocated to the CONT or FO treatment diets independently within blocks. Age, initial body weight, and initial body condition score were used to block mares into blocks of two. Data were measured at one time point during the study or serially over time. Statistical analyses for all data were performed using the Proc Mixed procedure of SAS 9.1 (Cary, NC).

Data measured at one time point throughout the study include the following: follicular fluid fatty acid and hormone measurements, length of interval from ovulation to a 35 mm follicle during the second and third estrous cycle, length of interval from a 25 mm follicle to ovulation during the second estrous cycle, length of interval from a 35 mm follicle to ovulation during the second estrous cycle, mean growth per day from a 25 mm follicle to a 35 mm follicle during the second estrous cycle, and mean growth per day from a 35mm follicle to one day prior to ovulation during the second estrous cycle. For this analysis, the model contained the factors treatment and block and F-tests were calculated to test treatment main effects. Means and standard errors were also calculated for treatments.

Data measured serially over time include serum fatty acid and hormone measurements, mean follicular size per day from five days prior to ovulation to one day prior to ovulation during the second estrous cycle, mean follicular size on the day of hCG administration and on the day of aspiration. An initial repeated measures analysis was conducted in the SAS GLM procedure to evaluate equal correlation between time points using the Greenhouse-Geisser and Huynh-Feldt adjustments to p-values. It was determined that the model with a split-plot in time was appropriate and those results are reported in the following results section. For this analysis, the model contained the factors block, treatment, block x treatment, day, treatment x day, and the split-plot error term. Treatment main effects, day main effects, and the treatment x day interaction were calculated by F-tests. Means and standard errors were also calculated for treatments, days, and the treatment x day interaction. When the treatment x day interaction was significant, CONT and FO means were compared for each day.

Results

Fatty Acids

Mare Serum

Changes in mare serum fatty acid concentrations were apparent on d 14, or two weeks after initiation of the diets. Linoleic acid concentrations were similar (P>0.05) between treatments for the majority of the study, but were lower (P<0.01) in the FO group on the final day of the trial (Figure 2.1). A treatment by day interaction (P<0.05) was evident. Arachidonic acid concentrations were significantly higher (P<0.05) in the FO group from d 14 to d 56 of the study (Figure 2.2). However on the final day of the trial, CONT horses had a higher (P<0.01) ARA serum concentration. An overall treatment effect was found (P<0.01) as well as a significant treatment by day interaction (P<0.01).

Similar to LA results, serum concentrations of ALA revealed fluctuations in both groups throughout the study (Figure 2.3). On d 28, ALA concentrations were significantly higher (P<0.01) in FO horses than CONT horses, but a trend (P=0.07) for concentrations to be higher in the CONT group was noted on the final day of the trial. A treatment by day interaction (P<0.05) was noted.

Figure 2.1 Serum linoleic acid (LA) concentration (LSMeans ± SE) in cycling mares fed a control diet (CONT) or a fish oil supplemented diet (FO) during the breeding season. [a] P<0.05

Figure 2.2 Serum arachidonic acid (ARA) concentration (LSMeans ± SE) in cycling mares fed a control diet (CONT) or fish oil supplemented diet (FO) during the breeding season. [a] P<0.05, [b] P<0.01

Figure 2.3 Serum alpha-linolenic acid (ALA) concentration (LSMeans ± SE) in cycling mares fed a control diet (CONT) or a fish oil supplemented diet (FO) during the breeding season. [b] P<0.01, [c] P=0.07

Eicosapentaenoic acid serum concentrations were higher (P<0.01) in the FO group by d 14 and remained significantly higher throughout the trial when compared to horses in the CONT group (Figure 2.4).   An overall treatment effect was found (P<0.01) as well as a significant treatment by day interaction (P<0.01). Serum concentrations of DPA were also found to be elevated (P<0.01) in the FO treatment group compared to the CONT group from d 14 to d 56 of the study, with no statistical difference being recorded on the final day of the trial (Figure 2.5). An overall treatment effect was found (P<0.01) as well as a significant treatment by day interaction (P<0.01).

Similar to EPA results, DHA serum concentrations were increased (P<0.01) in horses allocated the FO treatment by d 14 and remained significantly higher throughout the trial when compared to the CONT group (Figure 2.6). An overall treatment effect was found (P<0.01) as well as a significant treatment by day interaction (P<0.01). Although the EPA, DPA, and DHA concentrations were declining throughout the study from their peak on d 14, they were still elevated when compared to horses in the CONT group.
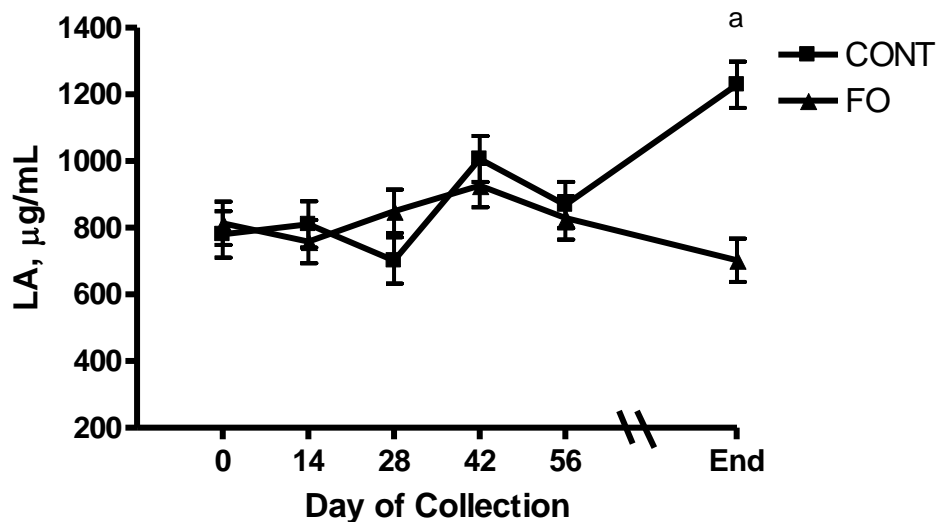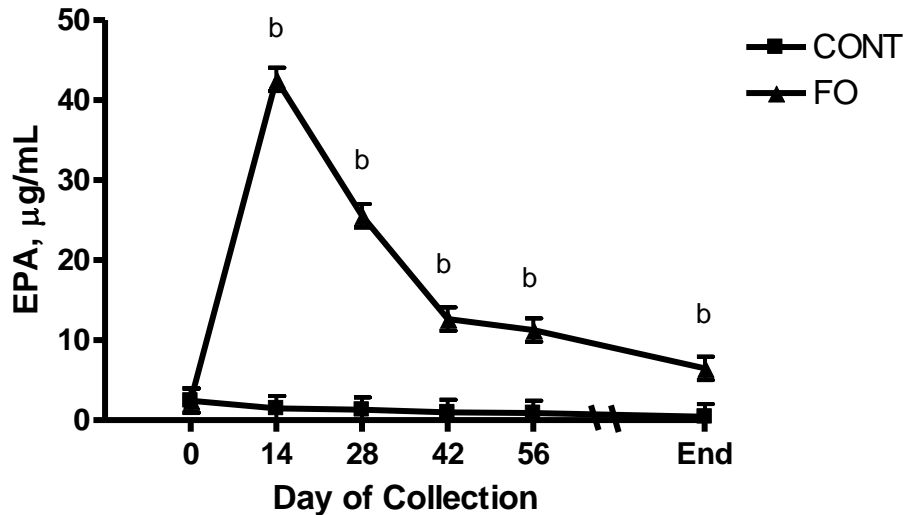

Figure 2.4 Serum eicosapentaenoic acid (EPA) concentration (LSMeans ± SE) in cycling mares fed a control diet (CONT) or a fish oil supplemented diet (FO) during the breeding season.
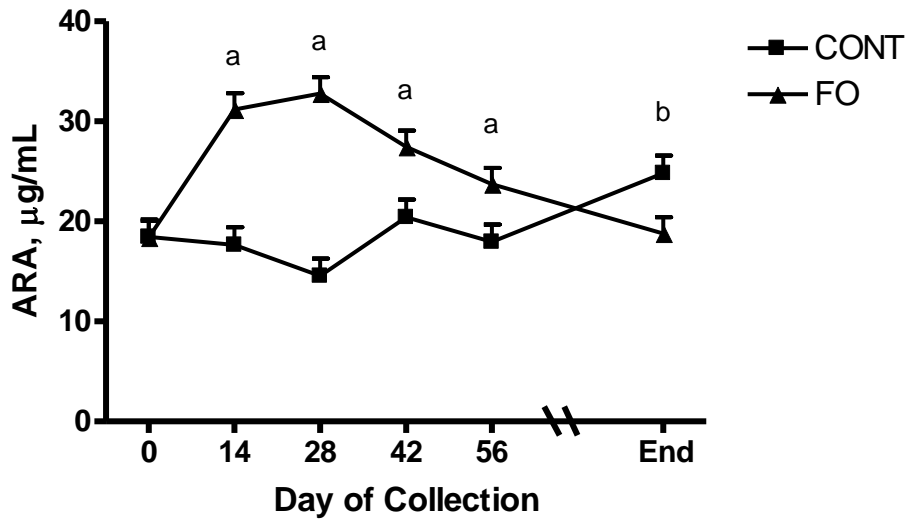[b] P<0.01
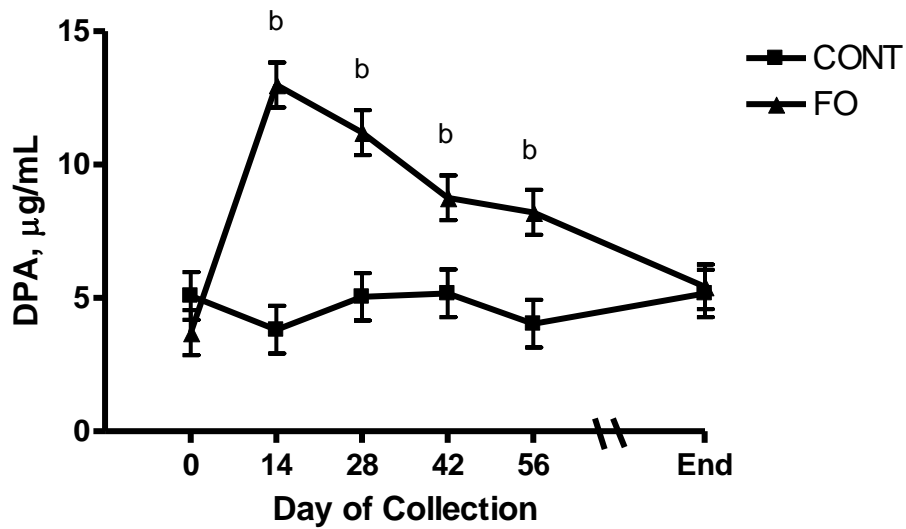
Figure 2.5 Serum docosapentaenoic acid (DPA) concentration (LSMeans ± SE) in cycling mares fed a control diet (CONT) or a fish oil supplemented diet (FO) during the breeding season.
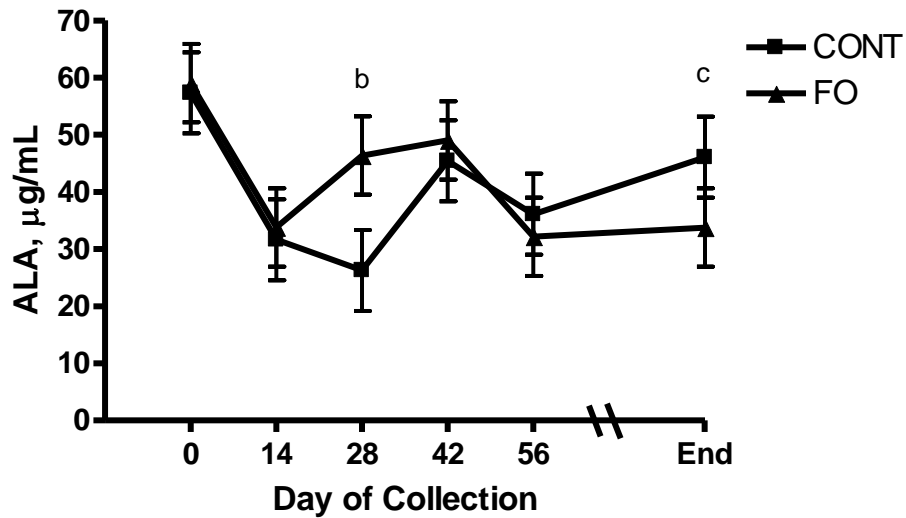[b] P<0.01
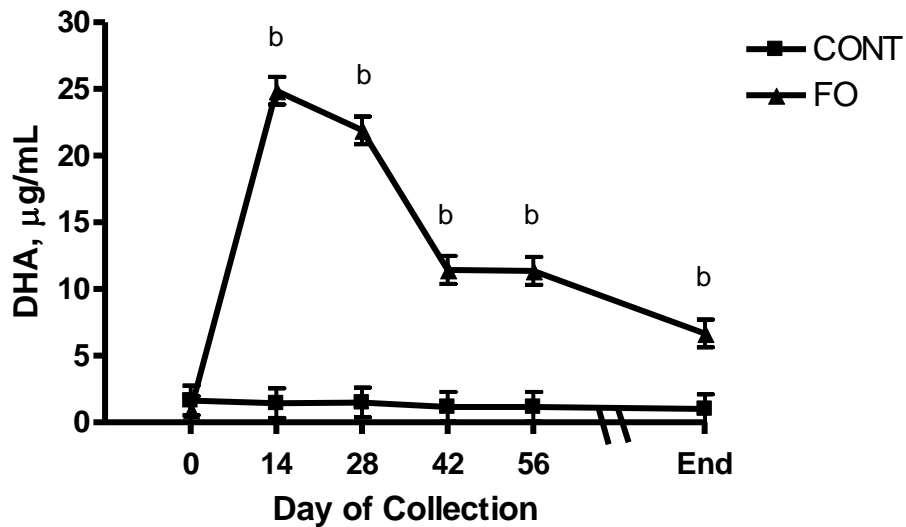
Figure 2.6 Serum docosahexaenoic acid (DHA) concentration (LSMeans ± SE) in cycling mares fed a control diet (CONT) or a fish oil supplemented diet (FO) during the breeding season.
[b] P<0.01

Mare Follicular Fluid

Equine follicular fluid, which was collected on the final day of the study, appeared to have a similar capability for dietary fatty acid uptake as serum, with n-3 fatty acid concentrations being altered by fish oil supplementation (Figure 2.7). A trend was revealed for ALA concentrations to be higher (P=0.06) in the FO group versus CONT horses. Additionally, concentrations of EPA, DPA, and DHA were higher (P< 0.01) in the follicular fluid of the FO group when compared to the CONT group. Concentrations of LA and ARA in follicular fluid were similar (P>0.05) between treatments (Figure 2.8).
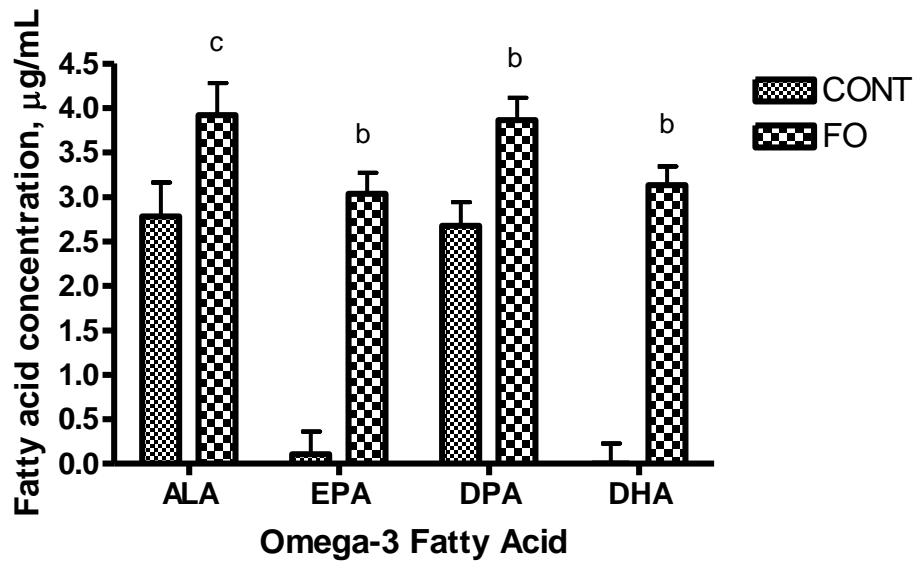
Figure 2.7 Concentrations of alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA) in follicular fluid collected from large (>35 mm) follicles (LSMeans ± SE) in mares fed a control diet (CONT) or a fish oil supplemented diet (FO). [b] P<0.01, [c] P=0.06
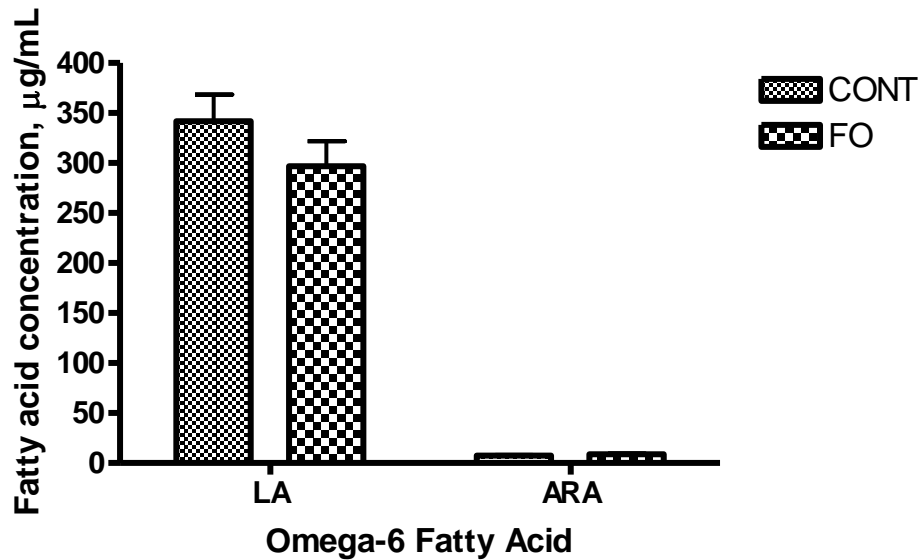
Figure 2.8 Concentrations of linoleic acid (LA) and arachidonic acid (ARA) in follicular fluid collected from large (>35 mm) follicles (LSMeans ± SE) in mares fed a control diet (CONT) or a fish oil supplemented diet (FO).

Hormone Concentrations

Mare Serum

2nd Cycle

Hormone samples collected during the second estrous cycle followed a similar pattern. However, some differences were noted between the two treatment groups. Estradiol-17β concentrations collected for 5 d prior to ovulation, on the d of ovulation, and on d 3 and 5 post-ovulation during the second estrous cycle were similar (P>0.05) between treatments (Figure 2.9). Serum concentrations of LH were not significantly different (P>0.05) between the FO and CONT groups until d 3 post-ovulation, when concentrations were higher (P<0.05) in CONT mares compared to the FO mares (Figure 2.10). Progesterone levels revealed a trend for FO concentrations to be higher (P=0.07) on d 3 post-ovulation (Figure 2.11). This trend became significant on d 5 post-ovulation, with higher concentrations (P<0.01) in FO mares compared with CONT mares. A treatment by day interaction (P<0.01) was also noted.
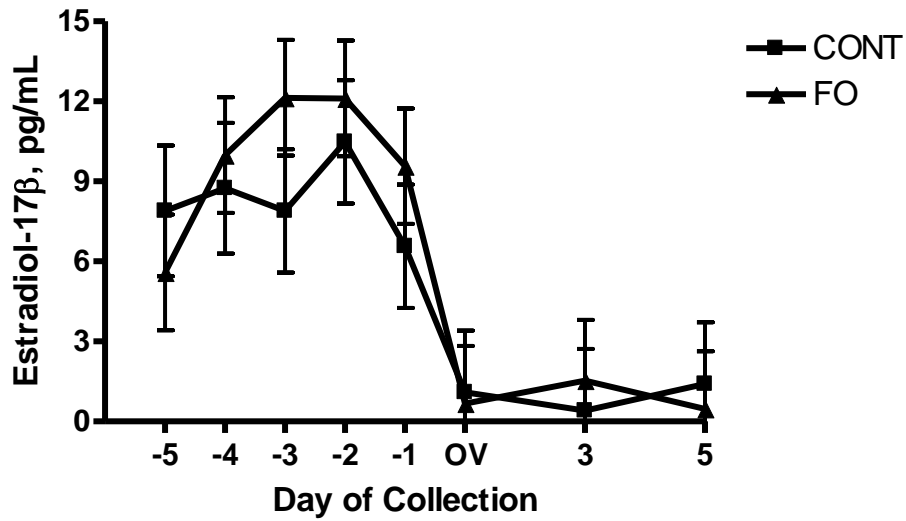
Figure 2.9 Serum estradiol-17β concentrations (LSMeans ± SE) in control mares (CONT) and mares supplemented with fish oil (FO) for 5 d prior to ovulation, at ovulation (OV), and on d 3 and 5 post-ovulation.

Figure 2.10 Serum luteinizing hormone (LH) concentrations (LSMeans ± SE) in control mares (CONT) and mares supplemented with fish oil (FO) for 4 d prior to ovulation, at ovulation (OV), and on d 3 post-ovulation. [a] $P<0.05$
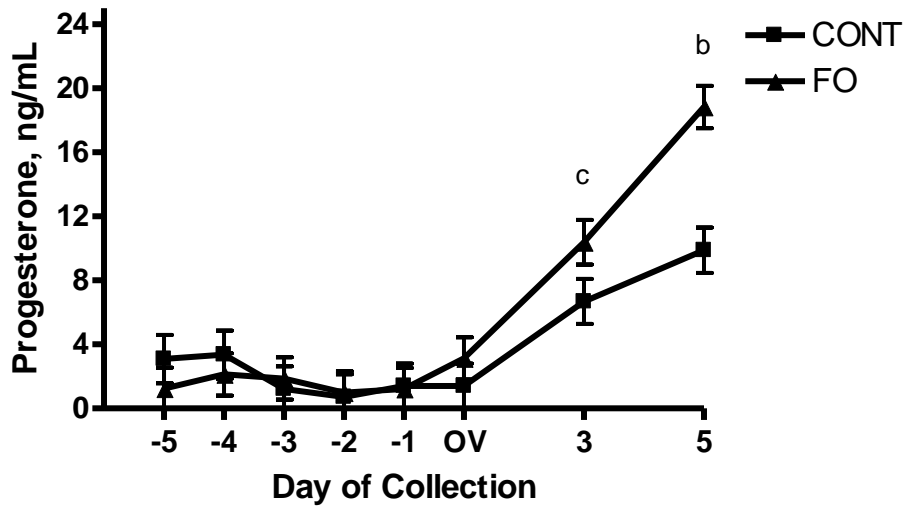
Figure 2.11 Serum progesterone concentration (LSMeans ± SE) in control mares (CONT) and mares supplemented with fish oil (FO) for 5 d prior to ovulation, at ovulation (OV), and on d 3 and 5 post-ovulation. [b] P<0.01, [c] P=0.07

3rd Cycle

Serum samples were collected during the third and final estrous cycle on the day a ≥ 35 mm follicle was first detected and hCG was administered, as well as on the following day when follicular aspiration was performed. Serum estradiol-17β, progesterone, and LH concentrations were not different (P>0.05) between FO and CONT mares on either day (Figures 2.12, 2.13, and 2.14). Serum IGF-1 concentrations were similar (P>0.05) on the day hCG was administered, but higher (P<0.05) in the CONT mares than the FO mares on the day the aspiration procedure was performed (Figure 2.15).
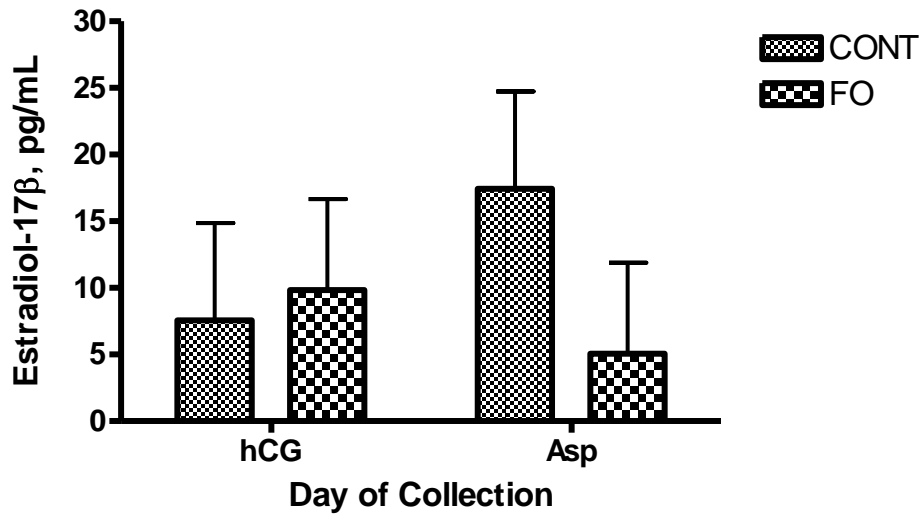
Figure 2.12 Serum estradiol-17β concentrations (LSMeans ± SE) in control mares (CONT) and mares supplemented with fish oil (FO) on the day a ≥ 35 mm follicle was first detected and hCG was administered, and the following day when follicular aspiration (Asp) was performed.
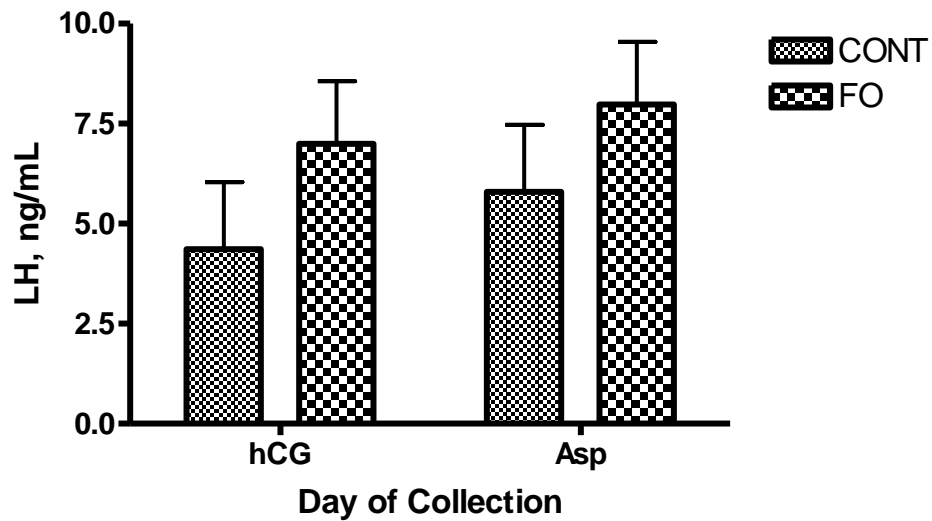
Figure 2.13 Serum luteinizing hormone (LH) concentrations (LSMeans ± SE) in control mares (CONT) and mares supplemented with fish oil (FO) on the day a ≥ 35 mm follicle was first detected and hCG was administered, and the following day when follicular aspiration (Asp) was performed.
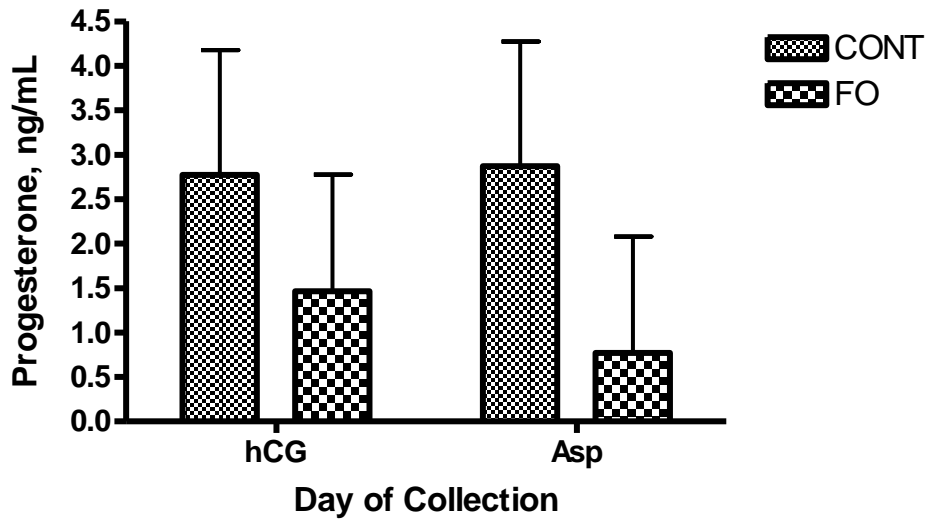
Figure 2.14 Serum progesterone concentrations (LSMeans ± SE) in control mares (CONT) and mares supplemented with fish oil (FO) on the day a ≥ 35 mm follicle was first detected and hCG was administered, and the following day when follicular aspiration (Asp) was performed.
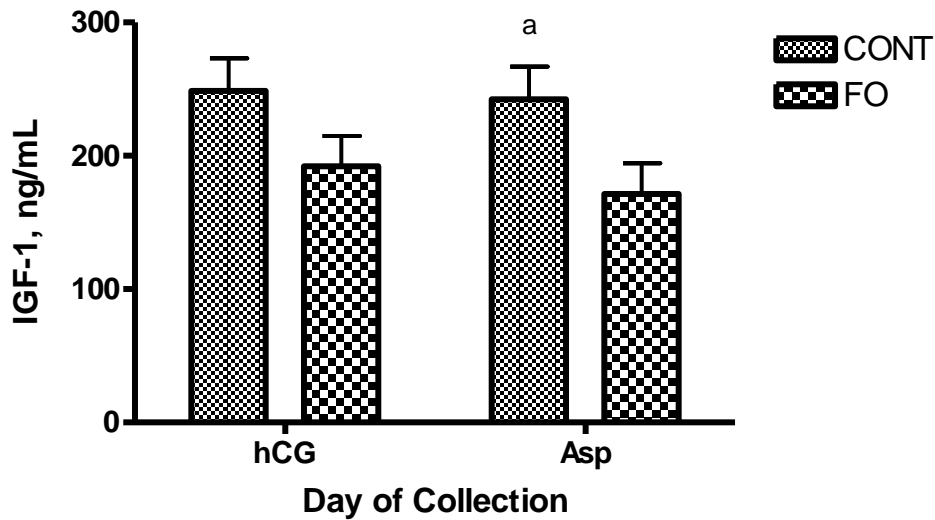
Figure 2.15 Serum insulin-like growth factor-1 (IGF-1) concentrations (LSMeans ± SE) in control mares (CONT) and mares supplemented with fish oil (FO) on the day a ≥ 35 mm follicle was first detected and hCG was administered, and the following day when follicular aspiration (Asp) was performed. [a] P<0.05

Mare Follicular Fluid

Concentrations of six hormones were measured in follicular fluid collected from a large preovulatory follicle during the third estrous cycle. There were no differences ($P>0.05$) found between the two treatments for estradiol-17β, progesterone, LH, $PGF_{2\alpha}$, and PGE_2 (Figures 2.16, 2.17, 2.18, and 2.19, respectively). However, IGF-1 results revealed that CONT mares had higher ($P<0.05$) concentrations than FO mares (Figure 2.20).
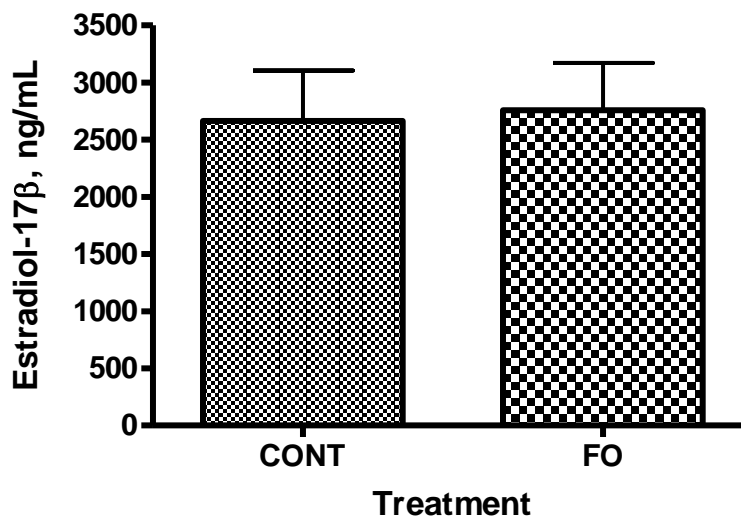
Figure 2.16 Follicular fluid estradiol-17β concentrations (LSMeans ± SE) in control mares (CONT) and mares supplemented with fish oil (FO) collected from a large (35 to 45 mm) preovulatory follicle one day following administration of hCG.
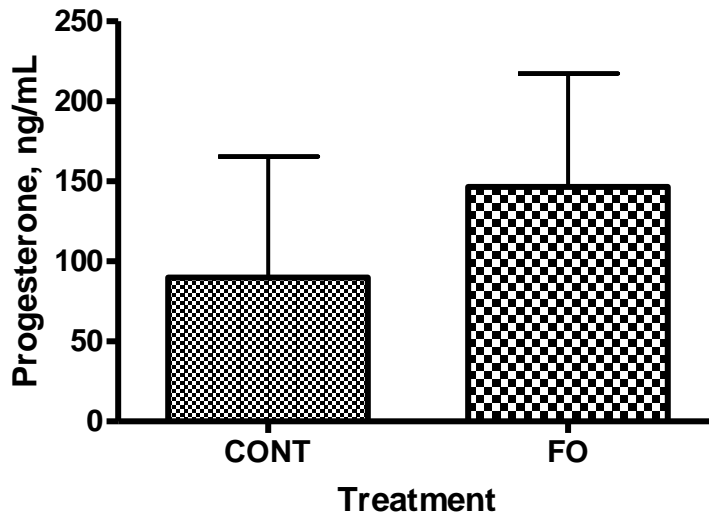
Figure 2.17 Follicular fluid progesterone concentrations (LSMeans ± SE) in control mares (CONT) and mares supplemented with fish oil (FO) collected from a large (35 to 45 mm) preovulatory follicle one day following administration of hCG.
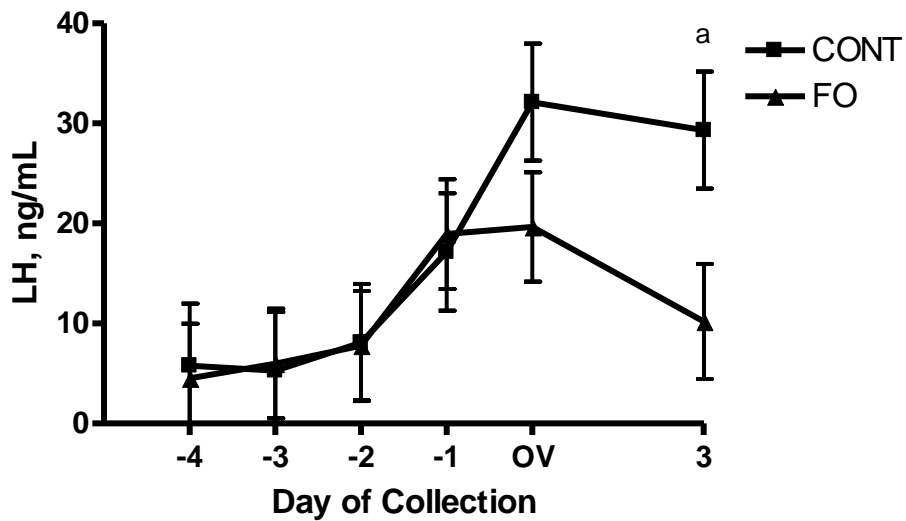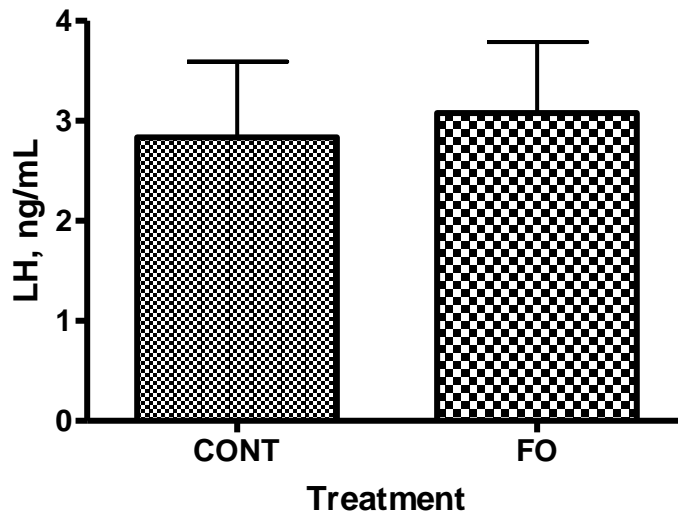

Figure 2.18 Follicular fluid luteinizing hormone (LH) concentrations (LSMeans ± SE) in control mares (CONT) and mares supplemented with fish oil (FO) collected from a large (35 to 45 mm) preovulatory follicle one day following administration of hCG.
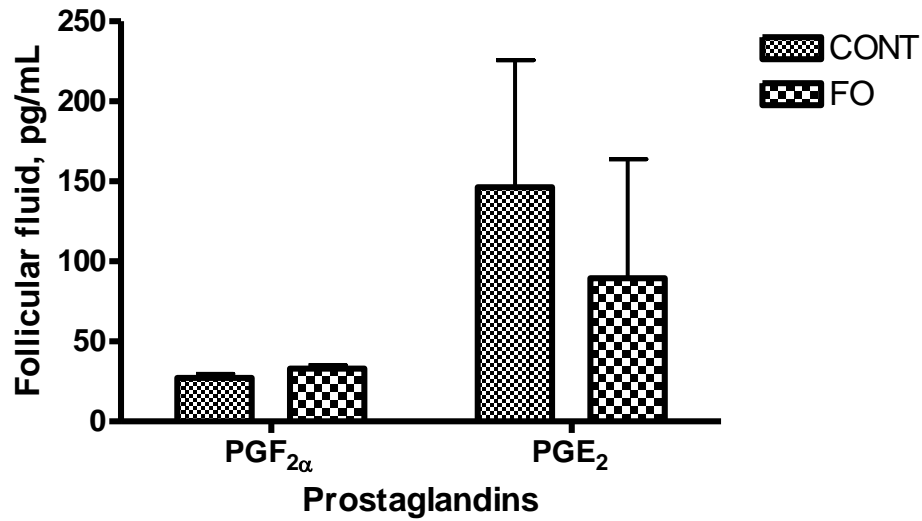
Figure 2.19 Follicular fluid concentrations (LSMeans ± SE) of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin E_2 (PGE_2) collected from a large (35-45 mm) preovulatory follicle one day following administration of hCG in control mares (CONT) and mares supplemented with fish oil (FO).
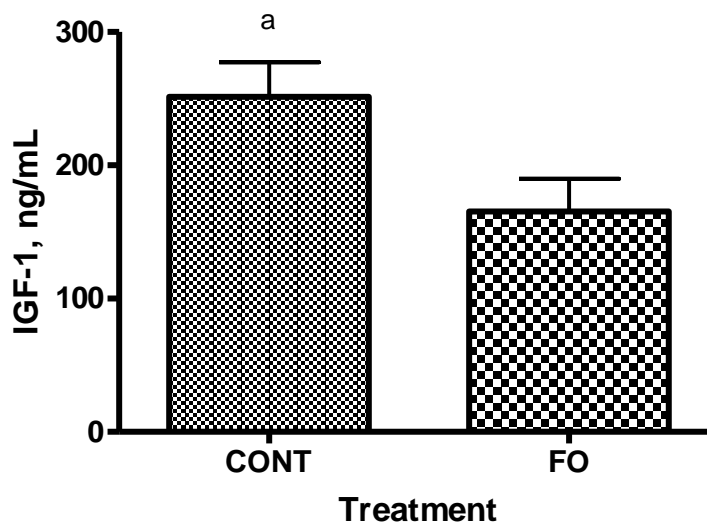
Figure 2.20 Follicular fluid insulin-like growth factor-1 (IGF-1) concentrations (LSMeans ± SE) collected from a large (35-45 mm) preovulatory follicle one day following administration of hCG in control mares (CONT) and mares supplemented with fish oil (FO).   [a] $P<0.05$

Follicular Dynamics

All mares ovulated after synchronization, which was denoted as the first estrous cycle, and ovulated naturally during the second estrous cycle. Additionally, no mare ovulated before being aspirated during the third estrous cycle following administration of hCG. One mare in the FO group developed and ovulated two follicles during the second estrous cycle. A different mare in the FO group also developed two preovulatory-sized follicles during the third estrous cycle and the largest follicle was aspirated.

2nd Cycle

Mean follicular growth per day was not different (P>0.05) between the FO and CONT groups from 5 to 1 d prior to ovulation, during early follicular growth (25 mm follicle to 35 mm follicle) or late follicular growth (35 mm follicle to ovulation) during the second estrous cycle. Additionally, no difference (P>0.05) was found between treatments in length in days from the first estrous cycle ovulation to a 25 mm follicle, the length in days from a 25 mm follicle to ovulation during the second estrous cycle, or the length in days from a 35 mm follicle to ovulation during the second estrous cycle. The mean size of the follicle ovulated was also similar (P>0.05) between treatments (Table 2.5).

3rd Cycle

No difference (P>0.05) was found between treatments in length in days from the second estrous cycle ovulation to a follicle measuring at least 35 mm during the third estrous cycle. The size of the follicle on the day hCG was administered did not vary between treatments, but the size of the follicle aspirated was greater (P<0.05) for the CONT group when compared to the FO group (Table 2.5). A treatment by day interaction (P<0.05) for the size of the follicle aspirated was also noted.

Table 2.5 Follicular traits of control mares (CONT) and mares supplemented with fish oil (FO) recorded during two consecutive estrous cycle during the breeding season

Follicular Trait	Treatment[a,b]	
2nd cycle	CONT	FO
Time from synchronized ovulation to $\geq$ 35 mm follicle (d)	17.3 ± 1.16	19.0 ± 1.09
Final size of preovulatory follicle (mm)	42.9 ± 1.98	47.5 ± 1.86
Time from 25 mm follicle to ovulation (d)	9.6 ± 0.59	8.5 ± 0.56
Time from $\geq$ 35 mm follicle to ovulation (d)	5.9 ± 0.59	5.9 ± 0.55
Growth rate (mm/d) from 25 mm to 35 mm follicle	2.6 ± 0.82	3.7 ± 0.77
Growth rate (mm/d) from 35 mm to ovulation	1.5 ± 0.34	2.08 ± 0.32
3rd cycle		
Time from ovulation in 2nd Cycle to follicle measuring $\geq$ 35 mm	19.6 ± 0.79	20.4 ± 0.74
Size of aspirated follicle (mm)	39.5 ± 0.50[c]	38.0 ± 0.47[d]
Avg d on trial	72.4	75.1

[a]Values are listed as LSmeans±SEM

[b]Average days on trial are listed as means

[c,d]Within a row, means without a common superscript differ (p<0.05)

Discussion

Fatty acids have long been recognized as a component in equine circulation. An early study identified the plasma fatty acid profile of horses fed a complete pelleted diet without hay, with 44.04% LA, 2.76% ALA, and 1.31% ARA being reported (Luther et al., 1981). Another study analyzed serum levels of horses being fed a control diet of first cut meadow hay and barley (Bergero et al., 2002). They reported total n-6 serum concentrations of $36.19 \pm 3.05\%$, with LA compromising $33.97 \pm 3.04\%$ and ARA compromising $1.27 \pm 0.41\%$ of the n-6 fatty acid total. Total n-3 serum concentrations of $1.72 \pm 0.68\%$ were also reported, with ALA compromising $1.03 \pm 0.36\%$ of the total n-3 concentrations (Bergero et al., 2002). It should be noted that in both of the previously mentioned studies, samples sizes were very small ($n \leq 6$) and the diet may have had additional dietary fat in the base diet affecting plasma and serum levels. Neither study reported measurable quantities (<1%) of EPA, DPA, or DHA. In both studies, the primary fatty acid found in equine circulation was LA.

Our serum fatty acid results revealed similar proportions of n-6 and n-3 fatty acids, with LA being the primary long chain fatty acid in mare circulation. Although significant differences were found at some time points between the two treatment groups, serum fatty acid profiles of LA and ALA showed no distinct pattern or difference overall due to supplementation. This may be due to the fact that supplementation was provided further down the PUFA pathway at the EPA and DHA level. By utilizing a supplement high in EPA and DHA instead of supplementing with a plant source high in ALA, such as flaxseed, dietary n-3s are absorbed and utilized within the body at a faster rate and in greater amounts by bypassing the rate-limiting reaction and loss due to bioconversion from ALA to DHA (Williams and Burdge, 2006).

Arachidonic acid serum concentrations were significantly greater in the FO group from d 14 to d 56 of the study. Although ARA is not typically involved in the n-3 fatty acid pathway, a previous report from this laboratory (Poland, 2006) also noted a significant increase in plasma ARA concentrations in supplemented pregnant and lactating mares, while Kruglik et al. (2005) noted no significant treatment effect in pregnant and lactating mares. More recently, a study in 2008 found increases in plasma ARA in light horse mares of differing breeds supplemented with a marine-derived source of EPA and DHA (King et al., 2008), supporting another study which documented a significant decrease in serum ARA concentrations in corn oil supplemented control geldings when compared to geldings supplemented with fish oil (O'Conner et al., 2007a).

A 2003 study in cattle reported an increase in ARA accompanied by an increase in EPA and total n-3 in plasma in response to n-3 supplementation (Burns et al., 2003), but a conflicting study noted a decreased ARA concentration in lactating dairy cows fed 5% fish meal diet (Heravi Moussavi et al., 2007). It appears that fish oil supplementation's effect on ARA serum concentrations is variable. In the current study, this increase may be due more to the level of ARA in the fish oil supplement (0.98% total fat) rather than as a consequence of the n-3 supplementation. Competition between metabolic n-3 and n-6 pathways for desaturase and elongase enzymes may also play a role. In diets high in n-6 fatty acids, LA and ARA serve as the dominant substrates and are incorporated into tissues in larger quantities than n-3 fatty acids. However, as dietary n-3 supplementation provides more EPA and DHA, enzymes are shifted toward the n-3 pathway and used to continue the metabolization process and incorporation into phospholipids, while any ARA that is present is not the dominant substrate and is not being metabolized further, perhaps leading to higher serum ARA concentrations. Additionally, n-3 fatty acids may be incorporated into tissues at a higher rate than ARA because they are more accessible, leaving ARA concentrations high in circulation.

Concentrations of serum EPA, DPA, and DHA in the FO mares peaked at d 14, but stayed significantly higher than the CONT group throughout the study. These increases due to fish oil supplementation in the equine are supported by previous studies in this laboratory (Kruglik et al., 2005; Poland, 2006), as well as other authors (O'Conner et al., 2001; Vineyard et al., 2005). The peak concentrations found at d 14 agree with results found by King et al. (2008) who reported an increase in plasma EPA and DHA concentrations by d 3 of marine-based n-3 supplementation, with peak concentrations being found as early as 7 d after initiation of supplementation in the equine. In contrast to King's results, our EPA and DHA concentrations stayed significantly higher in fish oil supplemented horses compared to control horses throughout the study. However, although still elevated, serum EPA, DPA, and DHA did decline after peak concentrations. It is possible that serum concentrations peak initially due to supplementation and then decline over time as n-3 fatty acids are absorbed into various fluids and tissues (Vineyard et al., 2010). From d 42 to the final day of the study, the decline of EPA, DPA, and DHA concentrations in serum becomes much more gradual, indicating that there may be a threshold level of n-3 fatty acids that the mare can absorb due to supplementation. The peak serum concentrations of EPA noted in this study are similar to peak plasma concentrations found

by King et al. (2008) where mares were fed a marine-based supplement with a similar concentration of EPA (17.81 g/mare/d). However, the supplement this study utilized had a higher concentration of DHA (21.32 g/mare/d) than our trial used, resulting in a peak plasma concentration of DHA that nearly doubled our recorded peak serum concentrations. In addition, a previous study from our laboratory that supplemented pregnant mares with comparable levels of EPA and DHA noted peak plasma EPA concentrations of nearly 25 µg/ml and peak plasma DHA concentrations of approximately 30 µg/ml (Poland, 2006). From these results, it appears that peak concentrations of fatty acids in circulation may partially depend on the amount supplemented. A human study involving fish oil capsule supplementation found that doubling the level of supplementation yielded a minimal additional response in plasma concentrations, suggesting that the ability for n-3 incorporation into phospholipids may become less efficient at higher levels of supplementation (Blonk et al., 1990).

This author is unaware of any previous reports on the PUFA content of equine follicular fluid or the effect of n-3 supplementation on equine follicular fluid. It appears that follicular fluid has a similar capacity to uptake dietary fatty acids as in circulation or other tissues. High-yielding dairy cows supplemented with flaxseed had approximately 5 fold greater ALA concentrations in follicular fluid than cows supplemented with sunflower oil and control cows (Zachut et al., 2010). Supplementation with fish oil in heifers resulted in increased EPA follicular fluid concentrations and decreased LA follicular fluid concentrations (Childs et al., 2008b). Our results reveal that the FO group showed an increase in n-3 fatty acids (ALA, EPA, DPA, and DHA) measured in follicular fluid when compared with the CONT group beginning at 59 days of n-3 supplementation. The follicular fluid sample was collected from a preovulatory follicle that was matured using hCG administration. This uptake of available fatty acids from circulation into the follicular fluid may occur gradually throughout the supplementation period or during the final maturation process just prior to ovulation. Collection of follicular fluid from various sizes of follicles, as well as preovulatory follicles, would be needed to pinpoint the time period when increases in follicular fatty acid content occurs in relation to the time of n-3 supplementation.

Serum hormone concentrations recorded for FO and CONT mares revealed that n-3 supplementation does have a limited effect on hormone levels in the mare. During the second estrous cycle, an increase in progesterone concentration was noted in the FO group at 3 and 5 d

after ovulation, although all values were in the normal range for this time period. This finding is supported by Furtney et al. (2009) who reported a tendency for increased progesterone plasma concentrations across estrous cycles in n-3 supplemented mares when compared to mares fed animal fat. Previous research in other species both supports and refutes this finding. Studies in cattle have reported no influence of n-3 supplementation on progesterone concentrations (Heravi Moussavi et al., 2007; Childs et al., 2008b), an increase in supplemented animals (Robinson et al., 1998; Staples et al., 1998), and a decrease in supplemented animals (Burke et al., 1997). A recent study in sows documented no effect of flaxseed supplementation on circulating progesterone concentrations (Farmer et al., 2010). The inconsistency in results could be due to numerous factors, including the varied supplementation levels, source of n-3 fatty acids, and physiological status of the animals used. In the current study, the increase in progesterone may be due to a more effective corpus luteum with luteal cells that have the ability to uptake more cholesterol to produce more progesterone when compared to horses fed a diet higher in n-6 fatty acids. It is possible that n-3 supplementation might also upregulate lipoprotein receptors present on luteal cells. The mechanism by which progesterone can be increased through n-3 supplementation is not clear, as n-3 supplementation has been documented to increase (Staples et al., 1998; Childs et al., 2008b) have no effect on (Heravi Moussavi et al., 2007) or even decrease (O'Connor et al., 2004; O'Conner et al., 2007b) circulating cholesterol and triglyceride levels, which act as precursors for steroid hormone production. Supplementation of n-3 fatty acids may also simply redistribute cholesterol stores, as fish oil is thought to decrease the efficiency of cholesterol ester transfer protein which serves to transport cholesterol esters from high-density lipoproteins (HDL) to low-density lipoproteins (LDL) (Nestel, 2000). Changes in enzymatic activity associated with steroidogenesis, via alteration of transcription factors, may be partially responsible (Wathes et al., 2007). Specifically, n-3 supplementation has been documented to inhibit COX-2 and increase steroid acute regulator (STAR) protein (Wang et al., 2003). Evidence also exists implicating an effect of n-3 supplementation on PPARs, which play a role in gonadal steroid synthesis (Wathes et al., 2007). Others have also speculated that increases in plasma progesterone due to fat-added diets may be due to a decreased clearance of progesterone from circulation rather than increased synthesis (Hawkins et al., 1995).

In contrast to progesterone, significant differences in estradiol-17β during the second estrous cycle were not found and all values were within the normal range at that point in the

estrous cycle. The differential effects of n-3 supplementation on steroid hormones shown in this trial may be due to selective alterations in steroidogenic enzymes that affect the conversion of cholesterol to progesterone but do not hinder or enhance the synthesis of estradiol-17β from testosterone.   Supplemented n-3 fatty acids may also be utilized in a different capacity prior to and during ovulation rather than influencing estradiol-17β production. Our estradiol results are in agreement with some work in cattle, in which plasma estradiol concentrations were not affected by fish meal supplementation (Heravi Moussavi et al., 2007; Childs et al., 2008b), but differs from another study in which plasma estradiol in lactating cows was increased with supplementation of a linseed-derived supplement providing ALA during the follicular phase (Robinson et al., 2002a). Conflicting results could be due to supplementation being provided in plant versus marine form as well as the physiological status of the supplemented animals.

Concentrations of LH during the second cycle were not different until d 3 post-ovulation, with LH decreasing in FO mares. Calcium soaps containing long chain fatty acids have been documented to increase (Sklan et al., 1994), decrease (Sklan et al., 1994), or have no effect (Lucy et al., 1992) on circulating LH concentrations in dairy cattle. Sklan et al. (1994) noted that PUFA supplementation increased circulating LH concentration in primiparious cows and decreased LH concentrations in multiparious cows during the follicular phase, indicating that the effect of PUFA supplementation on LH concentrations may vary with physiological status or age of animal. The difference noted in our trial may be due to individual mare differences, as abnormally high concentrations were also found in mares of both the CONT and FO groups compared to normal ovulatory concentrations during the estrous cycle. Additionally, differences in LH concentrations may relate indirectly to supplementation through IGF-1 concentrations, which will be discussed below.

During the third estrous cycle, serum hormone concentrations were not significantly different on the day of hCG administration. However, on the following day of aspiration, IGF-1 concentrations were significantly lower in the FO group when compared to the CONT group. This is the first time, to this author's knowledge, that an effect of n-3 supplementation on serum IGF-1 concentrations has been recorded in the equine. A recent study in heifers documented a dose-dependent increase in plasma IGF-1 concentrations in fish oil supplemented animals, with the highest concentration present at the highest level of supplementation (Childs et al., 2008b). Another trial in the same lab documented no effect of n-3 supplementation on plasma IGF-1

concentrations in beef heifers (Childs et al., 2008a). Supplementation levels and length of supplementation were similar between the two studies, but the study that found no effect of supplementation on IGF-1 concentrations utilized exogenous FSH to induce superovulation, possibly affecting final IGF-1 concentrations. The inconsistencies of the results cited above and results in the current study may be due to species differences or variations in supplementation levels and length. Insulin-like growth factor-1 is positively correlated with follicle selection and maturation, and is thought to be an important factor in the ovulatory process in the equine (Ginther et al., 2001; Ginther et al., 2008). Changes in IGF binding proteins in circulation have also been implicated as having a role in follicular growth and regression in cattle, sheep, and swine (Spicer and Echternkamp, 1995). Our finding may provide an alternate explanation to results found by Poland (2006), in which mares were fed a control diet, a DHA supplemented diet, and an EPA and DHA supplemented diet during pregnancy and early lactation. Poland noted that after parturition, EPA and DHA supplemented mares displayed a longer interval from foaling to the first postpartum ovulation than the DHA supplemented or control mares. Additionally, EPA and DHA mares held a preovulatory follicle for a longer period of time prior to ovulation than DHA supplemented or control mares. In addition to peripheral production, IGF-1 can also be synthesized within neurons of the brain and has been implicated to act as a direct regulator of GnRH neurons, generally having an excitatory or biphasic effect (Daftary and Gore, 2005). Lower IGF-1 concentrations in n-3 supplemented mares could affect gonadotropin and steroid hormone levels via reduced stimulation of the hypothalamo-pituitary-gonadal axis, subsequently delaying the final maturation process necessary to prepare a follicle for ovulation.

Serum estradiol-17β concentrations between the two groups during the third estrous cycle were not found to be significant, but a noticeable numerical difference on the day of follicular aspiration indicates that concentrations may have turned significant with a larger sample size or a longer supplementation period. It is tempting to deduce that IGF-1 and estradiol concentrations on aspiration day are related, as previous research in the equine has also noticed a positive correlation during follicular development (Bridges et al., 2002; Spicer et al., 2005). Additionally, a recent study noted that bovine endometrial cells treated with IGF-1 had an increase in estradiol-17β production, cell number, type 1 IGF-1 receptors, and follicle-stimulating hormone receptors (Murugan Mani et al., 2010).

Previous research evaluating equine follicular fluid has established hormone concentrations associated with preovulatory follicles (Gastal et al., 1999; Bogh et al., 2000; Tsukada et al., 2008). In large preovulatory follicles, estradiol-17β and prostaglandin levels are more concentrated than in circulation, along with detectable IGF-1 concentrations (Gerard and Monget, 1998; Davidson et al., 2002; Ginther et al., 2008). Follicular fluid samples were collected on the final day of the study to compare hormone concentrations with serum concentrations. Our results indicate that estradiol-17β and progesterone concentrations were within the normal range for a preovulatory follicle and not significantly different between the treatment groups, which contrasts with a recent study in ewes that noted increased progesterone concentrations in follicular fluid of n-3 supplemented ewes compared to n-6 supplemented ewes (Wonnacott et al., 2010). Follicular IGF-1 concentrations were lowered by n-3 supplementation, supporting the decreased serum IGF-1 concentrations that were also noted. This finding further implies that IGF-1 concentrations, which are important factors in the ovulatory process, can be altered by n-3 supplementation.

Follicular fluid concentrations of $PGF_{2\alpha}$ and PGE_2 were also similar between treatments and within the normal range for a preovulatory follicle. Theoretically, production of series 2 prostaglandins, like $PGF_{2\alpha}$ and PGE_2, would be suppressed in n-3 supplemented animals via the competitive nature of the n-6 and n-3 pathways. These separate pathways compete for the same enzymes and when n-3s are supplemented, enzymatic activity shifts toward the n-3 pathway at the expense of n-6 fatty acids (Mattos et al., 2000). Additionally, the PGHS-2 enzyme, which is essential to prostaglandin production, is known to convert EPA into series three prostaglandins in a slower and less efficient way than it converts ARA into series two prostaglandins (Kulmacz et al., 1994). Unless ARA production is significantly reduced, series two prostaglandins will continue to be synthesized at a normal level. The current study showed no difference between treatment groups in follicular ARA concentrations, which serve as the precursor fatty acid for series 2 eicosanoid production. This finding does not agree with our serum results, in which ARA was significantly higher in the n-3 supplemented group. This discrepancy between serum and follicular fluid results could point to a difference in how ARA is absorbed from serum and incorporated into other tissues compared to the n-3 fatty acids.

No previous studies were found documenting prostaglandin levels in follicular fluid of n-3 supplemented animals. Many studies have reported n-3 supplementation's effects on

prostaglandin in circulation or in endometrial tissue, but results in the literature are mixed. A study in exercising horses documented no effect of supplementation on plasma PGE_2 metabolites (Woodward et al., 2007), while leukocytes isolated from pregnant mares supplemented with n-3 fatty acids failed to stimulate an increase in PGE_2 in vitro in response to a lipopolysaccharide challenge, producing an anti-inflammatory effect (Skjolaas-Wilson et al., 2005). In contrast, intravenous infusion of n-3 fatty acids in sheep reduced plasma concentrations of PGE_2 within 4 days (Baguma-Nibasheka et al., 1999). Furthermore, an n-3 supplemented diet led to a decrease in serum PGE_2 concentrations in response to a lipopolysaccharide challenge in dogs (LeBlanc et al., 2008) and horses (Munsterman et al., 2005; LeBlanc et al., 2008). Subsequent research in rats revealed that rodents consuming EPA reduced ova release by 16%, had higher PGE_2 concentrations in ovarian tissue, and did not alter their expression of COX enzymes in the ovary (Broughton et al., 2009). In the bovine, previous research documented lower plasma $PGF_{2\alpha}$ or prostaglandin metabolite concentrations in n-3 supplemented groups in vivo in response to an oxytocin (Thatcher et al., 1997; Mattos et al., 2002; Mattos et al., 2004) or an oxytocin and estradiol challenge (Mattos et al., 2002). Lower prostaglandin production was also noted in n-3 supplemented bovine endometrial cells in vitro (Mattos et al., 2003). Supplementation with fish meal did not affect $PGF_{2\alpha}$ production in heifers with normal progesterone levels, but lowered synthesis in heifers with low luteal-phase progesterone (Wamsley et al., 2005). The discrepancies found in previous research may be due to slight differences in cycle length between animals, which may confound results (Wathes et al., 2007). Additionally, Heravi Moussavi et al. (2007) suggested that the net inhibition of $PGF_{2\alpha}$ by n-3 supplementation may depend on the amount of n-6 fatty acids reaching the target tissue.

In this trial, follicular data was collected via ultrasound during the second and third estrous cycle. During the second estrous cycle, follicular growth patterns measured were not different between the FO and CONT groups. These results contrast with Poland (2006) in which supplemented mares had an increased ovulatory interval and displayed a follicle measuring ≥35mm longer compared with the control group. The conflicting results may be due to the differences in mare physiological status, the n-3 supplementation level, length of supplementation, or the EPA to DHA ratio. In Poland's trial, supplemented mares were ultrasounded for follicular traits during their first postpartum estrous period, indicating that mares which have recently undergone parturition may mobilize their fatty acid stores differently

than open and cycling mares. Furthermore, mares from a comparable group in Poland's trial were supplemented beginning 60 d prior to the expected foaling date through first postpartum estrous cycle, providing slightly different ratios for a longer period of time.

Literature in other livestock species regarding n-3 supplementation and follicular responses has been mixed. Studies in cattle have noted both a stimulation in follicular activity as a result of supplementation (Lucy et al., 1992; Abayasekara and Wathes, 1999b), as well as no effect of n-3 supplementation on follicle number or size (Petit et al., 2002; Robinson et al., 2002b; Petit et al., 2004; Heravi Moussavi et al., 2007). Another study in n-3 supplemented cattle has documented a decrease in the interval from calving to the first postpartum ovulation when compared with controls (Hightshoe et al., 1991; Lucy et al., 1992), while dairy cows supplemented with flaxseed had a longer interval from $PGF_{2\alpha}$ administration to behavioral estrous, delaying the luteal phase of the subsequent estrous cycle (Zachut et al., 2010). Gilts fed a dietary n-3 supplement showed no difference in ovulation rate or litter size when compared with controls (Estienne et al., 2006), and sows supplemented with flaxseed had a similar weaning to estrus interval as controls (Farmer et al., 2010).

In the current trial, the lack of significant differences in follicular traits measured may also be due to the length of supplementation ($\approx$ 46 days) not being adequate enough to cause changes in follicular growth and ovulation time during the second estrous cycle. This theory is supported by a study assessing n-3 supplementation's effects on plasma, red blood cell membrane composition, and immune function in yearling horses in which the authors speculated that the time required for complete incorporation of dietary n-3 fatty acids into cell membranes may be longer than that of plasma (Vineyard et al., 2010). During the third estrous cycle, the size of the aspirated follicle was smaller in the FO mares when compared to the CONT mares in response to hCG administration. This may be an indication that supplementation affects final maturation of the follicle in some way, possibly by limiting IGF-1 production, or that an extended period of dietary supplementation may be necessary in the mare before differences can be noted.

Conclusions and Implications

In conclusion, n-3 fatty acids can be successfully incorporated into equine serum through dietary supplementation and can affect the equine species in multiple ways. With increased concentrations of EPA available for eicosanoid production, a shift from pro-inflammatory to anti-inflammatory products can occur. The suppression of inflammatory responses may have enormous potential for the treatment of equine joint ailments, developmental orthopedic diseases, and pulmonary inflammation (Munsterman et al., 2005; Khol-Parisini et al., 2007). In this study, n-3 supplementation increased ARA, EPA, DPA, and DHA levels in serum and increased ALA, EPA, DPA, and DHA in follicular fluid. No significant pattern was identified for LA and ALA in serum. With incorporation of dietary fatty acids, n-3s are more available as a substrate for enzymes in the omega fatty acid pathways. This allows for a shift in production towards the n-3 pathway.

Modifications in fatty acid profiles also altered hormone concentrations in serum and follicular fluid. The effect of dietary n-3 supplementation on hormone concentrations are potentially important to many systems in the body, but especially to the reproductive system. Changes in the hormonal balance have the potential to affect follicular growth and development, estrous cycle length, and also CL function and duration. Concentrations of IGF-1 were altered in both serum and follicular fluid, with supplemented mares having lower concentrations than control mares. Insulin-like growth factor-1 is an essential component to the ovulatory process, and decreasing concentrations prior to ovulation by feeding n-3s could postpone ovulation by affecting other hormonal levels or the maturation of the follicle directly. Delayed ovulation would make it more difficult for the timing of ovulation to be identified for breeding purposes, especially if using artificial insemination, with a potential negative consequence on pregnancy rates.

Although the effect of dietary n-3 supplementation on CL properties and the luteal phase were not noted in this study, serum progesterone concentrations increased in n-3 supplemented mares immediately after ovulation. Increased progesterone concentrations may indicate an increased capacity for the luteal cells of the CL to produce progesterone or a larger CL comprised of more luteal cells may be present. Supplementing older mares or mares that have a history of early pregnancy loss with n-3 fatty acids may be advantageous to increase pregnancy rates and decrease the need for a supplemental progesterone regimen during early pregnancy.

In the current trial, no changes in follicular dynamics were identified until the third estrous cycle, supporting the conjecture that a longer supplementation period, similar to the study by Poland (2006), may be needed to notice changes in follicular traits between the treatment groups. This author's recommendation for similar future research is to allow for supplementation over at least three full estrous cycles so that follicular traits can be documented at each estrous cycle and the length of supplementation necessary to induce any changes can be more accurately identified. Care should be taken to ensure that the mares are not transitional during the collection of data. On the day of aspiration, a smaller preovulatory follicle was measured in the FO group compared with the CONT group. This may indicate a slower maturation rate in supplemented animals in response to hCG, which may result in delayed ovulation.

A limited amount of research is currently available on marine-derived n-3 supplementation of horses and conflicting results prevent researchers from making general recommendations to the industry. Many supplements currently available are associated with high costs and storage requirements. Some odor and palatability issues have also been linked with marine-derived supplements, although most mares used in the current study readily accepted the fish oil supplement after a short acclimatization period during synchronization. Until researchers can provide more concrete answers, horse owners may be reluctant to pay for expensive supplements that are still an emerging area of research in the equine.

This study confirmed previous research in this laboratory and others that dietary n-3 supplementation rapidly alters serum fatty acid concentrations. Research on n-3 supplementation's effects on reproductive parameters has just begun to uncover multiple possibilities in the mare. Equine follicular fluid fatty acid profiles were also impacted through supplementation, with hormonal concentrations of both serum and follicular fluid being altered. Our results did not confirm the findings of Poland (2006) on follicular retention and delayed ovulation, but differences in preovulatory follicular size during the day of aspiration indicate that further research needs to be done to determine n-3 supplementation's effects on estrous cycle characteristics including the effect of supplementation duration on follicular dynamics. Additionally, future research with more intensive collection of serum and follicular fluid for hormone evaluation would be helpful in making further conclusions as to the mare's hormonal response to n-3 supplementation. Currently, no recommendations can be given in regards to

length of supplementation or the ideal combination of EPA and DHA in supplements as it impacts estrous cycle characteristics in mares. Unlimited opportunities are available for researchers to discover the impact of n-3 supplementation on the equine species and educate the industry on the potential of these marine-derived products.

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Appendix A - Fatty Acid Profiles

Table A.1 Fatty acid profile of FO supplement (expressed as percent total fat)

Fatty Acid[ab]	% Total Fat
C14:0	9.41
C14:1	0.15
C15:0	0.64
C16:0	20.02
C16:1	11.34
C17:0	0.42
C17:1	1.77
C18:0	3.41
C18:1t9	0
C18:1n9	6.77
C18:1n7	3.3
C18.2	**2.43**
C18:3n3t	**1.08**
C18:4n3t	2
C20:0	0.28
C20:1n9	1.02
C20:3n3	0.11
20:4n6	**0.98**
20:4n3	1.18
C20:5n3	**13.54**
C22:0	0.17
C22:1n9	0.18
C22:5n3	**2.43**
C22:6n3	**7.29**
C24.0	0.12
C24:1n9	0.23
C24.0	0.12
C24:1n9	0.23

[a]Analysis provided by JBS United, Sheridan, IN

[b]Primary n-6 and n-3 fatty acids of concern are bolded

Table A.2 Fatty acid profile of brome hay, grain, and corn oil (μg/g feed as is)

| Fatty Acid[ab] | Feedstuffs | | Supplements |
	Grain Mix	Brome Hay	Corn Oil
C6:0	25.50	10.16	303.37
C8:0	104.31	35.45	1033.08
C10:0	0.00	0.00	0.00
C11:0	18.80	13.14	0.00
C12:0	10.45	31.06	61.65
C14:0	60.05	22.32	376.95
C14:1	0.00	0.00	0.00
C15:0	19.34	8.00	120.80
C15:1	0.00	0.00	0.00
C16:0	5230.77	546.49	110022.91
C16:1	81.70	8.96	1087.03
C17:0	53.71	14.54	710.87
C17:1	0.00	0.00	0.00
C18:0	628.96	55.41	18700.34
C18:1n9t	0.00	0.00	21.83
C18:1n10t	0.00	0.00	0.00
C18:1n11t	0.00	0.00	190.05
C18:1n9c	8242.49	209.43	258352.44
C18:1n11c	334.63	14.78	6170.64
C18:2n6t	0.00	0.00	0.00
C18:2n6c	**13266.15**	**567.71**	**528143.00**
C20:0	136.30	47.37	4283.09
C18:3n6	0.00	0.00	0.00
C20:1	149.92	9.58	2517.09
C18:3n3	**859.80**	**456.24**	**11071.30**
CLA 9c, 11t	0.00	0.00	0.00
C21:0	16.38	7.28	106.43
CLA 10t, 12c	0.00	0.00	0.00
CLA 9c, 11c	14.20	25.93	0.00
CLA 9t, 11t	9.27	3.83	240.73
C20:2	24.03	0.00	570.71
C22:0	100.72	63.41	1461.83
C20:3n6	0.00	0.00	108.14
C22:1n9	22.68	4.17	109.99
C20:3n3	13.51	0.00	0.00
C20:4n6	**0.00**	**4.63**	**0.00**
C23:0	25.91	14.75	235.03
C22:2	0.00	0.00	0.00
C24:0	169.32	69.08	1763.72
C20:5n3	**0.00**	**0.00**	**0.00**
C24:1	17.75	6.51	237.03
C22:5n3	**0.00**	**0.00**	**0.00**
C22:6n3	**0.00**	**0.00**	**0.00**
Total Fatty Acids	29636.64	2250.24	948000.05

[a]Analysis done by gas chromotography

[b]Primary n-6 and n-3 fatty acids of concern are bolded