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DIETARY SUPPLEMENTATION OF OMEGA-3 FATTY ACIDS AND SUBSEQUENT EFFECTS ON FRESH, COOLED, AND FROZEN SEMINAL CHARACTERISTICS OF STALLIONS

A Thesis

by

SICILIA TATIANA GRADY

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

MASTER OF SCIENCE

August 2008

Major Subject: Animal Science

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Approved by:

Chair of Committee,	Clay A. Cavinder
Committee Members,	Steven P. Brinsko
	David W. Forrest
Head of Department,	Gary R. Acuff

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ABSTRACT

Dietary Supplementation of Omega-3 Fatty Acids and Subsequent Effects on Fresh, Cooled, and Frozen Seminal Characteristics of Stallions. (August 2008)

Sicilia Tatiana Grady, B.S., University of California-Davis

Chair of Advisory Committee: Dr. Clay A. Cavinder

The use of cooled and frozen/thawed semen offers many advantages to breeders. However, many stallions produce spermatozoa that are unable to endure the stresses of cooling/storage and freezing/thawing. Improving the quality and viability of equine spermatozoa via appropriate dietary manipulation could make these stallions commercially viable for cooling or cryopreservation. To evaluate whether spermatozoa quality and viability can be improved by supplementation of omega-3 fatty acids, and if improvements can be made by altering the sources of these fats, nine miniature stallions were placed into 1 of 2 treatment groups and fed either a fish- or algae/flaxseed-based supplement which was added to the basal concentrate. Motion characteristics, membrane integrity and morphology of spermatozoa in fresh, cooled/stored (24 and 48 h), and frozen/thawed semen samples were analyzed. When comparing spermatozoa obtained from stallions in each treatment, no differences were found ($P > 0.05$) in motility, percentage of membrane intact spermatozoa, and percentage of morphologically normal spermatozoa of stallions. Overall, omega-3 supplementation did not appear to have a beneficial effect on offsetting the harmful effects of the cooling and freezing processes.

However, when analyzing the data of one stallion that had < 40% progressive motility (PMOT) after 24 h of cooling and storage, a significant increase was observed in total motility, and progressive motility of fresh and 24 h cooled/stored spermatozoa was observed when supplemented with the fish-based supplement. Thus, omega-3 fatty acid supplementation may be most beneficial for stallions that produce lower quality ejaculates. However, further studies should be conducted, with a larger sample size, in order to substantiate these findings.

To my mother

"May all that I do in the remainder of this life assist in your understanding of how loved and admired you are, how much you have inspired me, and how greatly I value your support."

ACKNOWLEDGEMENTS

"Life will go on and we will all strive to become better people; make it a point to remember who helped you get there."

First of all, I wish to express my greatest appreciation to my graduate advisor, Dr. Clay A. Cavinder, for his patience, guidance, dedication and support throughout my graduate career at Texas A&M University. It has been an honor and pleasure working with such a great person, mentor, and researcher. I would like to thank the members of my graduate committee: Dr. Steven P. Brinsko, for his advice and help with data analysis, and Dr. David W. Forrest, for his help and support with my thesis research. I would also like to thank Dr. Jason Sawyer for his much appreciated help with data analysis, and Dr. Thomas Spencer for providing the facilities and equipment for fluorescence microscopy. Furthermore, I am extremely grateful to Sheila Teague for sharing her much needed advice and expertise during this project.

I would like to extend a sincere thank you to Jen Seale for her help and dedication to this project, and her many hours of hard work spent at the breeding barn. A special thank you is extended to Dr. Héctor Gutiérrez for his advice and help with statistical analysis, but most importantly for his friendship.

A very special thank you is offered to my father, William, and grandparents, H. Alan and Jean, for their endless support through my academic journey. I also thank my mother, Laura, and siblings William, John, and Jean for their love and encouragement. I realize that without all of you I would not be where I am today. Thank you to Celia for

giving me a “home away from home” and F. Tamm for his friendship, advice and for sharing his love for horses with me. A heartfelt thank you is intended for Anais, Jimena and Yara for their friendship throughout all these years.

Finally, I wish to thank Mr. and Mrs. Grizzaffi from Circle G Miniatures, Mr. and Mrs. Willette from RJ7 Miniatures, and Alyssa Plackemeier for providing use of the stallions utilized in this study and JBS United for donating the supplements.

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CHAPTER I

INTRODUCTION

In the United States, most breed registries have allowed the use of cooled and/or frozen/thawed semen for artificial insemination for many years. The use of these technologies has increased among horse breeders because of the various advantages that artificial insemination with cooled or frozen semen provides over natural mating. However, the widespread exchange of genetic material among breeding populations with preserved spermatozoa is limited by a relatively short-lived fertilizing capacity. Cooled semen from most stallions can be used with good results for approximately 2 d after collection and dilution [1]. Furthermore, about one-third of breeding stallions produce semen that has poor quality and reduced motility after cooling/storage and/or freezing/thawing [2] because spermatozoa are sensitive to many environmental factors, including light, temperature, physical damage, and a variety of chemicals [3].

The plasma membrane is a selectively permeable layer that covers the entire surface of spermatozoa and comprises their outermost component. The plasma membrane consists of three zones: a lipid bilayer, a phospholipids-water interface, and a glycocalyx [4]. The majority of the lipids of stallion spermatozoa are phospholipids which are largely composed of highly polyunsaturated fatty acids (PUFAs). Polyunsaturated fatty acids are essential for structure and function of the plasma membrane. The ratio of phospholipids with polyunsaturated acyl chains, and the nature

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This thesis follows the style and format of Theriogenology.

of the phospholipids determine the fluidity of the plasma membrane [5]. Integrity of the plasma membrane is of crucial importance for the functioning of spermatozoa. The plasma membrane forms a semipermeable barrier for molecules and maintains and modulates the intracellular composition. It protects the cells from extracellular influences, both in the male and the female reproductive tracts, and from non-physiological influences such as the addition of extenders during spermatozoa preservation. Adequate functioning of the plasma membrane is essential for survival of spermatozoa until fertilization [6]. This structure is also essential for normal capacitation, binding to the oocyte's zona pellucida, acrosome reaction, and actual fusion of the gametes [7,8].

Spermatozoa are unique in that they have limited biosynthetic capabilities; therefore, spermatozoa rely primarily on extracellular substrates to meet their energy requirements. Energy is used by spermatozoa to initiate catabolic processes such as glycolysis, and to maintain motility, ion balance, and vital cell functions. The energy used by spermatozoa is principally derived from carbohydrates; however, other exogenous substrates include lactic acid, glycerol, amino acids, and fatty acids [4]. The incorporation of PUFAs from the diet to the plasma membrane of mammalian [9,10,11,12] and fowl [13] spermatozoa has been shown to be effective. Thus, it is possible to alter the structure and function of the plasma membrane by making changes in dietary fat intake.

Spermatozoa from all mammals contain high levels of PUFAs, especially docosahexanoic acid (DHA; 22:3 n-3, an omega-3 fatty acid) and docosapentanoeic acid

(DPA; 22:5 n-6, an omega-6 fatty acid), but the phospholipid bilayer of spermatozoa from different species has a distinctive PUFA composition. In most mammals DHA is the major PUFA component of the membrane, but spermatozoa from stallions and boars have a higher proportion of DPA [14]. Studies with boars [15] and humans [16,17] have shown that a high DHA to DPA ratio in semen results in enhanced fertility, while higher DPA levels result in reduced fertility.

The objective of the present study is to compare the effects of two omega-3 fat supplements on the quality of fresh, cooled/stored and frozen/thawed stallion semen by evaluating motion and morphological characteristics, and membrane integrity of spermatozoa. We hypothesize that the addition of omega-3 fatty acids to stallion diets may enhance the quality of their spermatozoa.

Rationale for Present Research

Considerable interest exists in the equine breeding industry to breed mares using cooled or frozen semen. However, for many stallions the current techniques for processing, cooling, packaging and freezing semen result in a loss of viability and fertility. Furthermore, commercial diets fed to stallions commonly contain fat added in the form of corn grain or corn oil both of which are high in omega-6 fatty acids. If DHA is essential for optimal fertility in stallion spermatozoa, as it is in other species, then it is possible that standard stallion diets provide a suboptimal supply of DHA and its precursors for the maximal production of viable spermatozoa. The addition of PUFAs, in particular omega-3 fatty acids, through the dietary supplementation of fats may be an

alternative to increase semen quality in stallions. Moreover, comparisons of different fat sources and their effect on motility, morphology, and membrane integrity could lead to more efficient dietary fat supplementation of stallions. Eventually, research in this area could provide a better understanding of the mechanisms by which addition of PUFAs to the diet may enhance stallion seminal characteristics. Thus, the objective of the research presented herein is to determine and compare the effects of 2 dietary fat supplements on the seminal characteristics and quality of ejaculates collected from stallions by analyzing the quality of fresh, cooled/stored (24 and 48 h), and frozen/thawed semen samples.

CHAPTER II

LITERARY REVIEW

Spermatozoa Structure and Viability

For spermatozoa, which are end-differentiated cells with restricted biosynthetic capacity, limited ability to withstand the cryosurvival severely affects future fertility [18]. Exposure of spermatozoa to cooling and freezing causes disruption to the plasma membrane due to thermal, mechanical, chemical and osmotic stresses as well as intracellular changes brought about by dehydration associated with ice formation. In addition, components of the extender required for dilution not only alter the environment of the spermatozoa and the cell itself, but also the biological responses of spermatozoa to cooling and freezing [2].

In order for a spermatozoon to fertilize an ovum, it must retain at least six general attributes after cooling or freezing and thawing: normal metabolism for production of energy, progressive motility, intact cellular membranes, presence of acrosomal enzymes that are essential for penetration of the structures surrounding the egg, intact surface-associated proteins of the plasma membrane that are important for survival of the spermatozoon in the female reproductive tract and for attachment of the spermatozoon to the egg plasma membrane at fertilization, and uninjured nucleoprotein [2,3]. Destruction of spermatozoal components associated with one or more of these functions will reduce or abolish fertility, viability and motility of spermatozoa [19]. Most spermatozoal damage results from altered membrane structure due to thermal, mechanical and chemical stresses, osmotic shock, dehydration, salt toxicity, intracellular

ice formation, fluctuations in cellular volume/surface area, or metabolic imbalance [3,20].

Cooling

Spermatozoa from individual stallions differ in their ability to survive the cooling/storage and freezing/thawing processes by any given procedure [2]. These differences appear to be related to the individual composition of the plasma membrane [16,21]. The reasons for individual variation in resistance to cooling and freezing/thawing are still unclear, but the reduced fertilizing capacity of cooled and frozen semen can mainly be attributed to changes in membrane structure which result from the processes involved with cooling and freezing [20,22].

Although cooling stallion semen provides longevity of spermatozoal motility by reducing the metabolic activity of cells, the cooling process itself is not harmless [23]. When semen is cooled from 20˚ C to 1˚ C, a series of irreversible changes, collectively known as “cold shock”, occur in spermatozoa [20,24], thus causing damage to the cells. Cold shock damage to spermatozoa is attributed to a temperature-induced, fluid-ordered phase transition that causes lipid structural changes in the plasma membrane [2,3,12,25]. Lipid phase separation events also cause integral membrane proteins to cluster which is expected to alter function especially of proteins which undergo a structural modulation to carry out their function, such as ion channel proteins, which is why membrane permeability is increased after cooling [26,27]. Cold shock, which can be lethal, is evidenced by the presence of many spermatozoa swimming in circular paths, premature

loss of motility, decreased energy production, increased plasma membrane permeability, and loss of intracellular molecules and ions [24,28,29].

Cellular membranes must withstand several stresses during freezing and thawing. These stresses include the addition of cryoprotectants prior to freezing, volumetric changes and associated membrane stretching and shrinkage in response to hyperosmotic cryoprotectant solutions, as well as freeze-induced dehydration, thermotropic phase transitions in membrane phospholipids, and intracellular ice formation [20]. When semen is cooled below 0˚ C, extracellular crystals begin to form. This results in an increased concentration of salts in the extracellular fluid causing osmotic pressure differentials across all cell membranes. Water moves from the inside of spermatozoa to the extracellular environment and, thus, spermatozoa become dehydrated [2]. As a result of osmotic dehydration, the plasma membrane can undergo ultrastructural changes which result in deformation and increased permeability, and therefore, cellular damage [2,18]. In addition, if water cannot leave spermatozoa rapidly, intracellular ice formation occurs causing death or irreversible damage to the cells. To allow survival of spermatozoa through the freeze/thaw process, permeating cryoprotective agents, such as glycerol, must be added [2]. The water volume of a cryopreserved cell changes by contraction and then recovery when the molar concentrations of glycerol are added to the spermatozoa suspension, a second contraction when extracellular ice formation increases external salt concentration, return to the original volume as extracellular ice melts to return external salts to normal concentration, and expansion and recovery as glycerol is removed during processing/insemination [18]. Cell plasma membrane accommodation to

volume changes is not a simple matter because the elasticity of the bilayer is minimal. It can expand or contract by less than 5% before it bursts and loses its structural integrity [30].

Although cryoprotectants are essential for successful freezing of semen, they may also adversely affect cellular membranes [19,31] and render spermatozoa infertile even though the spermatozoa may be motile after thawing [19]. The major beneficial effects of cryoprotective agents such as glycerol are extracellular [2]. However, glycerol also enters and resides in the cell membrane and cytoplasmic interior and has a direct effect on the plasma membrane in addition to its osmotic effect on the cell [20]. Glycerol binds directly to phospholipid headgroups reducing membrane fluidity [32] and interacts with membrane-bound proteins and glycoproteins [33] causing clustering of intramembranous particles [34]. Any change in stallion spermatozoa induced by cold shock will exacerbate damages induced by freezing/thawing [35]. In addition to crystal formation, damage due to cryopreservation also includes loss of progressive motility [36,37,38,39], chromatin [40] and morphological alterations [41], and acrosome changes [37,42]. These alterations lead to a decrease in spermatozoa lifespan, thus decreasing the longevity and fertilizing capacity of spermatozoa when inseminated [37]. Furthermore, centrifugation, removal of seminal plasma, and resuspension of spermatozoa in freezing extender are processes often used to concentrate spermatozoa for freezing but may also reduce the percentage of motile spermatozoa or increase the percentage of cells with damaged plasma and/or acrosomal membranes [2]. During these steps, irreversible damage may occur to the plasma membrane of spermatozoa that is not detectable by

evaluation of motility [43].

Temperature is another factor that affects the structure and function of the plasma membrane causing changes in its flexibility or fluidity [2]. Membranes are “fluid” at body temperature, meaning that phospholipids are free to move laterally in the lipid bilayer [44,45,46]. It is suspected that the nature, position, and distribution of phospholipids, as well as the nature of their fatty acyl side chains, determines the fluidity of the membrane [5]. Phospholipids with highly unsaturated fatty acyl side chains normally have the general shape of an inverted cone. In the presence of cold temperatures their shape becomes more sharply conical resulting in structural changes that are considered to decrease the fluidity of the membrane [12] thus causing loss of membrane barrier function and increasing the permeability of the plasma membrane [2]. These changes in structure persist during further cooling and may not be reversed upon reheating [45].

Omega-3 fatty acids contain a double bond in the third position from the methyl group and because of their unique structural properties are crucial for cell membranes [47]. Omega-3 and omega-6 fatty acids cannot be interconverted [48] but when large amounts of long- chain omega-3 fatty acids are ingested, there is a high incorporation of eicosapentanoeic acid (EPA 20:5 n-3 an omega-3 fatty acid) and DHA in membrane phospholipids. By the increased amount of PUFAs, the physical characteristics as well as the function of the membranes may be altered [49]. Unsaturated fatty acids are essential because of PUFA incorporation in membrane phospholipids and their contribution to lipid structure [12]. Greater resistance to cold-shock and freeze-induced

damage has been noted for species with spermatozoa membranes characterized by a high degree of saturation in the phospholipid-bound acyl moieties [50].

The phospholipid membrane of mammalian spermatozoa has a characteristic fatty acid composition, the most distinctive feature of which is a very high proportion of long chain PUFAs. This characteristic varies by species as well as by individual [24]. Of particular interest are the fatty acids with 22 carbon atoms, synthesized by omega-3 and omega-6 acids, which are essential constituents of reproductive organs [51,52] and particularly spermatozoa [52,53]. However, the lipid composition, and especially the fatty acid composition of the membrane may vary by effects of different factors. Membrane lipids and their fatty acid composition change with the fatty acid concentration of the diet [12,53,54]. Dietary linoleic acid (LA; 18:2 n-6, an omega-6 fatty acid), the parent compound of DPA and other omega-6 fatty acids, and linolenic acid (ALA; 18:3 n-3, an omega-3 fatty acid), the precursor of DHA and other omega-3 fatty acids modify the fatty acid composition of phospholipids of different cells and tissues in diverse ways [54]. Linoleic and linolenic acids are not synthesized in mammals but when they are included in the diet they are metabolized to longer chain omega-6 and omega-3 fatty acids [55,56]. These acids are then made available for phospholipid biosynthesis [55]. High concentrations of linoleic acid are found in vegetable oils such as corn and soybean oils [57]. Linolenic acid, on the other hand, is found in fish oils and linseed oils [47]. As previous reasons state, modifications of the fatty acid composition of the lipid membrane, and thus its physiological function can occur through dietary fat intake [12,53,54]. For example, rats normally have high levels

of omega-6 and low levels of omega-3 fatty acids in their testes but this can be reversed by fish oil feeding. Fish oil-fed rats exhibited greater testes maturation as evidenced by greater spermatogenesis. This was associated with an increase in DHA from 0.6 to 10.8% and a decrease in 22:5 n-6 from 19.9 to 8.6% suggesting that DHA can functionally replace 22:5 n-6 in the rat testes [58]. Recent research also suggests that DHA is important for normal spermatozoa function.

Several studies with fats containing high DHA concentrations have shown to increase certain spermatozoa characteristics, including percentage of motile cells, of normal cells, and of cells with normal acrosome scores when fed in the diets of stallions [57,59], boars [15], roosters [60,61], and humans [16,17]. Feeding a DHA-enriched nutriceutical to stallions resulted in improvements in motion characteristics in cooled/stored and frozen/thawed semen of stallions with marginal fertility [57]. Mean spermatozoa concentration in ejaculates of stallions being fed the nutriceutical was 1.8 times higher than when stallions were fed the control diet. After 48 h of cooling/storage, total and progressive motion characteristics were improved by feeding the nutriceutical (43 versus 54%, $P = 0.07$ and 33 versus 43%, $P = 0.06$, respectively). Both total and progressive motility of frozen/thawed semen were also improved by treatment (21 versus 25%, $P = 0.03$ and 17 versus 20%, $P = 0.03$, respectively) [57]. Stallions with poor quality ejaculates have also benefited from supplementation of fatty acids from marine sources [59]. Supplementation resulted in increased daily spermatozoa output (46%, $P < 0.05$) and higher ($P < 0.05$) percentage of morphologically normal spermatozoa. Supplementing boar diets with tuna oil significantly increased the proportion of

progressively motile spermatozoa (69.2 versus 74.5%, $P < 0.01$) and the proportion of spermatozoa with a normal acrosome score (44.3 versus 49.9%, $P < 0.001$), and reduced the proportion of spermatozoa with abnormal morphologies (12.1 versus 5.4%, $P < 0.01$) [15]. Evaluation of the ratio of dietary omega-6 to omega-3 fatty acids on the reproductive performance of roosters using fish oil supplementation resulted in linear increases ($P < 0.05$) of semen volume ($R^2 = 0.88$), spermatozoa motility ($R^2 = 0.91$) and vigor ($R^2 = 0.93$) in the roosters consuming diets containing higher levels of omega-3 fatty acids [60]. Dietary supplementation with linolenic acid has also resulted in a significant increase in fertility of roosters (83 to 97%) which corresponded with an increased amount of 22:5 n-3 and a decreased n-6:n-3 ratio. Reductions in the amount of DHA in spermatozoa were associated with reduced spermatozoa numbers and impaired motility and fertilizing ability [61]. In human spermatozoa, omega-3 fatty acids have been found to enhance optimal fertility [16]. Reductions in the amount of omega-3 fatty acids in the plasma membrane of human spermatozoa are used as a marker of impaired fertility in men because these reductions are correlated with decreased spermatozoal concentration and reduced number of spermatozoa with progressive motility and normal morphology. A linear correlation between the amount of DHA in semen and the density of spermatozoa along with a linear correlation between DHA content, and the number of motile spermatozoa have also been shown to exist in humans [17].

Motility

Spermatozoal motility has been considered a major criterion of semen analysis for predicting potential male fertility and is, therefore, emphasized heavily in stallion fertility examinations. In evaluating motility with most species, spermatozoa are classified as non-motile, progressively motile, or non-progressively motile. A progressively motile spermatozoon swims forward in essentially a straight line, whereas a non-progressively motile spermatozoon swims in an abnormal path such as in tight circles [4]. After passage through the epididymis, spermatozoa are motile cells. Motility becomes critical at the time of fertilization because it allows and facilitates passage of the spermatozoon through the zona pellucida. Motility indirectly evaluates the ability of midpiece mitochondria to propel the cell [62]. Hence, assessing the motile fraction of spermatozoa in a population is perhaps the most widely-used measure of semen quality. Traditionally, motility has been assessed by visual estimation using a microscope. However, the recent development of computerized systems for evaluation of motility have provided an objective approach for measuring selected motion characteristics in stallions, thus eliminating the element of subjectivity [63].

Motile spermatozoa are not always fertile [19]. Although motile spermatozoa almost certainly have an adequate production of energy, other important aspects affecting cell functionality may be altered during the cooling and freezing processes [2]. As a result, cooled stallion spermatozoa tend to be both less motile and fertile than spermatozoa used fresh [64]. An important reason for the decrease in fertility of stallion semen during storage is the peroxidation of spermatozoa membrane lipids [65].

Immobile or poorly motile spermatozoa produce more lipid peroxides and exhibit a higher rate of endogenous lipid peroxidation than spermatozoa classified as normal and fully motile. However, ejaculates with poorly motile spermatozoa tend to have a lower concentration of DHA than those with good motility [17]. Polyunsaturated fatty acids are highly susceptible to peroxidation and lipid peroxides are highly toxic to living cells [66,67]. Spermatozoa are very susceptible to lipid peroxidation due to their high content of polyunsaturated fatty acids [68]. Peroxidation of spermatozoa has been shown to cause extensive structural alterations especially in the acrosomal regions of spermatozoa and irreversible loss of motility and fertilizing ability [69]. Lipid peroxides inhibit or destroy certain enzymes [70,71]. The loss of activity of membrane-bound enzymes is due to the loss of membrane structure [72]. Although there is data that demonstrates the important role of PUFAs in the maintenance of membrane integrity and how a relatively small amount of peroxidation of PUFAs leads to extensive loss of enzyme activity (50% of enzyme inactivation results from destruction of only 8% of DHA content) [73], the mechanism of lipid peroxidation has not been completely understood yet [17]. It has been reported, however, that peroxidation of PUFAs is the major cause for the loss of spermatozoal motility and fertilizing ability [74,75] by altering membrane fluidity and fuctional integrity [76].

Morphology

Morphological examination of spermatozoa is a valuable method for evaluating potential stallion fertility, but it requires objective classification and strict standards for

classifying abnormal spermatozoa [77]. Spermatozoal morphology can easily be evaluated in the liquid-preserved state using buffered formol-saline (BFS) under a phase [6] or a differential interference contrast (DIC) microscope [78,79]. These two techniques are most reliable because they reveal the structure of the heads, acrosomes, and mid-pieces in addition to abnormalities such as bent or coiled tails [6,78,79]. Typical features of normal stallion spermatozoa include an asymmetrical head, abaxial position of the tail, an acrosome of small volume, and presence of microtubules in the neck [80]. Abnormalities such as bent or coiled tails, which are the most obvious signs of structural damage [81], may be caused by stress such as osmotic shock [82] or may be due to structural malformation of organelles of the tail [83] and are generally considered to be incapable of fertilization. Most ejaculates of good fertility contain only a small proportion of abnormal spermatozoa resulting mainly from errors of spermatogenesis [81]. Abnormalities of spermatozoa can also be caused by structural changes occurring after spermatogenesis and usually after maturation of spermatozoa in the epididymis. These initially involve degeneration of organelles such as the acrosome or mitochondria, or bending or coiling of otherwise structurally normal tails [82]. Bent and coiled tails can also result from the plasma membrane around the tail swelling and exerting pressure on the motor apparatus thus altering its shape during the increase in volume of the cells [82,84]. The process is a response to adverse conditions such as osmotic stress [82], inhibition of metabolic activity [85], or dilution [86]. Bending and coiling do not appear to be reversible, so the process is most likely followed by death [81].

Membrane Integrity

The integrity of the plasma and acrosomal membranes is of crucial importance for the functioning of spermatozoa. The plasma membrane protects the cell from the extracellular environment, either in the male or female genital tract, and from non-physiological influences such as addition of extenders during spermatozoa preservation. Its integrity and adequate functioning are essential for survival of spermatozoa until fertilization [6]. Assessment of membrane integrity is a crucial step in the evaluation of the viability of spermatozoa due to its role, not only as a cell boundary, but also for its function in cell-to-cell interactions (for example between spermatozoa and the female genital tract, and between spermatozoa and the ovum) [7]. Alteration of the membranes affects spermatozoal function in the genital tract (specifically attachment to oviductal epithelial cells, capacitation, acrosome reaction and binding to and penetration of the oocyte's zona pellucida) [8].

Membrane integrity can be evaluated with fluorescent stains which demonstrate that the cell membrane is intact [21]. A combination of a live/dead fluorescent stain, such as propidium iodide (PI) and SYBR-14 can be used to assess cell viability. SYBR-14 is a membrane permeant nuclear stain which causes the nuclei of living cells to fluoresce green throughout [62,87]. Propidium iodide is employed to detect the proportion of dead cells in a population. Propidium iodide only stains structures containing double helix nucleic acids. Since the intracellular amount of double helix RNA is negligible, the red fluorescent signal emitted is produced by the intercalation of PI with nuclear DNA when it is no longer bound to nuclear proteins due to cellular death

and degeneration. The intact plasma membrane of spermatozoa is impermeable to PI, and thus it can only enter and stain damaged (permeable) cells [88,89].

Extending and Packaging

If spermatozoa are maintained at body temperature (37˚C) extensive cellular death will occur [90]. Cooling stallion semen reduces metabolic activity and prolongs longevity of spermatozoal motility [91]; however, the cooling process itself is not harmless. The deleterious effects of cooling can be diminished when a suitable extender is added to the semen, which is then slowly cooled and stored at 4 to 5˚C [92,93].

Dilution ratios of at least three parts extender to one part semen for whole ejaculates have been shown to maximize spermatozoal survivability in vitro and optimize spermatozoal motility in cooled/stored stallion semen provided that the final concentration remains between 25 to 50 x 10^6 progressively motile spermatozoa/mL [3,94,95,96]. This dilution ratio ensures that the final seminal plasma concentration in extended semen is $\leq 20\%$ [3]. Several investigators have reported that removing some seminal plasma from stallion spermatozoa before cooling and storage is beneficial [94,96,97,98,99,100,101,102,103,104]. However, completely removing seminal plasma from spermatozoa before cooling can have detrimental effects. Leaving 5 to 20% seminal plasma in semen maintained the highest spermatozoal motility [94,98,105] and acrosomal integrity [96]. Seminal plasma is removed by either collecting only the spermatozoa-rich fraction of the ejaculate or by centrifugation [95,99,106,107]. The process of centrifugation to remove seminal plasma can be detrimental to spermatozoal

survivability [108,109,110,111], but can be minimized by using low centrifuge forces [102,111,112]. The concentrations recommended for cooling stallion spermatozoa, however, are too low for cryopreservation, since a great number of straws would be needed for each insemination [113]. Therefore, centrifugation is necessary to enable resuspension of spermatozoa into a freezing extender at a concentration high enough to enable packaging of all spermatozoa required for one insemination dose in an individual plastic straw [2]. It has been determined that spermatozoa frozen at a concentration of 200 x 10^6 spermatozoa/mL have higher percentages of total motile and progressively motile spermatozoa compared to spermatozoa frozen at higher concentrations [114]. When cryopreserving stallion semen, spermatozoa can be packaged in 0.5, 2.5 or 5 mL straws. Smaller volume straws have a higher surface area to volume ratio, which permits spermatozoa to cool, freeze, thaw and warm at a more uniform and optimal rate [115].

Stallion Size

Testicular size or volume is a direct measure of the amount of testicular parenchyma present, which in turn determines potential spermatozoa production [116]. Small testicular size therefore corresponds to reduced spermatozoa production [117]. However, provided the testicular parenchyma is normal, one would not expect parameters such as the percentage of motile cells and percentage of morphologically normal cells to differ greatly between stallions with different size testicles since these values are independent of testicular mass. The number of spermatozoa produced per

gram of testicular parenchyma is consistent regardless of testicular size or horse breed [118].

CHAPTER III

MATERIALS AND METHODS

Animals and Treatments

Miniature stallions (n = 9) ranging in age from 3 to 13 yr were used from June to October 2007. Stallions used in this study were maintained under the approval of the Texas A&M University Institutional Agricultural Animal Care and Use Committee using guidelines set forth by the Federation of Animal Science Societies [119]. Stallions were housed in individual stalls at the Texas A&M University Horse Center and had *ad libitum* access to fresh water throughout the study. Each stallion was turned out every other day in outside pens in order to exercise. Stallions were each fed 1.50% of their body weight (BW) per day of alfalfa hay and 0.5% BW per day of concentrate (Table A-1). At the beginning of the experiment, the stallions' body weights ranged from 69.9 to 109.3 kg. Stallions were weighed every 2 weeks and their concentrate intake was adjusted accordingly.

Stallions were placed into treatment groups based on the ability of their extended spermatozoa to maintain progressive motility after cooling and storage. Four stallions produced spermatozoa that were ≥ 60% progressively motile after 24 h of cooling and storage, two that ranged from 40 to 59%, and three that were ≤ 39%. One stallion from each pair was then assigned to one of two treatments so that an equal distribution of motion characteristics was allotted to each treatment. One of the three stallions that had ≤ 39% progressively motile spermatozoa was randomly assigned to one of the two

treatments.

Each treatment group received supplementation of omega-3 fatty acids to the basal diet. However, the omega-3 fatty acid source differed. Stallions in treatment 1 (n = 5) received 60 g per day of a flake blend of flaxseed and algae-based omega-3 fatty acids (Goldfat®, JBS United, Inc., Sheridan, IN); while stallions in treatment 2 (n = 4) received 150 g per day of a pelleted blend of fish-based omega-3 fatty acids (Magnitude®, JBS United, Inc., Sheridan, IN). The fatty acid composition of both diets is shown in Table A-2.

Supplemented diets were fed for 84 d to insure inclusion of one full spermatogenic cycle. One month prior to the onset of the study, semen was collected once per week with an artificial vagina (AV, Missouri model) to familiarize the stallions with the collection process. Once the study began, semen was collected from the stallions for three consecutive days every other week for a total of nine collections. The first two consecutive collections of each sample period were discarded in order to aid in depletion of extragonadal spermatozoa reserves and to help ensure uniformity of ejaculates. The third consecutive collection was processed for analysis. Semen collection for analysis occurred on days -28, -14, 0, 14, 28, 42, 56, 70 and 84 with day 0 being the onset of dietary fat supplementation. The semen obtained from days -28, -14 and 0 served as a control for each stallion and was used to compare seminal characteristics before and after dietary fat supplementation. Ejaculates were analyzed as fresh, cooled/stored (24 and 48 h), and frozen/thawed samples.

Semen Processing

The gel-free volume of each ejaculate was determined using disposable serological pipettes (Fisher Scientific Inc., Pittsburgh, PA) to avoid contamination of samples while measuring volume. Spermatozoa concentration was assessed using a densimeter (Animal Reproduction Systems, Chino, CA). The number of total spermatozoa in each ejaculate was determined by multiplying the total gel-free volume of semen by the concentration. A raw semen sample was preserved in BFS (buffered formol-saline) for morphological analysis. The remaining fresh semen was diluted at least 3:1 (extender to semen) with a commercial semen extender (INRA-96®, Breeder's Choice, Aubrey, TX) in order to achieve a final concentration of 25 to 50 x 10^6 spermatozoa/mL. Extender was warmed to 37˚ C prior to collection and was maintained at this temperature until use. All the materials used in handling ejaculates were maintained at 37˚ C in an incubator until time of use.

Three aliquots, each containing 1.5 mL of extended semen, were separated and stored in microcentrifuge tubes. One of the aliquots was kept at 37˚ C and analyzed for motion characteristics and membrane integrity within 30 min of collection. The other 2 aliquots underwent standard processing for cooled transported semen. These aliquots were stored in separate cooling devices (Equitainer-II®, Hamilton-Thorn Biosciences, Beverly, MA) and analyzed after 24 and 48 h of storage. The remaining semen was transferred to 50 mL conical centrifuge tubes at a final volume of 35 mL per tube and centrifuged at 500 x G for 12 min at room temperature. After centrifugation, the supernatant was removed and the spermatozoa pellet was resuspended with freezing

extender (EZ-Freezin LE®, Animal Reproduction Systems, Chino, CA). Prior to suspension of spermatozoa with freezing extender, the extender was centrifuged at 2400 x G at room temperature for 10 min and passed through both a 5.0 and a 1.2 µm filter. This was done to homogenize the extender and to filter any large particles that might interfere with the motility analysis. The resuspended semen was then packaged in five 0.5 mL straws at a concentration of 200 x 10^6 spermatozoa/mL and frozen in liquid nitrogen vapor by placing the straws on a rack 3 to 4 cm above the liquid-gas interface for 5 min. After 5 min, the straws were plunged into liquid nitrogen and stored in a standard storage dewar (Model XC Millenium 20, Animal Reproduction Systems, Chino, CA) at -196˚ C for future spermatozoa analysis.

Spermatozoa Analysis

The percentage of total motile spermatozoa, progressively motile spermatozoa as well as other motion characteristics including path velocity (VAP), progressive velocity (VSL), track speed (VCL), lateral amplitude of head (ALH), beat frequency (BCF), straightness (STR), and linearity (LIN) were determined via computer-assisted sperm analysis (CASA, Ceros Motility Analyzer, Hamilton-Thorne, Beverly, MA). During the analysis of motion characteristics, a minimum of 500 cells were counted. The settings used by CASA are shown in Table A-3. Fresh samples were analyzed 15 min after dilution. After removal from the storage containers, cooled semen samples were placed in an incubator for 15 min at 37˚ C prior to motility and membrane integrity analysis to ensure maximum reactivation of spermatozoal motility [94]. After at least 48 h of

storage in liquid nitrogen, frozen samples were removed from the dewar and immediately placed in a 37˚ C water bath for 30 seconds [2] and diluted to 25 x 10^6 spermatozoa/mL with INRA-96® extender.

Membrane integrity was evaluated with a fluorescent microscope (Nikon Eclipse E1000, 40X objective) using a live/dead sperm viability kit (Invitrogen Molecular Probes, Eugene, OR) of SYBR-14 and propidium iodide. SYBR-14 was diluted with DMSO at a ratio of 1:5 (SYBR-14:DMSO); 2.0 µl of this dilution were then added to 15.0 µl of the semen sample to be analyzed, along with 3.6 µl of PI, and 40 µl of Garner's solution. A minimum of 200 cells were counted and a percentage of intact and damaged cells was obtained. Morphology of the fresh samples preserved in BFS was analyzed using DIC microscopy (Olympus BX60, 100X objective). One-hundred cells per sample were evaluated. Cell abnormalities were recorded and quantified by counting the presence of each abnormality.

Statistical Analysis

Data were analyzed by repeated measures in time using PROC MIXED (SAS v 9.1; SAS Inst. Inc., Cary, NC). Treatment, time, and treatment x time interactions were included in the model. Differences were considered significant at a probability level of $P < 0.05$.

CHAPTER IV

RESULTS

Motion characteristics, morphology and membrane integrity of fresh, cooled/stored (24 and 48 h) and frozen/thawed semen samples were assessed. No treatment effects were found in any of the endpoints measured, meaning there were no significant differences between treatment 1 (Goldfat®) and treatment 2 (Magnitude®). There were also no day effects within the groups. However, treatment x day effects did exist in some of the endpoints evaluated. When evaluating data from individual stallions, only one out of the three whom at the beginning of the trial produced ≤ 39% progressively motile spermatozoa exhibited significant improvements in total and progressive motility of fresh and cooled spermatozoa throughout the trial.

Ejaculate Parameters: Volume and Concentration

Although there were no significant differences (P = 0.16) between the two treatments, a trend toward a decrease in the gel-free seminal volume of the ejaculates was observed within treatments. Stallions from both treatment groups produced a numerically higher ejaculate volume at the beginning of the trial which slowly decreased throughout the trial. A treatment x day effect was observed at d 42 (10.08 ± 9.18 mL and 4.50 ± 2.65 mL, treatment 1 and 2, respectively) and d 56 (8.78 ± 4.47 mL and 3.55 ± 2.72 mL, treatment 1 and 2, respectively) of the trial (Fig. 1).

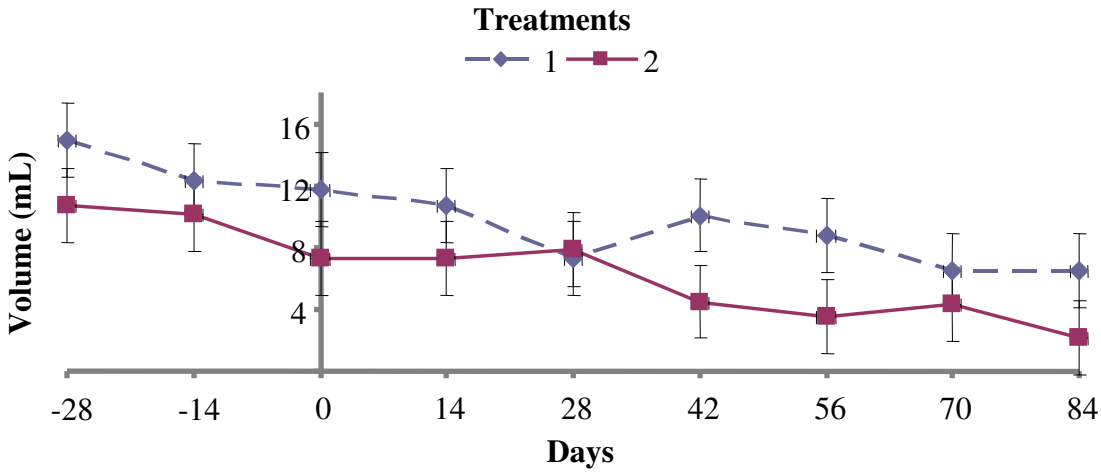

Fig. 1. Mean (± S.D.) gel-free semen volume observed for treatments 1 and 2 throughout the trial (d 0 = start of supplementation).

Mean spermatozoa concentration in the ejaculates did not differ (P = 0.91) between treatments; nevertheless, stallions from both treatments produced a numerical increase in the concentration of spermatozoa in the ejaculate throughout the trial (d -28 to d 84). No treatment, day or treatment x day effects were observed (Fig. 2).

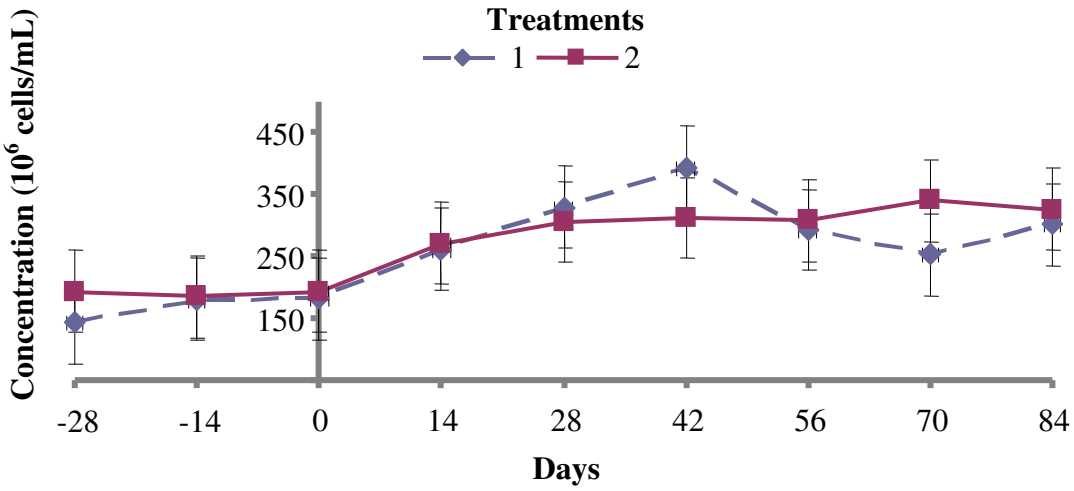

Fig. 2. Mean (± S.D.) spermatozoa concentration observed for treatments 1 and 2 throughout the trial (d 0 = start of supplementation).

Although not statistically different, the mean seminal volume was lower in treatments 1 and 2 during the supplementation period (d 14 to 84) compared to the pre-supplementation period (d -28 to 0) (P = 0.28). Mean spermatozoa concentration in the stallions' ejaculates was greater for both treatment groups during the supplementation period versus the pre-supplementation period (P = 0.83, comparison between treatments). However, the mean total spermatozoa produced for both treatment groups did not differ at any collection day (P = 0.20). Total spermatozoa (mean ± S.D.) produced by stallions in treatment 1 before supplementation was 2.19 ± 0.21 billion versus 2.53 ± 0.37 billion after supplementation. Total spermatozoa (mean ± S.D.) produced by stallions in treatment 2 before supplementation was 1.69 ± 0.26 billion versus 1.54 ± 0.66 billion after supplementation (Table 1).

Table 1
Mean (± S.D.) gel-free semen volume and spermatozoa concentration throughout the trial.

	Treatment 1 (n = 5)		Treatment 2 (n = 4)		Trt P-value
Day	-28 to 0	14 to 84	-28 to 0	14 to 84	
Volume (mL)	12.97 ± 1.73	8.30 ± 1.81	9.38 ± 1.80	4.96 ± 2.22	0.20
Concentration (10^6 cells/mL)	168.47 ± 22.42	305.37 ± 51.51	190.50 ± 5.20	310.96 ± 23.35	0.91
Total spermatozoa in ejaculate (10^9 cells/mL)	2.19 ± 0.21	2.53 ± 0.37	1.69 ± 0.26	1.54 ± 0.66	0.20

Motility

Total motility (TMOT) and PMOT in fresh samples was not improved by treatment. When spermatozoa were cooled and stored for 24 and 48 h, TMOT and PMOT were also not improved by treatment. Motion characteristics of frozen spermatozoa were less than optimal in both treatments and were not improved by omega-3 supplementation. The mean total motility of frozen samples in the pre-treatment period was 7.07% for treatment 1 and 6.33% for treatment 2; these values decreased in the post-treatment period to 4.63% in treatment 1 and 2.96% in treatment 2. The mean progressive motility of frozen samples also declined in both treatments throughout the trial from 5.40 to 4.07% in treatment 1 and 4.83 to 3.08% in treatment 2. In addition, none of the motion characteristics analyzed differed between treatments, nor were they improved by treatment (Table A-4).

During the trial, TMOT and PMOT for both treatments followed similar trends but there were no treatment or day effects; however a treatment x day effect did exist. Fresh TMOT (mean ± S.D.) was not significantly different between the two treatments with the exception of d 0 (74.8 ± 19.04% and 54.0 ± 24.21%, treatment 1 and 2, respectively) (Fig. 3).

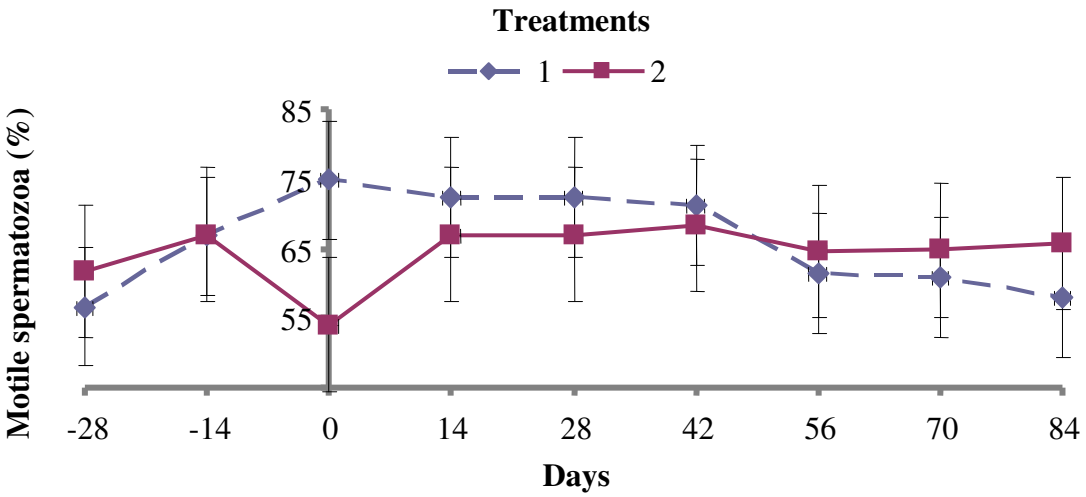

Fig. 3. Mean (± S.D.) total motility of fresh spermatozoa observed for treatments 1 and 2 (d 0 = start of supplementation).

Differences in fresh PMOT (mean ± S.D.) were observed at d 0 (66.80 ± 16.95% and 41.75 ± 21.17%, treatment 1 and 2, respectively) and d 28 (36.40 ± 31.09% and 59.50 ± 7.14%, treatment 1 and 2) (Fig. 4).

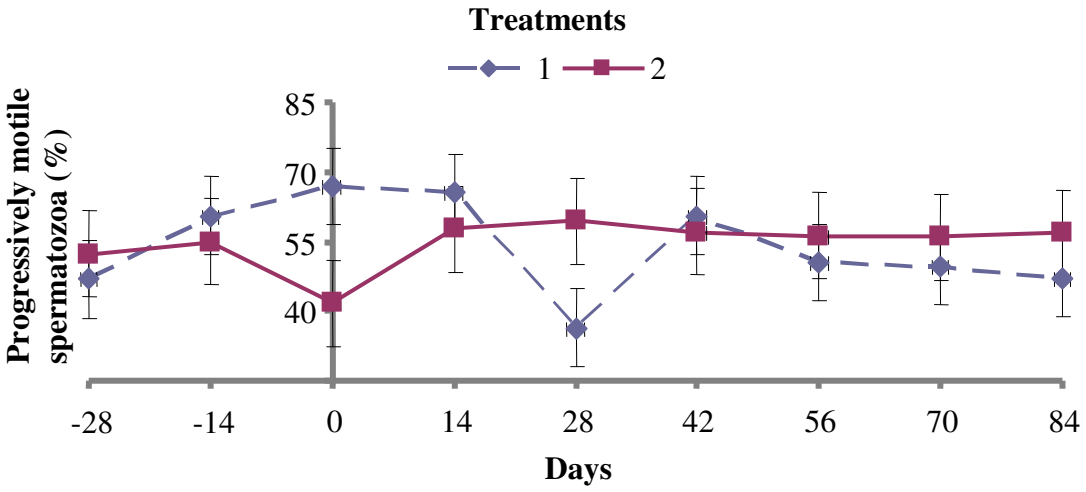

Fig. 4. Mean (± S.D.) progressive motility of fresh spermatozoa observed for treatments 1 and 2 (d 0 = start of supplementation).

After 24 h of cooling and storage, differences in TMOT (mean ± S.D.) were observed at d 0 (63.40 ± 26.78% and 40.50 ± 26.41%, treatment 1 and 2, respectively) (Fig. 5). Progressive motility was different at d 0 (53.00 ± 24.53% and 31.75 ± 24.23%, treatment 1 and 2, respectively) and d 28 (34.60 ± 35.44% and 58.00 ± 6.98%) (Fig. 6).

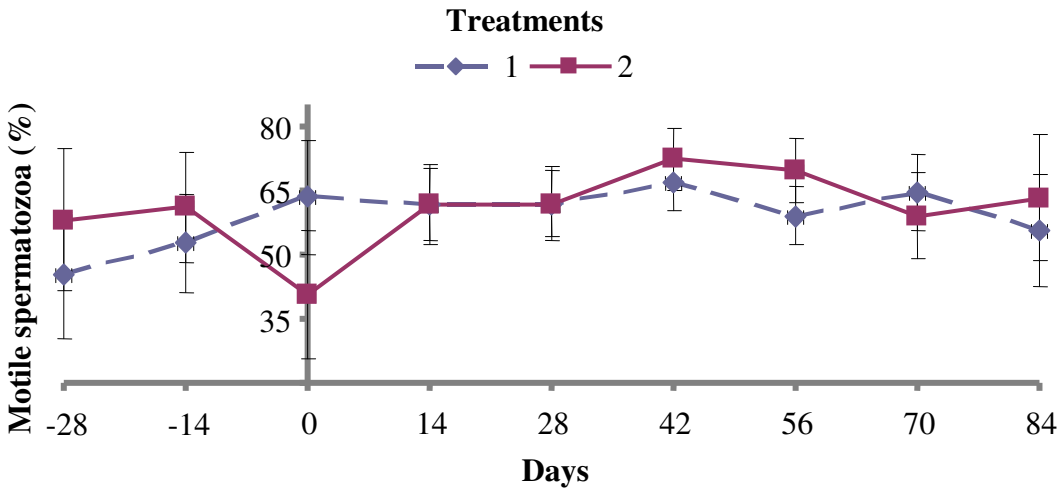

Fig. 5. Mean (± S.D.) total motility after 24 h of cooling and storage in treatments 1 and 2 (d 0 = start of supplementation).

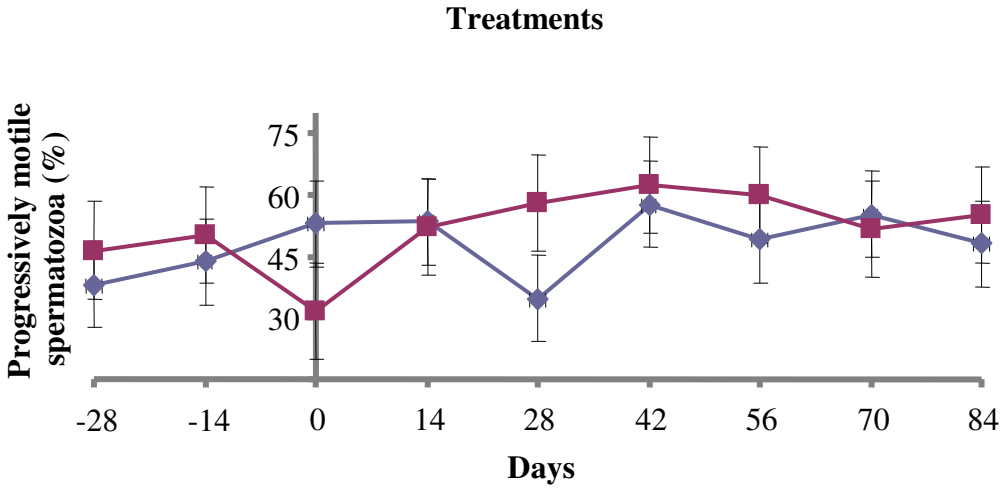

Fig. 6. Mean (± S.D.) progressive motility after 24 h of cooling and storage in treatments 1 and 2 (d 0 = start of supplementation).

The trends observed for the motion characteristics of spermatozoa cooled for 48 h are shown in Fig. 7 and 8. There were no significant differences at any days for TMOT or PMOT between or within treatments.

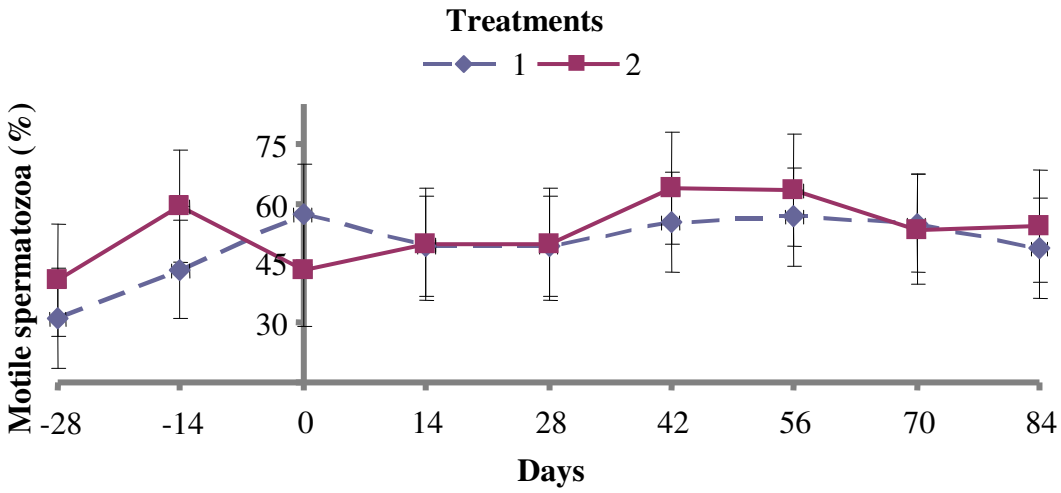

Fig. 7. Mean (± S.D.) total motility after 48 h of cooling and storage in treatments 1 and 2 (d 0 = start of supplementation).

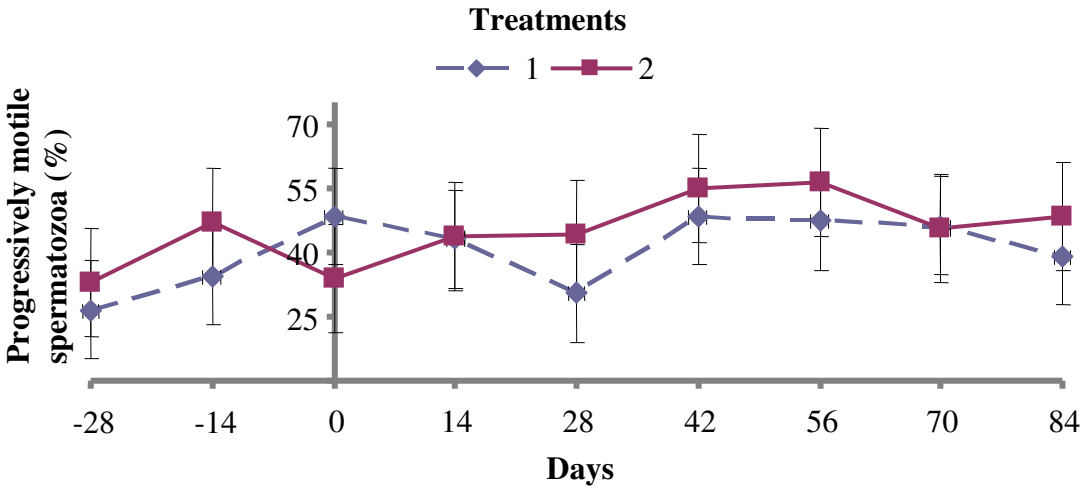

Fig. 8. Mean (± S.D.) progressive motility after 48 h of cooling and storage in treatments 1 and 2 (d 0 = start of supplementation).

When evaluating the motion characteristics of individual stallions, of the three that produced ≤ 39% progressively motile spermatozoa after 24 h of cooling and storage at the beginning of the trial, significant increases in only one of the stallions (stallion 3) were observed when comparing pre- (d -28 to d 0) to post-treatment (d 14 to d 84) data; this stallion was in treatment group 2. Mean TMOT of fresh spermatozoa increased from 53.00 to 65.30% (P = 0.03) (Fig. 9). Whereas mean progressive motility of fresh spermatozoa increased from 46.30 to 59.50% (P = 0.01) (Fig. 10).

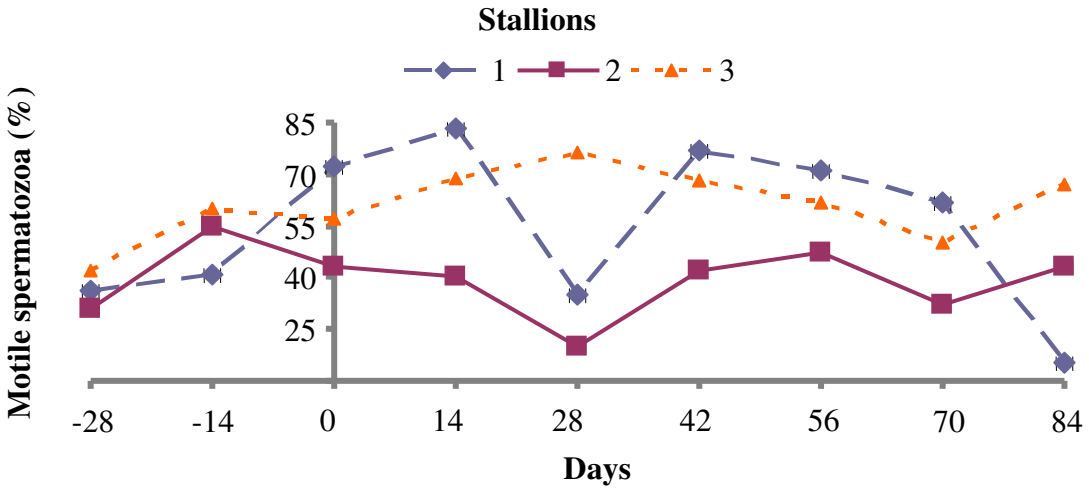

Fig. 9. Mean (± S.D.) total motility of fresh spermatozoa from stallions having ≤ 39% progressively motility after 24 h of cooling and storage (d 0 = start of supplementation).

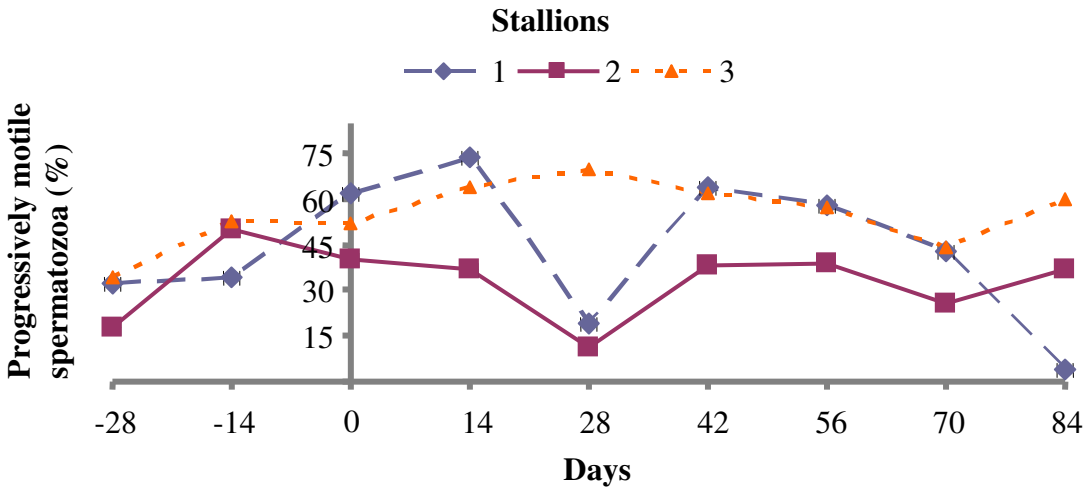

Fig. 10. Mean (± S.D.) progressive motility of fresh spermatozoa from stallions having ≤ 39% progressive motility after 24 h of cooling and storage (d 0 = start of supplementation).

The mean TMOT of stallion 3 after 24 h of cooling and storage increased from 43.70% in the pre-treatment period (d -28 to d 0) to 64.30% in the post-treatment period (d 14 to d 84) (P = 0.02) (Fig. 11).

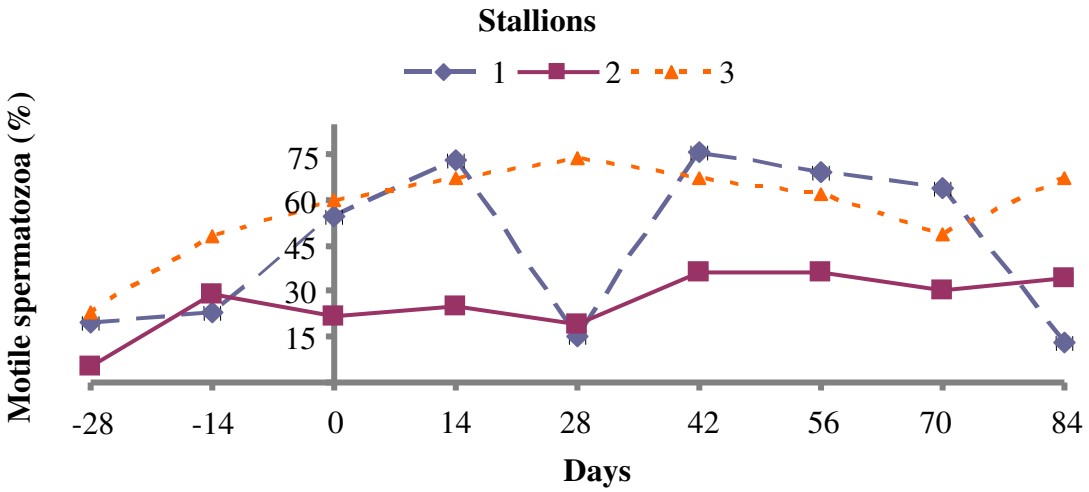

Fig. 11. Mean (± S.D.) total motility after 24 h of cooling and storage in stallions having ≤ 39% progressive motility after 24 h of cooling and storage (d 0 = start of supplementation).

The mean PMOT of stallion 3 after 24 h of cooling and storage increased from 35.00 to 55.00% (P = 0.04) (Fig. 12).

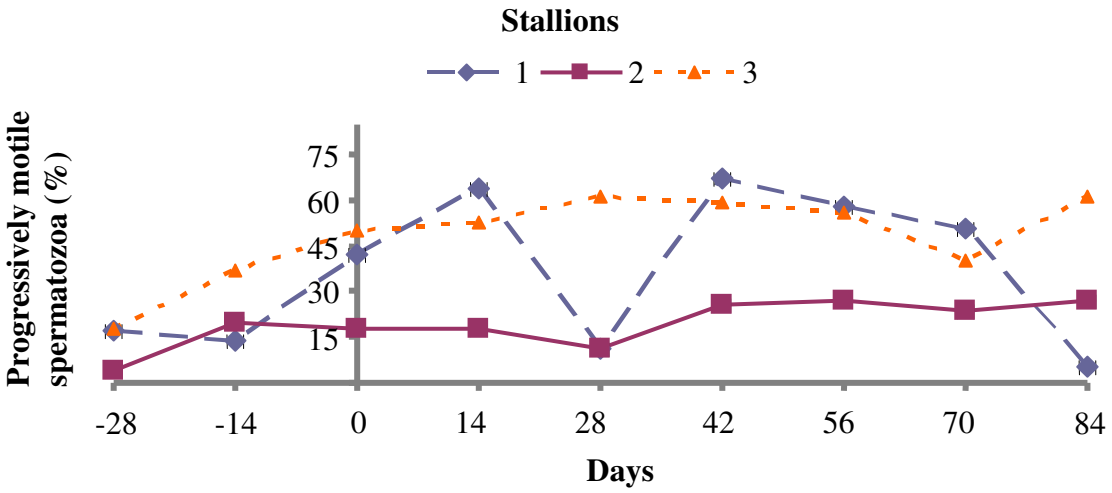

Fig. 12. Mean (± S.D.) progressive motility after 24 h of cooling and storage in stallions having ≤ 39% progressive motility after 24 h of cooling and storage (d 0 = start of supplementation).

Although the mean TMOT of stallion 3 after 48 h of cooling and storage increased from 35.3 to 56.0% (pre- vs. post-treatment), this increase was not statistically significant (P = 0.13) (Fig. 13).

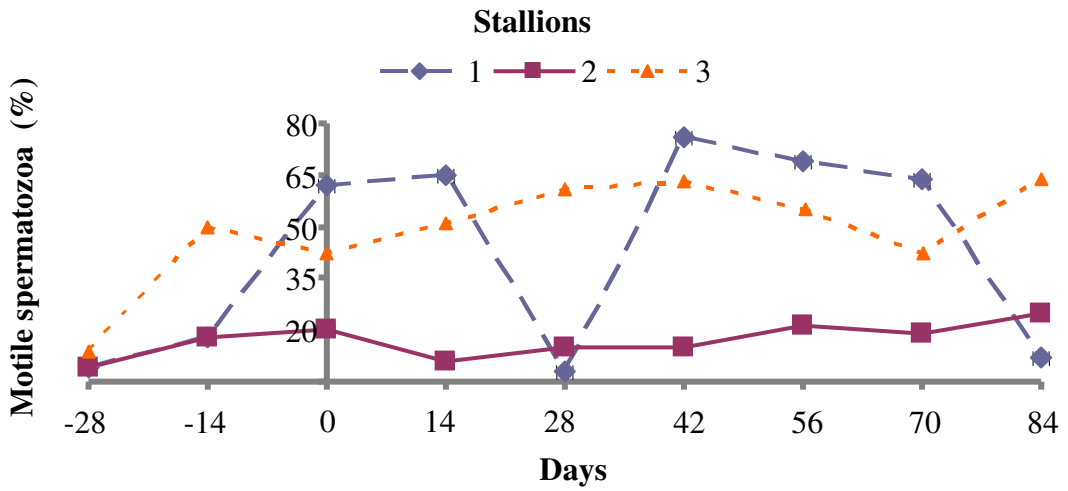

Fig. 13. Mean (± S.D.) total motility after 48 h of cooling and storage in stallions having ≤ 39% progressive motility after 24 h of cooling and storage (d 0 = start of supplementation).

The mean PMOT of stallion 3, however, did increase significantly from 24.70 to 47.50% (P = 0.06, pre- vs. post-treatment) (Fig. 14).

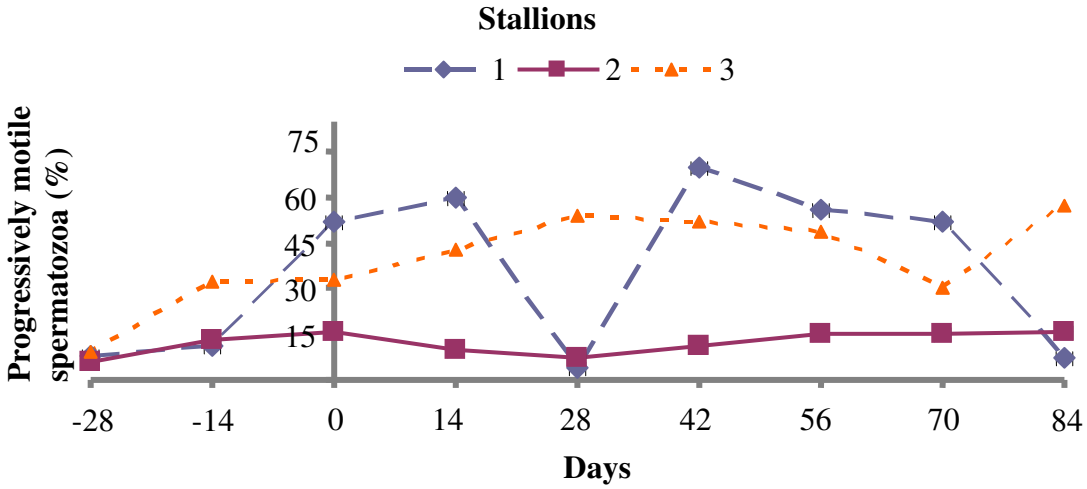

Fig. 14. Mean (± S.D.) progressive motility after 48 h of cooling and storage in stallions having ≤ 39% progressive motility after 24 h of cooling and storage (d 0 = start of supplementation).

Membrane Integrity

The percentage of live, acrosome-intact spermatozoa in fresh, cooled and frozen/thawed semen is shown in Table 2. The percentage of spermatozoa with intact membranes was unaffected by treatment, although a day effect in the fresh spermatozoa of stallions in treatment 1 was observed at d 42 of the trial.

Table 2
Percentage (mean ± S.D.) of membrane intact spermatozoa in fresh, cooled/stored, and frozen/thawed semen samples

	Treatment 1 (n=5)		Treatment 2 (n=4)		Trt P-value
Day	-28 to 0	14 to 84	-28 to 0	14 to 84	
Semen sample					
Fresh	34.16 ± 22.89	16.21 ± 6.55	29.93 ± 24.98	24.61 ± 10.93	0.29
24 h cooled	21.99 ± 13.96	15.56 ± 8.81	24.90 ± 21.87	18.71 ± 7.66	0.48
48 h cooled	18.67 ± 1.96	16.13 ± 6.55	16.83 ± 1.25	15.53 ± 5.26	0.78
Frozen/thawed	2.86 ± 0.08	2.29 ± 0.97	2.54 ± 0.80	1.80 ± 1.00	0.55

Morphology

Spermatozoal morphology was also unaffected by treatment. No treatment, day, or treatment x day effects were observed (Table 3).

Table 3
Percentage (mean ± S.D.) of normal spermatozoa in fresh semen samples preserved in BFS.

	Treatment 1 (n = 5)	Treatment 2 (n = 4)	Trt P-value
Day			
-28 to 0	71.73 ± 5.61	73.17 ± 0.88	0.65
14 to 84	72.23 ± 3.57	77.88 ± 2.10	

CHAPTER V

DISCUSSION

Normal Seminal Parameters

In order to establish normal semen parameters of miniature stallions, a previous study evaluated semen from 216 miniature stallions during the months of March and April, from 1992 through 1996 [117]. The ejaculates were filtered to remove the gel fraction and the volume of the ejaculate, along with progressive motility, concentration, and morphology were determined. The percentage of total motile spermatozoa was reported to be 63.8 ± 0.7%, the percentage of normal spermatozoa was 54.28 ± 1.05%, the gel-free volume was 24.18 ± 0.76 mL, and the concentration of spermatozoa in the ejaculate was 233.60 ± 0.76 x 10^6 cells/mL. These values are similar to those reported from a group of more than 400 normal, full-size stallions where the percentage of motile spermatozoa was 55%, the percentage of morphologically normal spermatozoa was 54%, and the average concentration was 200 x 10^6 spermatozoa/mL [120]. The data reported in the present study are similar to the normal parameters established for miniature and full-size stallions. At the beginning of the present study, the stallions used had a total percent motility of 63.50 ± 3.64%, concentration of 179.49 ± 15.58 x 10^6 cells/mL (Table 1), percentage of normal spermatozoa of 72.15 ± 1.02% (Table 3), and volume of 11.18 ± 2.54 mL (Table 1). The differences in the concentration of spermatozoa and volume of the ejaculates reported throughout the study could be due to the different months during which the collections were performed (March to April

versus June to October). Because stallions are seasonal breeders, the total semen volume and spermatozoal output are greatly influenced by season, among other factors [121]. The total semen volume will begin to increase between the months of March and April until reaches a peak in May, and will start decreasing in late June or early July until late December or early January when the lowest ejaculate volumes are observed. Other reports state that the volume of stallion ejaculates increases by approximately 40% from the low months to the peak of the breeding season and spermatozoa output during the lowest months is approximately 50% of the higher month's output. Previous reports have also indicated that the motion characteristics of spermatozoa are not affected by seasonal effects [122].

Miniature stallions have smaller testicles than is commonly accepted as normal in full-size stallions [117]. Testicular volume, estimated by testicular measurements, has been correlated with spermatozoa production [116,118, 123]. Small testicles therefore yield lower spermatozoa output. However, similarities in the percentage of motile spermatozoa, percentage of morphologically normal spermatozoa, and concentration are expected regardless of the size of the stallion since these values are independent of testicular mass, provided the testicular parenchyma is normal [117]. Therefore, it is safe to assume that miniature stallions can be used as a research model in comparison to full-size stallions.

Sources of Omega-3 Fatty Acids

Omega-3 and omega-6 fatty acids cannot be interconverted [47], but it is well established that these 2 families of fatty acids are metabolized to longer chain fatty acids that are then made available for membrane lipid biosynthesis [55]. It has been recognized that competition between omega-3 and omega-6 fatty acids for a common desaturase plays a major role in determining the amounts of each type of fatty acid that are produced for lipid biosynthesis [55]. Nonetheless, omega-3 fatty acids with very long-chain fatty acids are the precursors of signal molecules that are important for several cellular functions including cell motility. Flaxseed contains approximately 57% of alpha-linolenic acid (ALA) but does not contain any very long-chain (more than 18 carbon atoms) fatty acids such as DHA and EPA. On the other hand, omega-3 fatty acids with more than 18 carbon atoms are highest in marine animals. It is believed that the original source of these fatty acids is algae, which then transfers these fatty acids via the nutrition chain to higher animals including fish [47]. To our knowledge, this is the first reproductive study performed using an algae/flaxseed blend and a fish source to determine if there is a difference in the origin of the omega-3 fatty acids on the seminal parameters of stallions. Although the body can convert ALA to DHA and EPA, this requires more metabolic work and could be a reason why absorption of fatty acids may be greater from animal rather than plant sources as previously reported [47]. Because there is no DHA or EPA in flaxseed, the combination of algae with flaxseed is expected to provide both the ALA found naturally in flaxseed, and DHA and EPA from algae.

Motility

Although it would be hypothesized that the addition of omega-3 fatty acids to the diets of stallions may enhance, among other parameters, the motion characteristics of spermatozoa, data from the current research suggest that motion characteristics were unaffected by treatment. These findings are in agreement with previous reports with stallions supplemented with long-chain omega-3 fatty acids [59], and with a DHA-enriched nutriceutical [57] where motion characteristics of fresh and cooled/stored spermatozoa were also not improved by treatment. Similar reports are also found with boars supplemented with cod liver oil. No significant differences were found in the motion characteristics determined in four different steps of the freezing process [124]. However, the present research does not agree with the DHA-enriched nutriceutical report where significant improvements in the TMOT and PMOT of frozen/thawed spermatozoa were observed [57]. In the present study, the motion characteristics of frozen/thawed spermatozoa were less than optimal in all the stallions including those with high fresh motility and remained low throughout the trial. The motility of processed spermatozoa (cooled/stored or frozen/thawed) differs greatly among stallions and ejaculates from the same stallions [2]. Although the cause of differences in motility after processing has not been established, the change in motility after cooling/storing and freezing/thawing is not proportionate to the value before processing [43]. Also in disagreement with the current findings is another study in boars supplemented with tuna oil where increases in viability, and the proportions of spermatozoa with progressive motility were reported [15]. However, these increases occurred in both the control and

the tuna oil fed diets, which indicates that the changes observed may be related to factors other than dietary supplementation of omega-3 fatty acids. The present data of individual stallions indicate that only one of the three stallions classified as “poor coolers” at the beginning of the study showed significant increases in total and progressive motility of fresh and cooled/stored spermatozoa. This is similar to what was observed in stallions, also considered “poor coolers,” that were supplemented with a DHA-enriched nutriceutical [57] and with long-chain omega-3 fatty acids [59] where increases in total [57] and progressive [57,59] motility were reported after 48 h of cooling. The stallion in which significant increases were observed was in treatment group 2 (fish-based diet) whereas the other 2 were in group 1 (algae/flaxseed-based diet).

A prior study comparing the effect of different oil sources, including sunflower, soybean, canola, flaxseed, and fish/soybean, and of the omega-3:omega-6 ratio in the reproductive performance of cockerels has indicated that the fatty acid content of spermatozoa, physical seminal characteristics, and fertility rate are influenced by the oil source in the diet [60]. Conversely, the current data do not indicate that there was a significant difference between the two sources of omega-3 fatty acids utilized in the improvement of spermatozoa motility when comparing treatment groups 1 and 2. However, fish rather than algae/flaxseed sources of omega-3 fatty acids may be more effective at improving the motion parameters of stallions with lower quality ejaculates. These different findings might be due to variations in the omega-3:omega-6 ratios fed. In the present research, both diets fed contained an omega-3:omega-6 ratio of approximately 2:1 (Table A-2). In comparison of different rations, the study using

cockerels reported that a dietary ratio from 6:1 to 9:1 resulted in desirable proportions amongst the omega series and maximized the fertility rate. It is important to note, however, that when comparing the effects of different oil sources in the reproductive performance of cockerels, the addition of flaxseed oil to the diet resulted in the lowest content of PUFAs in the spermatozoa cells and was associated with the lowest fertility rates. Nonetheless, the effect on the fertility rate was reverted by vitamin E supplementation [60]. Thus, further research with stallions supplemented with the algae/flaxseed supplement used in the present study along with vitamin E supplementation would be of interest. The omega-3:omega-6 ratio in flaxseed oil is high, due to its high content of ALA. Therefore, the high degree of polyunsaturation of this oil source results in a high susceptibility of spermatozoa to lipid peroxidation, with the consequent risk of damage to the cellular structures [47,60]. Consequently, highly unsaturated acids in the diet increase vitamin E requirements, in order to prevent oxidation of unsaturated lipid materials within the cells [125,126]. The use of fish/soybean oil in the diet of cockerels resulted in the lowest total antioxidant status of the semen. However, when vitamin E was added to the fish/soybean-oil-based diet, linear increases in semen volume, motility and spermatozoa vigour were observed. This linear effect was also due to the increase in vitamin E requirement [60]. Although the mechanisms involved in the delivery of fatty acyl components from the circulation to the developing spermatozoa and in the selective incorporation of specific fatty acids into the spermatozoa phospholipids are not completely understood, transfer of PUFAs from the diet has been shown to occur in several species including humans [10,17], fowl [60,61],

boars [15,124] and stallions [57,59]. However, it is clear that there is a need for a balanced omega-3:omega-6 ratio in the diet, and further research with stallions would be valuable in determining the optimal ratio to increase spermatozoa quality and viability. If a high omega-3:omega-6 ratio is essential for optimal fertility in equine spermatozoa, as it is in cockerel spermatozoa, then it is possible that increasing the dietary omega-3:omega-6 ratio in addition to an anti-oxidant additive (such as vitamin E) could potentially increase the quality of equine spermatozoa. Because not all stallions produce spermatozoa that withstand the stresses involved with cooling and freezing/thawing, appropriate dietary manipulations that could possibly increase the viability and resistance of spermatozoa to different processing techniques would allow breeders to benefit from the many advantages that the use of processed semen offers.

Membrane Integrity

In the present study there did not appear to be a proportionate change between the percentage of membrane-intact spermatozoa and the percentage of motile spermatozoa. Similar findings have been reported in boars supplemented with cod liver oil [124] where a significant correlation between the number of motile normal spermatozoa and the number of membrane-intact spermatozoa could not be confirmed. Because the organelles concerned with motility and penetration of an ovum are located in different parts of a spermatozoon [127], and because composition of membranes overlying the rostral spermatozoal head differs from that of the plasma membrane over the middle piece [128,129], it has been previously hypothesized that damage may occur

to the acrosome or overlaying plasma membrane of spermatozoa that is not detectable by evaluation of spermatozoal motility [43]. It is now generally admitted that phospholipids are asymmetrically distributed in membranes [12], meaning that the composition and localized distribution of phospholipids and the nature of their fatty acyl side chains differ from membrane to membrane and even within domains (regions) of a given membrane [2]. Therefore, it is not surprising that several studies have demonstrated that acrosomal and plasma membrane changes are not always related to changes in motility of spermatozoa [130,131,132,133] because during the cooling and freezing/thawing procedures the motility apparatus or the metabolic processes in spermatozoa can be injured independent of one another [43]. In non-motile spermatozoa, for example, damage of the metabolic processes such as mitochondrial injury can lead to insufficient energy for all cellular functions, or a shift in the available energy from spermatozoal motion to maintenance functions without disrupting the motility apparatus itself. And because of the different composition of the spermatozoal membranes many non-motile spermatozoa with an intact plasma membrane may be detected. The low correlations between the percentage of sperm with intact plasma membrane and the percentage of motile or percentage of progressively motile spermatozoa is indirect evidence that many non-motile spermatozoa possess an intact plasma membrane [43] and that it is possible for many motile spermatozoa to have a damaged plasma membrane. This could be a reason why low fertility and conception rates often result even when processed semen (cooled/stored or frozen/thawed) used for artificial insemination contains an adequate number of progressively motile spermatozoa [2].

Morphology

Contrary to data previously reported in a study with boars supplemented with tuna oil [15], the percentage of morphologically normal spermatozoa in this study was not affected by omega-3 supplementation. However, the percentage of normal spermatozoa in both treatments 1 and 2 was already high prior to supplementation (Table 3) which is why it is not surprising that it remained unchanged throughout the trial. Furthermore, the increases in the percentage of normal spermatozoa reported in boars [15] occurred both in the control and tuna oil-containing diets and, thus may be related to either seasonal effects or to the inclusion of antioxidants in both the control and the tuna oil-containing diets.

CHAPTER VI

SUMMARY

In this study, the analyses of spermatozoa quality between stallions supplemented with omega-3 fatty acids from two different sources (fish or algae/flaxseed) show that the motion characteristics, the percentage of membrane intact spermatozoa, and the percentage of morphologically normal spermatozoa of fresh, cooled/stored, and frozen/thawed spermatozoa are not significantly altered by such supplementation. Overall, the results indicate that adding omega-3 fatty acids to stallion diets may not enhance the quality of spermatozoa significantly nor have a beneficial effect on cold shock or freezability of equine spermatozoa. However, the data of individual stallions suggest that fish sources might be more effective at improving the motion parameters of spermatozoa from lower quality ejaculates.

The lack of improvement in spermatozoal morphology was not unexpected. All of the stallions on the project had a high percentage of normal spermatozoa at the onset. Thus, any improvement of this spermatozoal characteristic would have been difficult to achieve in these stallions. The disproportionate changes observed between the motion characteristics and the membrane integrity of spermatozoa may have resulted because the current semen processing procedures are not equally successful in preserving motility and integrity of the membranes.

Further research with a larger number of stallions is needed to make more definitive explanations of the mechanisms involved in potentially altering spermatozoa

motility and viability. In addition, further research with different basal diets and supplements, and different omega-3:omega-6 ratios is needed in order to determine the optimal ratio and source of omega-3 fatty acids that could potentially enhance spermatozoa quality and viability. The diet used in the present research was a typical equine formulation containing corn oil which contains omega-6 fatty acids. Using a basal diet with a higher omega-3 fatty acid content would increase the omega-3:omega 6 ratio and could result in a greater enhancement of spermatozoa quality. Since the vitamin E requirements are dependent of the dietary fatty acid composition, determining the requirements of this antioxidant for different sources of omega-3 fatty acids may also prove to be beneficial.

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APPENDIX

Table A-1
Composition of basal concentrate used in feeding all stallions.

Ingredient name:

Milo
Wheat midds
Soybean meal-48
Soybean hulls
Liquid binder
Horse premix #6886
Ground lime
Salt mixing

Nutrient	Units	Amount
Weight	lbs	1.00
Protein	%	13.00
Fat	%	2.90
Fiber	%	10.00
Ca	%	0.70
P	%	0.50
ADF	%	13.77
NDF	%	30.69
Lys	%	0.62
Met	%	0.18
K	%	0.87
DM	%	88.61
S	%	0.17
Mg	%	0.25
Mn	PPM	99.44
Fe	PPM	156.30
Cu	PPM	32.47
Co	PPM	0.79
Zn	PPM	115.13

I	PPM	0.59
Se	PPM	0.44
Vit A	IU/lb	3,019.00
Vit D	IU/lb	210.00
Vit E	IU/lb	38.10
Chol	MG/lb	412.51
Ribo	MG/lb	1.57
Niac	MG/lb	27.94
Pant	MG/lb	6.80
B 12	MCG/lb	3.20
Biot	MCG/lb	149.44
Pyrd	MG/lb	2.50
Thia	MG/lb	5.77
Fo A	MG/lb	0.26
Zn:Cu	PPM	3.55
Dig Lys	PPM	0.34
DE H	Kcal/lb	1,289.62
Ca:P	Ca:P	1.40
Vit A added	IU/lb	2,080.45
Vit D added	IU/lb	27.43
Valine	%	0.42

Table A-2

Fatty acid profile of concentrate and treatments expressed as g consumed per day.

Fatty acid (FA)	Concentrate[a]	Treatment 1	Total FA	Treatment 2	Total FA
C18:2 n-6 (LA)	4.86 ± 0.80	ND	4.86 ± 0.80	1.57	6.43 ± 0.80
C20:4 n-6 (AA)	ND	0.10	0.10	0.31	0.31
C18:3 n-3 (ALA)	0.41 ± 0.06	ND	0.41 ± 0.06	0.50	0.91 ± 0.06
C20:5 n-3 (EPA)	ND	0.24	0.24	2.21	2.21
C22:5 n-3 (DPA)	ND	ND	ND	0.48	0.48
C22:6 n-3 (DHA)	ND	8.06	8.06	7.48	7.48
Total n-6	4.86 ± 0.80	0.10	4.96 ± 0.80	1.88	6.74 ± 0.80
Total n-3	0.41 ± 0.06	8.30	8.71 ± 0.06	10.70	11.08 ± 0.06
n-3:n-6 ratio	0.41:4.86	8.30:0.10	1.76:1	10.70:1.88	1.64:1

[a]Variation due to differences in concentrate consumption.

ND: none detected

Table A-3
Analysis setup of CASA.

Apply sort:	0
Frames acquired:	45
Frame rate:	60 Hz
Minimum contrast:	70
Minimum cell size:	4 pixels
Minimum static contrast:	30
Straightness threshold:	50.00%
Vap cutoff:	15.0 µm/s
Prog. min VAP:	30.0 µm/s
VSL cutoff:	0.0 µm/s
Cell size:	6 pixels
Cell intensity:	106
Static head size:	0.60 to 2.00
Static head intensity:	0.20 to 2.01
Static elongation:	31 to 85
Slow cells motile:	No
Magnification:	1.89
Video frequency:	60
Bright field:	No
LED ilumination intensity:	2200
IDENT ilumination intensity:	3000
Temperature, set:	37.0 ˚C
Chamber depth:	20
Chamber position:	6.8 mm
Chamber position B:	26.2 mm
Chamber position C:	0.0 mm
Chamber position D:	0.0 mm
Chamber type:	Leja
Field selection mode:	Select
IDENT fluorescent option:	Off
Integrating time:	1 frames

Table A-4
Mean motion characteristics analyzed by CASA.

Sample	Motion characteristic	Treatment 1 (n = 5)	Treatment 2 (n = 4)	P-value
Fresh	TMOT (%)	66.04	65.45	0.95
	PMOT (%)	52.55	56.13	0.72
	VSL (µm/s)	56.87	59.74	0.69
	VAP (µm/s)	69.29	74.67	0.63
	VCL (µm/s)	121.97	133.4	0.52
	ALH (µm)	5.21	5.26	0.93
	BCF (Hz)	36.91	38.2	0.56
	STR (%)	81.63	80.98	0.85
	LIN (%)	48.05	47.88	0.97
24 h Cooled	TMOT (%)	60.39	62.95	0.84
	PMOT (%)	48.99	54.57	0.67
	VSL (µm/s)	58.3	63.89	0.53
	VAP (µm/s)	69.74	77.91	0.5
	VCL (µm/s)	117.88	134.26	0.39
	ALH (µm)	4.6	4.98	0.59
	BCF (Hz)	38.78	39.99	0.23
	STR (%)	82.58	82.2	0.88
	LIN (%)	51.36	50.83	0.9
48 h Cooled	TMOT (%)	51.17	54.61	0.83
	PMOT (%)	41.51	47.24	0.70
	VSL (µm/s)	59.53	63.69	0.65
	VAP (µm/s)	72.81	76.93	0.72
	VCL (µm/s)	125.78	136.25	0.51
	ALH (µm)	5.13	5.27	0.81
	BCF (Hz)	38.23	38.78	0.14
	STR (%)	80.57	82.45	0.52
	LIN (%)	47.98	49.19	0.69
Frozen	TMOT (%)	4.98	3.44	0.34
	PMOT (%)	4.26	3.33	0.56
	VSL (µm/s)	45.49	48.89	0.59

VAP (µm/s)	53.1	57.25	0.61
VCL (µm/s)	89.5	91.69	0.88
ALH (µm)	4.23	3.4	0.74
BCF (Hz)	30.92	32.62	0.46
STR (%)	76	77.58	0.68
LIN (%)	46.89	50.52	0.17

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