Effect of Omega 3, 6 and 9 Fatty Acids and Vitamin E on Canine Semen Concentration, Motility, Morphology and Cryopreservation

By

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Abstract

There are few studies in dogs investigating the efficacy of male fertility supplements, most notably fatty acids. The objective of this study was to investigate the effects of a commercial fatty acid supplement (OM3 Gold 1000; Spectrum Veterinary; Phoenix, Arizona), on canine semen quality parameters and cryopreservation survivability. Each capsule contained 144 mg linoleic acid, 500 mg alpha-linolenic acid, 203 mg oleic acid and 20 IU vitamin E. Dogs were assigned to either the treatment (n=8) or control group (n=4). All treatment dogs received one capsule of the supplement twice daily for 8 weeks. Ejaculates were collected prior to initiation of treatment then every two weeks for 8 weeks. Ejaculates were cryopreserved at time zero and 8 weeks and post-thaw motility was assessed. The treatment group was then further subdivided into normal dogs (n=4) and sub-fertile dogs (n=4) based upon initial post thaw motilities being greater or less than 30%, respectively. After 8 weeks, sub-fertile treated dogs had an average post thaw motility that increased from 19% to 51% (P = 0.043). Average post thaw motility for normal treated dogs changed from 52% to 58% (P = 0.55) and control dogs from 45% to 53% (P = 0.51). Fatty acid analysis via gas chromatography was performed on the serum and frozen thawed semen initially and 8 weeks after treatment. In all treatment dogs, there was a decrease of omega-6 linoleic acid (P = 0.013) in the serum compared to controls. No change was detected in fatty acid concentrations in frozen thawed semen samples. In summary, supplementation with a fatty acid supplement may help improve post-thaw motility and decrease the concentration of pro-inflammatory omega-6 fatty acids.

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List of Abbreviations

CASA – Computer assisted semen analysis

CBC – Complete blood cell count

TSN – Total spermatozoa number

LA – Linoleic acid

AA – Arachidonic acid

ALA – Alpha-linolenic acid

EPA – Eicosapentaenoic acid

DHA – Docosahexaenoic acid

DPA – Docosapentanoic acid

OA – Oleic acid

PUFA – Polyunsaturated fatty acid

VCL – Curvilinear velocity

VAP – Average path velocity

VSL – Straight line velocity

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1. Literature Review

1.1 Canine Spermatogenesis

Spermatogenesis is the process of transformation of spermatogonial germ cells into spermatozoa. This takes place within the seminiferous epithelium of the testicles and is comprised of many different phases. The first phase is the proliferation phase which consists of all the mitotic divisions of spermatogonia. The meiotic phase allows for genetic diversity and concludes with the production of haploid (1N) spermatids. The final phase is differentiation where the round undifferentiated spermatid undergoes transformation into a fully differentiated spermatozoa (1). The cycle of the seminiferous epithelium is the progression through a complete series of stages at one location along a seminiferous tubule (1). The mean duration of the seminiferous epithelium cycle for the dog is reported to be 13.73 ± 0.03 days (2). Approximately 4.5 cycles are necessary for the spermatogenic process to be completed thus, the total length of spermatogenesis is reported to be 61.9 ± 0.14 days for the dog (2). If a cross section is taken along any point of the seminiferous tubule, all different stages would be noted. This is referred to as the spermatogenic wave. The importance of the spermatogenic wave is to provide a relatively constant supply of spermatozoa to the epididymis, creating a pool for ejaculation (1). During the transit along the epididymis, spermatozoa undergo morphological and functional changes that allow the spermatozoa to acquire progressive motility and the ability to fertilize an oocyte (1). In the head of the epididymis, the spermatozoa have a proximal cytoplasmic droplet, they are non-motile and not fertile. As the spermatozoa move through the epididymis, the cytoplasmic droplet translocates to a distal position and should eventually be lost during ejaculation. The spermatozoa in the tail of the epididymis should have expression of normal motility and fertilizing potential. In the dog, the epididymal transit time is 15 days from caput to cauda (3).

Spermatozoa are then stored in the tail of the epididymis until ejaculation and it usually contains enough spermatozoa for several ejaculations (1).

1.2 Evaluating canine semen

1.2.1 Motility

Analysis of canine spermatozoa motility is most often a subjective assessment. A sample of raw or diluted semen is placed on a microscope slide and examined for the percentage of the population that is motile. Motility is assessed using one of two methods: visual or computer assisted. The visual or manually assessed motility is usually performed by one or two people who have experience in looking at sperm motility, such that their experience is able improve their accuracy. Motility can also be assessed using computer assisted semen analysis (CASA) software. The computer takes video images of the spermatozoa and stores them for analysis. The system recognizes motile from non-motile sperm and other organic debris by comparing luminosity (gray-scale intensity) and size of the object. There are also preset user-defined thresholds for size and luminosity that helps to prevent mistaking other cells and debris for non-motile sperm (4). Computerized systems have been shown to be more accurate than subjective visual assessment of spermatozoa motility in human, equine, bovine and canine studies (4). The CASA is able to provide different classifications on motility. Progressive motility is the percentage of spermatozoa moving actively, either linearly or in a large circle, regardless of speed (5). Progressive motility can be further classified as rapid or slow progressive, based on speed. Non-progressive motility is all other patterns of motility with an absence of progression, i.e., swimming in small circles or hyperactive motility. Immobility is no spermatozoa movement

seen (5). Progressive motility is important, as it is those forward-moving spermatozoa that are more likely to fertilize an oocyte. Normal canine spermatozoa should have a progressive motility of 70% or greater (6). However, motility assessment alone has not provided the definitive answer for measuring the fertilizing potential of spermatozoa.

Other motility parameters measured by CASA include the following: curvilinear velocity (VCL) which is velocity measured over the actual sperm track and includes all deviations of sperm head movement. Also measured is average path velocity (VAP) or velocity over a calculated, smoothed path, and straight-line velocity (VSL) which is velocity over the straight-line distance between the beginning and end of the sperm track. VSL, VAP, and VCL are all measures of sperm velocity over specified paths and indicate that the sperm classified as high motility swam faster than did those classified as lower motility (7). While the CASA is a great tool, it does have some limitations. Spermatozoa that are swimming backwards due to a bent midpiece or tail may be classified as progressively motile due to its movement pattern, however, that spermatozoa will be unable to successfully fertilize an egg due to the morphological abnormality.

1.2.2 Morphology

Morphology is an assessment of the structure of individual spermatozoa noting any defects that may be present. These preparations are typically examined with a light microscope on oil immersion and 100 to 200 spermatozoa are counted. Background stains (e.g., eosin-nigrosin, India ink) are the most widely used stains because of their ease of application. Because of the stain osmolality, it can result in stain artifacts in spermatozoa, such as coiled or bent tails (8) and this must be considered during the evaluation. Visualization of the structural detail of sperm can

be enhanced by fixing the cells in buffered formal saline and then viewing the unstained cells as a wet mount with either phase-contrast or differential interference contrast (DIC) microscopy. The incidence of artifactual changes is reduced in comparison with stained smears.

The eosin-nigrosin stain is most commonly used. It allows spermatozoa to be readily visualized, and is also a "live-dead" stain, allowing the membrane integrity to be assessed at the same time as morphology (9). The nigrosin stain produces a dark background on which the spermatozoa stand out as lightly colored objects.

Abnormalities in spermatozoal morphology in the dog are typically recorded as the percentage of each specific morphologic defect, e.g., abnormal heads, proximal droplets, distal droplets, abnormal midpieces, coiled tails, etc. This method of classification is thought to be superior because it reveals more specific information regarding a population of spermatozoa (10). In most cases the percentages of morphologically normal spermatozoa have a relatively high correlation to the percentage of progressively motile spermatozoa (4). Spermatozoa structure is related to fertility, however, the impact of specific spermatozoa morphologic features on fertility remains unclear. Investigators are generally in agreement that the percentage of morphologically normal spermatozoa is positively correlated with fertility (6). Normal canine ejaculates should have 80% or greater normal spermatozoal morphology (8).

1.2.3 Volume

The volume of a canine ejaculate can be highly variable depending on the amount of the third fraction (prostatic fluid) that is collected during the ejaculation process. On its own, volume is

not a useful parameter or an indicator of semen quality and is only used to calculate total spermatozoa number (TSN). (e.g. volume (mL) x concentration (million sperm/mL) = TSN).

1.2.4 Concentration

The concentration of a canine ejaculate will vary depending on whether an estrus bitch is present or only a scent swab is used (4). Sperm concentration refers to the number of spermatozoa per unit volume of semen and is a function of the number of spermatozoa emitted and the volume of fluid diluting them (5). Concentration will also vary based on the volume of ejaculate collected, so concentration alone is not a reliable indicator of semen quality and is only used to calculate total spermatozoa number, as described above. Concentration can be measured via several different methods: hemocytometer, photometric and automated cell counters.

The hemocytometer is a glass slide onto which a precision grid has been etched. Since the dimensions of the grid squares and depth of sample chamber are known, it is a simple matter to calculate cell concentration. The hemocytometer method is more time consuming but highly accurate (4).

Photometric methods, such as using a Densimeter (Animal Reproduction Systems, Inc.; Chino CA), measures the optical density of the solution to count the number of sperm cells in the sample. Photometric methods tend to have highly repeatable results, however the accuracy is affected negatively by contamination of the ejaculate with debris such as urine, blood, or an opaque extender such as a yolk-based extender (4).

The NucleoCounter SP-100 analyzer (ChemoMetec; Allerød, Denmark) is an example of an automated cell counter. The NucleoCounter determines sperm concentration, based on

intercalation of the fluorescent dye, propidium iodide, with DNA of sperm after permeabilizing the sperm membranes with a detergent solution to expose the DNA to the dye. The treated sperm emit fluorescence when excited with green light from the fluorescence microscope within the instrument. Studies have demonstrated that this instrument can reliably evaluate spermatozoa concentration, even when semen is pre-mixed in a variety of opaque and non-opaque media (11).

1.2.5 Total Spermatozoa Number

Volume of ejaculate in mL x concentration of spermatozoa in million per mL = total sperm number (TSN) in millions. Testicular volume is highly correlated with daily spermatozoa output in stallions (10). While total testicular volume is routinely measured in stallions and scrotal circumference used in bulls, these measurements are not commonly done in dogs. Normal dogs should produce about 300 million to 2 billion spermatozoa in an ejaculate. Larger testicles are able to produce more spermatozoa as they possess a larger mass of spermatogenic tissue (i.e. larger breed dogs with larger testicles produce higher total sperm numbers than small breed dogs (8). A general guideline for canine total sperm number in an ejaculate is 10 million sperm cells per pound of body weight (4).

1.3 Fatty acids

The majority of fatty acids are found as one of three main classes of esters: triglycerides, phospholipids, and cholesterol esters. Phospholipids are a major component of all cell membranes, including cell membranes of mammalian spermatozoa.

Fatty acids are classified as saturated or unsaturated fatty acids (Figure 1). Unsaturated fatty acids can be further classified into monounsaturated and polyunsaturated fatty acids. Monounsaturated fatty acids contain one double bond and most notably include the omega-9 fatty acids. Polyunsaturated fatty acids (PUFA) contain multiple double bonds and are the omega-3 and omega-6 fatty acids. The number of double bonds affects the melting point of a fatty acid, with more double bonds lowering the melting point (12).

Linoleic acid (omega-6) and alpha-linolenic acid (omega-3) are considered essential fatty acids. This means that the animal is unable to produce them and must receive them from their diet. These two fatty acids cannot be synthesized by animals because they lack the desaturase enzymes required for their production (12) (Figure 2). These fatty acids are responsible for the functions of many different body systems. Omega-6 fatty acids are considered pro-inflammatory whereas omega-3 fatty acids are anti-inflammatory (13).

Linoleic acid (LA) can be converted into arachidonic acid (AA) (Figure 3) and alpha-linolenic acid (ALA) can be converted to EPA and DHA, although metabolism of alpha-linolenic acid to EPA and DHA in humans and animals, is limited (14-16) and in dogs the conversion rate can be less than 10% (17). Linoleic and alpha-linolenic acid are in direct competition for the enzymes that produce the longer more unsaturated fatty acids (16) (Figure 3). Therefore, dietary excess of LA versus ALA will favor more omega-6 long chain fatty acids and dietary excess of ALA versus LA will favor more omega-3 long chain fatty acids. (12). Animals will preferentially incorporate the longer chain fatty acids (e.g. DHA or AA) before the shorter chain precursor fatty acids (e.g. ALA or LA) (16). Hence, direct supplementation of DHA is more effective.

1.4 Spermatozoa plasma membrane composition

The spermatozoa plasma membrane is composed of a bilayer of phospholipids, with the saturated or unsaturated fatty acids as the functional units (18). Fatty acids accumulate in testicular cells through two distinct processes: passive diffusion through the lipid bilayer and/or protein-facilitated transport mediated by CD36 glycoprotein, which is widely expressed in Sertoli cells. Lipids are pivotal and function as fuel for Sertoli cells. They are also used in membrane remodeling of developing germ cells (19). However the plasma membrane lipid content can be modified during spermatogenesis and spermatozoa maturation (20). While passing through the head of the epididymis, the spermatozoa plasma membrane is flexible, facilitating lipid remodeling (21). Across the other epididymal segments (body and tail), the spermatozoa becomes more stable and able to withstand the damages of the storage area situated at the tail of the epididymis (22). The lipid organization of the spermatozoa during epididymal maturation is mediated by protein and lipid constituents of the epididymal fluid (18, 23). Hence, the spermatozoa plasma membrane adjusts in accordance with the environment in which the spermatozoa are located. In comparison to spermatozoa from the head of the epididymis, there is an increase of longer unsaturated fatty acid residues in the spermatozoa of the tail of the epididymis. This particularly applies for docosahexanoic (DHA) and docosapentanoic (DPA) acids that are both markedly increased, whereas arachidonic acid decreases. These data suggest that spermatozoa membranes become more flexible during epididymal maturation due to a higher content of double bonds in the fatty acids (24). At the final stage of epididymal maturation, the plasma membrane reaches its final composition consisting of approximately 70% phospholipids, 25% neutral lipids (mainly cholesterol) and 5% glycolipids (20, 25). The spermatozoa plasma membrane in dogs contains approximately 96% saturated fatty acids, 2%

monounsaturated fatty acids, and 2% polyunsaturated fatty acids (26), 50% of which are DHA (27).

Simultaneously with the aforementioned changes in the spermatozoa lipid membrane, spermatozoa motility parameters gradually change along the epididymal transit, from immobility to progressive movements (28, 29). During the transition from spermiation to the epididymal head, spermatozoa are immotile. Progressive motility, speed and linearity are acquired starting in the epididymal body (30). The spermatozoa's ability to acquire motility depends on changes in the plasma membrane composition and is directly related to spermatozoa motility (31-33). For example, cholesterol and polyunsaturated fatty acids are associated with spermatozoa motility while also providing flexibility to the spermatozoa membrane (34). Additionally, the presence of DHA in the spermatozoa membrane positively correlates to spermatozoa motility in pigs and humans (35). Similarly, low concentrations of such fatty acids were observed in asthenospermic spermatozoa cells in man (32). Incorrect changes in the composition of the spermatozoa plasma membrane phospholipids and fatty acids may culminate in membrane fluidity alteration which may lead to impaired motility, disruption of plasma and acrosomal membranes and, finally, disruption of fertilizing capacity (36).

DHA is essential for the fluidity of the plasma membrane, and thus for the acquisition of spermatozoa motility and the acrosome reaction (37). Moreover, DHA is related to the physiological occurrence of oxidative stress in spermatozoa cells (38). The sperm plasma membrane is rich in polyunsaturated fatty acids (PUFAs), which are sensitive to the attack of reactive oxygen species. Such lipid peroxidation is related physiologically to several important steps of the fertilization process such as spermatozoa hyperactivation, capacitation, the acrosome reaction and finally, fertilization (38). The plasma membrane plays a crucial role during the

acrosome reaction by forming mixed vesicles with the acrosomal membrane forming a continuous membrane structure during fertilization (36).

Maturation of spermatozoa in the epididymal duct is an important event to increase membrane fluidity and flexibility (23). Although it is a crucial prerequisite for the acquisition of spermatozoa motility, it also increases the risk of oxidative damage (24, 39). The presence of unsaturated fatty acids which provides greater fluidity to the membrane and is one of the main targets of the lipoperoxidative process (23, 33). The double bonds, typical of unsaturation, makes them more susceptible to chemical reactivity and may favor the action of free radicals and lipid peroxidation, which may result in membrane damage and loss of motility (36, 40). Their degree of unsaturation is therefore an essential parameter in the ability of spermatozoa to maintain equilibrium in an oxidative environment (23).

1.5 Reactive Oxygen Species (ROS)

Oxidative stress is known to be involved in the pathology of many diseases, and reactive oxygen species are able to deteriorate the fertilizing ability of spermatozoa (41). Since mammalian spermatozoa comprises such large amounts of highly unsaturated fatty acid residues, particularly docosahexanoic acid (DHA) and docosapentanoic acid (DPA) (42) spermatozoa are very sensitive to oxidation by atmospheric oxygen or by the more harmful reactive oxygen species (ROS) that are endogenously generated (24). The major ROS generated by spermatozoa is superoxide anion (O_2-). This electron-reduced product of O_2- reacts with itself via dismutation to generate hydrogen peroxide (H_2O_2) (43) (Figure 4). ROS-induced damage to spermatozoa is mediated by oxidative attack of bis-allylic methylene groups of spermatozoa phospholipid-bound

polyunsaturated fatty acids (PUFAs), leading to lipid peroxidation and inducing DNA damage (44, 45). The oxidative attack occurs do to a free radical, most likely hydroxyl radical, where it can abstract a hydrogen atom from the polyunsaturated acyl radical, thereby initiating the lipid peroxidation chain (46).

ROS can be toxic for spermatozoa and can significantly affect spermatozoa motility (47). The sources of ROS in the genital tract are the spermatozoa themselves during cellular respiration and in the mitochondria, leukocytes during inflammatory processes, and hypoxia caused by vascular diseases (23). The generation of ROS has been correlated with spermatozoa pathologies. In fact, increased concentration of ROS are readily detectable in a large percentage of infertile human patients, while they are found at very low concentrations in fertile subjects (43). Experiments have shown that degenerate cells isolated from percoll gradients are the main source of ROS and consist mostly of leukocytes and damaged spermatozoa (23). ROS production in animals with low motility is most likely to be the cause of the low motility rather than the effect of the decreased motility (43).

Excess free radical generation frequently involves errors in spermatogenesis resulting in the release of spermatozoa with large amounts of cytoplasmic droplets (48). Cytoplasmic droplets contain enzymes that fuel production of reactive oxygen species by the spermatozoa's plasma membrane redox systems. The consequences of such oxidative stress include loss of motility and fertilizing potential, and the induction of DNA damage in the spermatozoa nucleus. The loss of spermatozoa function is due to the peroxidation of unsaturated fatty acids in the spermatozoa plasma membrane. As consequence of this, the membrane loses its fluidity and the cells lose their function (48).

Reactive oxygen species (ROS) are very important during the normal spermatozoa capacitation process (49). These positive effects are strictly related to the equilibrium between ROS and the scavenger systems (23). ROS are normally produced at low levels; however, in some cases of infertility, they are produced in much higher amounts. ROS interrupt spermatozoa function by causing peroxidative damage to the plasma membrane and thereby impair motility, the acrosome reaction (exocytosis), and disrupt spermatozoa-oocyte fusion (50). Spermatozoa are particularly susceptible to oxidative stress due to the reduced cytoplasm content with the consequent limited amount of enzymatic antioxidant and the composition of plasma membrane, rich in polyunsaturated fatty acids, which are more easily oxidized (51). Furthermore, important spermatozoa functions are impaired, such as DNA integrity, lipid, carbohydrate and protein functionality, which may lead to apoptosis and cellular death (51). Oxidative stress may also cause mitochondrial DNA and nuclear genome damage (52).

To avoid damage caused by ROS, antioxidants are present in the epididymal plasma (47). These substances, in low concentrations, delay or prevent the oxidation of a substrate. Living organisms are known to physiologically produce a number of antioxidants that may minimize the effect of ROS (53, 54). Plasmalogens, a phospholipid that contains a vinyl ether linkage, may serve a protective role during oxidant-induced stress, functioning as an endogenous antioxidant (55). Plasmalogens are at least as important as alpha-tocopherol in scavenging free radicals within the cell (55). Due to the considerable time period in which spermatozoa remain in transit through the distinct segments of the epididymis, the increased cell density of this environment may potentially boost ROS generation (24). These substances are believed to play an important role in spermatozoa protection against oxidative stress. Among the enzymatic antioxidants previously found in ejaculated semen, superoxide dismutase (SOD), catalase (CAT) (Figure 4), glutathione

(GSH), glutathione reductase (GR), and glutathione peroxidase (GPx) are the most important (56). In dogs, however, little is known regarding the specific profile and the region in which these antioxidants are added to the semen (i.e. male sexual accessory glands or epididymis) (57). The activity of SOD, based on concentration is known to correlate with several spermatozoa functions, such as motility, for both fresh (58) and cryopreserved semen (59).

In mature spermatozoa, the high concentration of unsaturated lipids is associated with a relative scarcity of oxyradical scavenger enzymes such as superoxide dismutase, catalase and glutathione peroxidase (60). This relative deficiency is probably due to the virtual absence of cytoplasm in mature spermatozoa cells. This, however, is offset by the powerful antioxidant system in seminal plasma. Several studies have demonstrated that, in contrast to other biological fluids, seminal plasma contains significant levels of SOD, catalase and glutathione peroxidase together with significant concentrations of chemical antioxidants such as ascorbic acid (vitamin C) and tocopherol (vitamin E). Vitamin E is a chain-breaking antioxidant and its role is to terminate the free radical cascade in cellular membranes (23).

According to Tramer et al. (61), antioxidants are essential to ensure protection during the acquisition of motility and the modifications that occur in the plasma and acrosomal membranes during spermatozoa maturation (62). This assertion is also suspected in dogs due to the positive correlation between SOD and motility found in the epididymal tail and body (57).

It has been hypothesized that the increase of reactive oxygen species (ROS) during cryopreservation and the decrease of antioxidant activity of the spermatozoa cause the peroxidative damage to the spermatozoa plasma membrane and affect DNA integrity (63-65).

ROS are a delicate balancing act as far as spermatozoa are concerned: a low level of ROS exposure is physiologically required to drive the processes associated with sperm capacitation and fertilization, whereas over-exposure to such metabolites leads to a state of oxidative stress that decreases the fertilizing potential (66).

2. Studies using fatty acids

It was noted that human men that had lower concentrations of omega-3 fatty acids in seminal plasma were more likely to be infertile (42, 67). Another study evaluated dietary intake of polyunsaturated fatty acids as it related to spermatozoa quality. It was determined that those men who consumed higher levels of omega-3 fatty acids in their diets had better spermatozoa quality (67). Additional human studies have also evaluated the use of omega-3 fatty acid supplements with varying success (68-70).

A study in stallions showed improvement in cooling and freezing ability of spermatozoa in stallions that received a supplement that supplied 30% of the total fatty acids as omega 3, 25% of which was DHA in their diet for 14 weeks. The spermatozoa also showed higher swimming velocity while stallions were fed the DHA supplement. This study also found that stallions that had initial poor spermatozoa quality showed greater improvement in cooling and freezing while receiving the DHA, indicating that the benefit of supplementation may be greater in stallions with poorer semen quality (71). It was also mentioned that stallion spermatozoa may be more resistant to the effects of cold shock if the omega-6 to omega-3 fatty acid ratio was further reduced by incorporating more omega-3 fatty acids in the diet (71). Another study performed in stallions has shown that an increase in motility does correlate with an increase in fertility, therefore any supplement that can demonstrate an improvement in motility can therefore hope to increase subsequent fertility (72).

Scientific published reports evaluating the use of fatty acids to improve semen motility, morphology and cryopreservation in dogs are lacking. Michael et al. added different antioxidants directly to canine semen prior to freezing and evaluated the effect on post thaw parameters. In this study, Vitamin E was noted to have a slight beneficial effect (73). In another study, dexamethasone was administered to dogs to mimic a stress response leading to decreased spermatozoa quality. Dogs were treated with 500mg of Vitamin E orally for 1 week prior to dexamethasone treatment and compared to dogs receiving only dexamethasone. The dogs that were pre-treated with Vitamin E had decreased percentages of head and acrosomal abnormalities due to dexamethasone than dogs that were not pre-treated with Vitamin E (74).

The essential role of polyunsaturated fatty acids in membrane constitution and in the fertilization process has been confirmed by experimental data on rats fed an essential fatty acid deficient diet. Together with decreased concentrations of polyunsaturated fatty acids in both red blood cells and serum, these animals showed a degeneration of the seminiferous tubules, a progressive decrease in germinal cells and an absence of spermatozoa in the lumen of the seminiferous tubules and epididymis (75).

The majority of the fatty acid studies, mentioned above, use DHA instead of shorter chain precursor (ALA). Is it possible that ALA can be as effective as DHA, considering the slow conversion to DHA.

3. Hypothesis and Objectives

3.1 Hypothesis

The primary hypothesis evaluated in this study is that the use of an omega 3, 6 & 9 fatty acid and vitamin E supplement will improve at least one of the study variables (concentration, motility, morphology, or post thaw motility) in the dog.

3.2 Objectives

The objective of this study is to track changes in semen parameters (concentration, motility, morphology and post thaw motility), before and after treatment with a fatty acid/Vitamin E supplement.

4. Materials and Methods

4.1 Dog inclusion criteria

Twelve healthy client-owned dogs between the ages of one and six years of age were enrolled. All dogs were of average body size (25 kg to 31 kg) and consisted of ten Labrador retrievers, a German shepherd dog and a Siberian husky. Dogs were given full physical and reproductive examinations, ensuring that all dogs had 2 scrotal testes and no other health problems. A complete blood cell count (CBC) and serum biochemical analysis was performed to establish normal physiological health. Finally, a negative Brucellosis test was required for all dogs enrolled into the study. All dogs were to maintain their current commercial diet and exercise habits and were not allowed to be on any other supplements at least 2 months prior or for the duration of the study. The dogs remained with their owners throughout the study period and traveled to small animal

Theriogenology service for semen collections. The owners of dogs in the treatment group were responsible for administration the supplement.

4.2 Semen collection procedure and categorization of treatment groups

Dogs were collected via digital manipulation into disposable plastic cones using estrus scent swabs as a tease source. Each dog had semen collected every two to three days for four to five collections to ensure that they depleted reserves of stored spermatozoa within the epididymis (76).

The dogs were randomly distributed into two study groups, a treatment group (n=8) and a control group (n=4). The treated dogs were further categorized into sub-fertile dogs (n=4) and normal dogs (n=4), based on initial post thaw motility. Treated dogs with initial post thaw motility of less than 30% (n=4) were classified as sub-fertile and those with motility greater than 30% were classified as normal. None of the dogs in the control group were classified as sub-fertile, as their initial post thaw motilities were 30% or higher.

Following the initial collection, dogs within the treatment group received one capsule of the supplement (linoleic acid 144 mg, alpha-linolenic acid 500 mg, oleic acid 203 mg, vitamin E 20 UI) by mouth twice daily for eight weeks. This dose was based on the manufacturer's recommendation. The sources of the fatty acids are the primula flower, borage flower, soybean and sunflowers.

4.3 Data collection

All 12 males were collected at time zero and each ejaculate was analyzed to determine volume, spermatozoa concentration using a NucleoCounter SP-100 analyzer (ChemoMetec; Allerød, Denmark), total spermatozoa number, total and progressive motility using computer assisted spermatozoa analysis (CASA, SpermVision SAR, MOFA Global; Verona, Wisconsin) and velocity (CASA). Normal and abnormal morphology of the spermatozoa cells was manually performed by counting 100 spermatozoa on oil immersion by an experienced theriogenologist using an eosin-nigrosin stain. All morphological defects for each sperm were recorded. Collection at time zero was also cryopreserved and post thaw motility was assessed subjectively by two different observers.

Total spermatozoa number (TSN) was calculated by multiplying the volume of the ejaculate in milliliters by the spermatozoa concentration, as determined by the NucleoCounter SP-100 analyzer (ChemoMetec; Allerød, Denmark). Progressive motility was performed using fresh extended semen and all samples were extended to a concentration between 25 to 50 million/mL, based on the manufacturer's recommendation. The curvilinear velocity was assessed by the CASA software (SpermVision SAR, MOFA Global; Verona, Wisconsin).

All 12 dogs then underwent semen collection and analysis every two weeks for the 8 week trial. At these visits, spermatozoa concentration and progressive motility was assessed within five minutes of collection. Serum and seminal plasma were also collected at each visit and stored frozen at -80 C for future fatty acid analysis. All collections and evaluations were performed by the same individual, who was blinded to which dogs received the supplement. After the eight week study period, the supplement was discontinued. A second CBC and serum biochemical analysis was performed after the supplement was discontinued to determine that the overall

health of each animal had not changed. The last ejaculate obtained prior to discontinuing the supplement (8 week time point) was cryopreserved, and the post thaw motility was compared to the initial cryopreserved sample. All dogs were followed every 2 weeks for an additional 4 weeks with semen analysis and physical examinations.

Immediately after collection, the semen sample was transferred into a conical tube and volume was recorded. Motility, morphology, total spermatozoa number and post thaw motility were measured at the initial start of the study and again after 8 weeks. Motility parameters and total spermatozoa number in the ejaculate were monitored every two weeks. After above parameters were measured, the semen was centrifuged at 2000 rpm for 10 minutes. The seminal plasma (supernatant) was immediately separated and frozen (-80 ^{o}C) until analysis. A venous blood sample was drawn from each dog immediately prior to the start of the study and repeated every two weeks for the remainder of the study. Blood samples were collected by jugular venipuncture and placed into a plain red top tube. Blood was allowed to clot, and then centrifuged at 3000 rpm for 10 minutes. Serum was immediately separated, and frozen (-80 ^{o}C) until fatty acid analysis.

4.4 Semen freezing

At time zero and 8 weeks, semen was cryopreserved on all dogs using Irvine Scientific (Santa Ana, CA) refrigeration and cryopreservation media. A concentration was obtained on the neat sample. The ejaculate was then mixed at a ratio of 1:1 with refrigeration medium and centrifuged at 2000 rpm for 10 minutes. After centrifugation, the supernatant was discarded and the sperm pellet was resuspended with fresh refrigeration media to a concentration of 400 million spermatozoa per milliliter. The semen was allowed to equilibrate at 5°C for one hour. After equilibration, freezing media was added to the semen at a ratio of 1:1, decreasing the total

concentration to 200 million spermatozoa per milliliter. The Irvine Scientific freezing media contains 20% egg yolk and 12% glycerol. The sample was gently mixed and then loaded into 0.5mL straws and vapor cooled 3 cm above liquid nitrogen for 10 minutes before being plunged into the liquid nitrogen. Post thaw motility was obtained after the freezing process. The spermatozoa were thawed in a water bath at 37ºC for 30 seconds. Post thaw motilities were visually assessed by two experienced clinicians within 5 minutes after thawing, as the freezing media was too optically dense to be read accurately with the CASA. The remainder of the semen straws were stored in dewars until further fatty acid analysis.

4.5 Gas chromatography analysis

Fatty acid methylation was carried out for all samples using in situ transesterification with 0.5N methanolic NaOH followed by 14% boron trifluroide (BF_3) in methanol as described by Park and Goins (77). Two hundred microliters of spermatozoa, 300 μL of serum or 400 μL of seminal plasma were added to glass tubes. One hundred microliters of methylene chloride and 1mL of 0.5N NaOH in methanol were added to each tube. The tubes were flushed with nitrogen, capped and placed into a 90°C water bath for 10 minutes. The samples were allowed to cool briefly then 1 mL of 14% BF_3 in methanol was added. The tubes were again flushed with nitrogen, capped and placed into a 90°C water bath for an additional 10 minutes. The samples were allowed to cool to room temperature. Then 1 mL of distilled water and 300 μL of hexane were added. The tubes were vortexed for 30 seconds and then centrifuged at 2000 rpm for 10 minutes. The top layer, representing the fatty acid methyl ester (FAME) was transferred to gas chromatograph (GC) vials. The fatty acid methyl esters were separated using a TR-FAME column (P/N: 260M153P, 60 m x 0.22 mm, 0.25um film thickness) from ThermoFisher Scientific (Bellefonte, PA, USA) and

quantified using a gas chromatograph (Agilent Technologies 7890A GC system; Santa Clara, CA, USA). The GC was fitted with a flame ionization detector and hydrogen (31 psi) was used as the carrier gas. The injector temperature was set at 240°C and the detector temperature was 290°C. The column oven was held at an initial temperature of 100°C for 2 minutes and then programmed to increase at a rate of 3.0°C per minute to a final temperature of 220°C, which was held for 6 min (total run time 48 min). For the identification and determination of the fatty acid composition, the standard retention times of fatty acid methyl esters were used as a reference (Food Industry FAME Mix, VWR).

5. Statistical Analysis

Descriptive statistics for all variables were analyzed through SAS 9.4. Morphology, post thaw motility, and GC related variables (LA, OA, ALA, AA, EPA, and DHA) had only two time points of measurement per subject. The change from baseline value was calculated for each subject. The unpaired t- test implemented in SAS 9.4 was employed for comparing the average change across groups. The mixed model ANOVA with repeated measures using proc mixed in SAS 9.4 were applied for total spermatozoa number and progressive motility, which were measured in seven time points. Significance was set at 0.05 for all parameters.

6. Animal Welfare

All animals were privately owned and remained with their owners for the duration of the study. This study was approved by the Clinical Research Review Committee of the College of Veterinary Medicine (CRRC) and the Institutional Animal Care and Use Committee (IACUC),

Office of Animal Resources, Auburn University. All procedures performed were in compliance with both entities.

7. Results

7.1 Total spermatozoa number

In the control group, TSN at the beginning and end of the study was 624.6 million ± 114.3 million and 700.7 million ± 366.6 million, respectively. For normal treated dogs, TSN at the beginning and end of the study was 458.1 million ± 204.5 million and 609.2 million ± 237.8 million, respectively. For sub-fertile treated dogs, TSN at the beginning and end of the study was 634.7 million ± 364.9 million and 807.2 million ± 304.1 million, respectively. No significant difference was found between TSN at Time 0 vs end of study in any of the groups, though the sub-fertile dogs were trending towards significance (P = 0.063). (Table 1)

Table 1: Total spermatozoa numbers at time 0 (initial) and after 8 weeks. The sub-fertile dogs were trending towards significance P = 0.063.

Group		Initial TSN (in millions)	8 weeks TSN (in millions)
Control dogs		624.6 ± 114.3	700.7 ± 366.6
Treated dogs	Normal	458.1 ± 204.5	609.2 ± 237.8
	Sub-fertile	634.7 ± 364.9	807.2 ± 304.1

7.2 Progressive motility (Fresh ejaculate)

In the control group, the progressive motility at the beginning and end of the study was 72.3% ± 8.1% and 80% ± 7.8%, respectively. For normal treated dogs, progressive motility at the beginning and end of the study was 77% ± 9.9% and 85.5% ± 3.3%, respectively. For sub-fertile treated dogs, progressive motility at the beginning and end of the study was 73.3% ± 18.6% and 78.5% ± 6.6%, respectively. Progressive motility was not significantly different in any of the groups between Time 0 and end of study (Table 2).

Table 2: Progressive motility (PM) at time 0 (initial) and after 8 weeks. There was no significant difference between the control and treatment dogs.

Group		Initial PM (in percent)	8 weeks PM (in percent)
Control dogs		74.3 ± 8.1	80.0 ± 7.8
Treated dogs	Normal	77.0 ± 9.9	85.5 ± 3.3
	Sub-fertile	73.3 ± 18.6	78.5 ± 6.6

7.3 Curvilinear velocity

In the control group the curvilinear velocity at the beginning and end of the study was 130.7 um/sec ± 23.05 um/sec and 140.95 um/sec ± 15.78 um/sec, respectively (P = 0.02). For the normal treated dogs, the curvilinear velocity at the beginning and end of the study was 142.05 um/sec ± 19.96 um/sec and 139.93 um/sec ± 20.54 um/sec, respectively. For the sub-fertile treated dogs, the curvilinear velocity at the beginning and end of the study was 133.33 um/sec ± 20.58 um/sec and 140.63 um/sec ± 17.63 um/sec, respectively. Only the control dogs had a significant increase in curvilinear velocity. (Table 3)

Table 3: Curvilinear velocity of the dogs at time 0 (initial) and after 8 weeks. The * denotes significance.

Group		Initial (um/sec)	8 weeks (um/sec)
Control dogs		130.7 ± 23.05	140.95 ± 15.78*
Treated dogs	Normal	142.05 ± 19.96	139.93 ± 20.54
	Sub-fertile	133.33 ± 20.58	140.63 ± 17.63

7.4 Morphology

The morphology was examined by a single theriogenologist and was assessed using eosin nigrosin stain. In the control group, normal morphology was assessed at beginning and end of the study (Time 0 and 8 weeks) and was 56.8% ± 8.5% and 53.3% ± 19.1%, respectively. For normal treated dogs, normal morphology at the beginning and end of the study was 56.8% ± 12.3% and 59.5% ± 20.8%, respectively. For sub-fertile treated dogs, normal morphology at the beginning and end of the study was 64.5% ± 15.9% and 74.3% ± 6.7% (P = 0.058), respectively. (Table 4). The most common morphological abnormalities did not change for any of the groups during the treatment period.

Table 4: Normal morphology of the dogs at time 0 (initial) and after 8 weeks, morphology slides were made on the same days that cryopreservation was performed. No significant difference was found between control or treatment dogs.

Group		Initial normal morphology (% normal)	8 weeks normal morphology (% normal)
Control dogs		56.8 ± 8.5	53.3 ± 19.1
Treated dogs	Normal	56.8 ± 12.3	59.5 ± 20.8
	Sub-fertile	64.5 ± 15.9	74.3 ± 6.7

7.5 Post thaw progressive motility

In the control group the post thaw motility at the beginning and end of the study was 45% ± 12.9% and 52.5% ± 12.5%, respectively. For normal treated dogs, post thaw motility at the beginning and end of the study was 51.7% ± 10.7% and 57.5% ± 8.6%, respectively. For sub-fertile treated dogs, post thaw motility at the beginning and end of the study was 19% ± 11.1% and 51.3% ± 4.7% (P=0.043), respectively Figure 5.

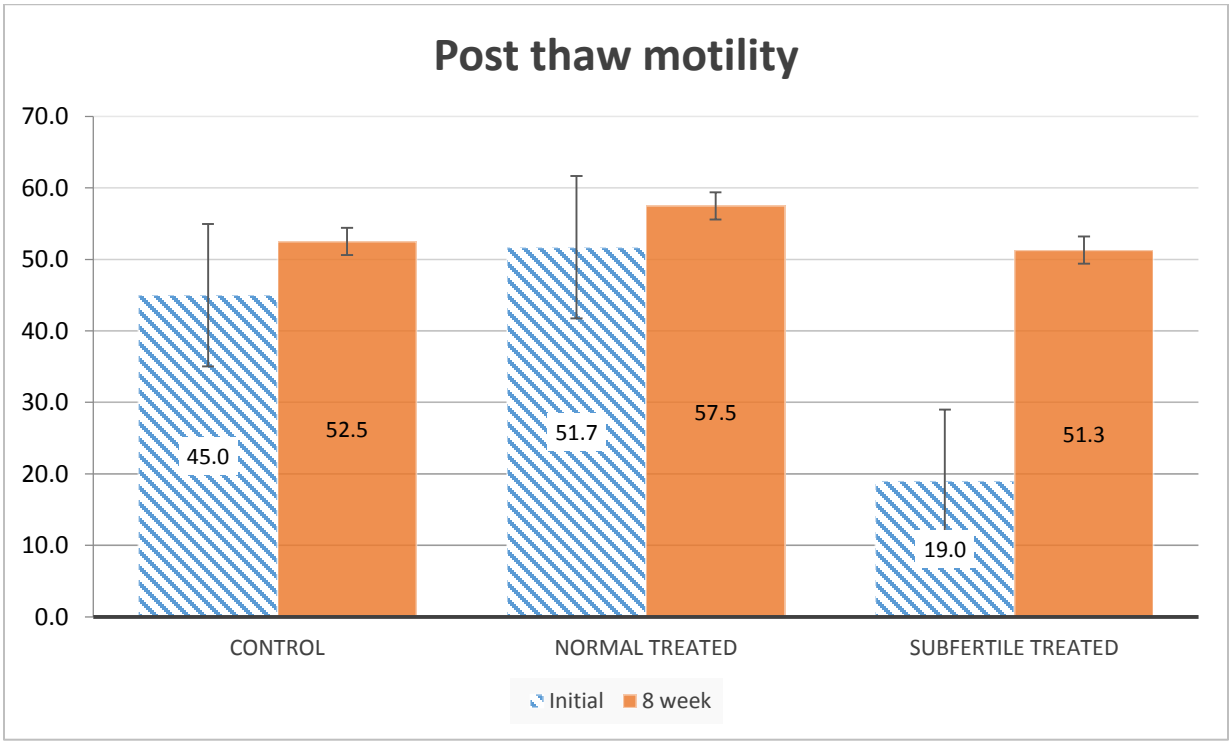

Figure 5 – Post thaw motility at time 0 (initial), in stripes, and after 8 weeks, in solid.

7.6 Gas chromatography

7.6a – Serum fatty acid measurement

There was no significant difference in the levels of arachidonic acid (AA), alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and oleic acid (OA) in the serum between treatment and control dogs at any time point. For the omega-6 fatty acid, linoleic acid (LA), at the beginning of the study levels were equivocal between groups but after 8 weeks there was an overall decrease in all treatment dogs (P=0.013) Table 5 and Figure 6.

Table 5: Gas chromatography of the serum fatty acids at time 0 (initial) and after 8 weeks. The asterisk (*) denotes significance.

Fatty acids	Time	Control dogs	Treated dogs
Omega-6 Linoleic acid	Initial	23.8	27.14
	8 weeks	25.14	22.42 *
Omega-6 Arachidonic acid	Initial	14.53	15.60
	8 weeks	14.47	13.97
Omega-3 Alpha-linolenic acid	Initial	0.35	0.21
	8 weeks	0.77	0.39
Omega-3 DHA	Initial	3.17	2.99
	8 weeks	2.82	2.72
Omega-3 EPA	Initial	3.23	3.38
	8 weeks	2.55	3.27
Omega-9	Initial	10.80	9.04

Oleic acid	8 weeks	13.56	8.02

7.6b – Frozen thawed semen fatty acid measurement

There was no significant difference in the levels of linoleic acid (LA), arachidonic acid (AA), alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and oleic acid (OA) in the frozen thawed semen between treatment and control dogs (Table 6).

Table 6: Gas chromatography of the frozen thawed semen fatty acids at time 0 (initial) and after 8 weeks. There was no significant difference found between the control and treatment dogs.

Fatty acids	Time	Control dogs	Treated dogs
Omega-6 Linoleic acid	Initial	20.05	19.78
	8 weeks	20.74	20.64
Omega-6 Arachidonic acid	Initial	2.27	2.49
	8 weeks	2.35	2.43
Omega-3 Alpha-linolenic acid	Initial	0.39	0.39
	8 weeks	0.38	0.37
Omega-3 DHA	Initial	0.60	0.64
	8 weeks	0.60	0.62
Omega-3 EPA	Initial	0.00	0.00
	8 weeks	0.00	0.00
Omega-9 Oleic acid	Initial	29.95	33.83
	8 weeks	36.59	37.02

7.6c – Seminal plasma fatty acid measurement

For the Omega 6 fatty acids in the seminal plasma; linoleic acid (LA) levels decreased in all treatment dogs (P=0.038). The levels of arachidonic acid (AA) decreased in all control dogs (P=0.011). There was no significant difference in the levels of alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and oleic acid (OA) in the seminal plasma between treatment and control dogs at any time point (Table 7).

Table 7: Gas chromatography of the seminal plasma fatty acids at time 0 (initial) and after 8 weeks. The asterisk (*) denotes significance.

Fatty acids	Time	Control dogs	Treated dogs
Omega-6 Linoleic acid	Initial	1.64	6.11
	8 weeks	7.34	0.07*
Omega-6 Arachidonic acid	Initial	8.60	2.99
	8 weeks	0.80*	0.46
Omega-3 Alpha-linolenic acid	Initial	0.00	0.02
	8 weeks	0.11	0.20
Omega-3 DHA	Initial	6.82	12.76
	8 weeks	0.70	0.37
Omega-3 EPA	Initial	0.00	1.10
	8 weeks	0.00	0.00
Omega-9 Oleic acid	Initial	10.93	5.35
	8 weeks	6.71	1.44

8. Discussion

Cryopreservation is an incredible tool that has allowed genetics of highly desirable animals to be maintained for future use. Breeding with frozen semen in both dogs and horses has lower pregnancy rates than either natural cover or fresh artificial insemination (78, 79). Reduced fertility of frozen semen is attributed mainly to the altered membrane structure and function during the freezing and thawing process (9). The fatty acid supplement tested in this study helped to improve the post thaw motility after cryopreservation of dogs with sub-optimal post thaw motility compared to control dogs. This is similar to what was previously described in horses (71). The fatty acids are incorporated into the spermatozoa plasma membrane (80), which makes the membrane more malleable and able to better withstand the cryopreservation process. Polyunsaturated fatty acids help to maintain the integrity of the cellular membrane's lipid bilayer. DHA may contribute to the membrane fluidity necessary for the motility of spermatozoa tails (23, 81).

The main targets for reactive oxygen species are DNA and membrane lipids; canine spermatozoa membranes are rich in polyunsaturated fatty acids (32). Antioxidants help to combat these reactive oxygen species. The vitamin E in the product and any increase in superoxide dismutase activity due to the increased amount of EPA and DHA in the seminal plasma may have helped minimize the free radicals that were produced during the freezing process and mitigate oxidative damage done to the spermatozoa plasma membrane. The generation of reactive oxygen species leads to a marked decline in spermatozoa motility (82). However there may be unseen damage that is not detected when performing a simple motility assessment, except for an overall decreased motility. These unseen damages may also prevent motile spermatozoa from being able to successfully undergo the acrosomal reaction. Additionally, even when oocytes are fertilized successfully, there may be nuclear damage due to reactive oxygen species that can lead to early embryonic death. The

structural integrity of the spermatozoal plasma membrane plays a pivotal role in successful fertilization (54).

Spermatozoa have high levels of DHA compared to other cells in the body (83), as DHA is associated with Sertoli cells (84, 85). Aksoy et al. reported higher omega-6:omega-3 ratios in spermatozoa of infertile vs. fertile men, and inadequate DHA concentration was associated with low-quality spermatozoa (32). Traditionally, animal diets are higher in levels of omega-6 fatty acids (80) and decreasing the ratio of omega-6 to omega-3 fatty acids may increase the fertility of a given animal. Owners in our study were not asked to feed a diet different from what they were already feeding. Commercial dog food has an average omega-6 to omega-3 ratio of 8:1 (86). Research in dogs has suggested that 6:1 is a more optimal dietary omega-6/omega-3 fatty acid ratio (87). In our study, the dogs receiving the supplement had a decrease in their omega-6 to omega-3 ratio to approximately 6:1, based on the average ratio of commercial dog foods. The supplement used has a good omega-6 to omega-3 ratio, which makes it an ideal supplement for dogs. Since it is plant based supplement, it avoids allergies and palatability issues for some dogs.

After supplementation, levels of omega-6 fatty acids (most notably linoleic acid) decreased in the serum of all dogs that received the supplement. Although a decrease in omega-6 was seen the concomitant increase in omega-3 was not seen. This may be due to the slow conversion of ALA into DHA. There were not any changes noted in the fatty acid concentrations in the frozen thawed semen. This could be due to low level changes that were difficult to detect or due to the fact that the spermatozoa were not washed off of the freezing media before analysis

In this study, there was a trend towards increased total spermatozoa number and increased normal morphology. While significance was not found, this may have been due to a small sample size in this study. It is possible that significance was not found due to the fact that the dogs had been

repeatedly collected. After being collected many times, the dogs were comfortable with the procedure and therefore gave better collections. The spermatogenic cycle in the dogs is 62 days (2). However, waves that occur during spermatozoa production may allow for some changes to be seen sooner than 60 days. As noted in the equine study, some stallions were showing improvement before a full spermatogenic cycle (71). An increase in the progressive motility of fresh extended samples was not found in this study but was likely due to the fact that all dogs already showed good initial motility. Good motility is defined as having at least 70% of the spermatozoa moving in a forward or progressive direction (6). A similar study in dogs also found no increase in progressive motility after treatment with a fatty acid supplement for 60 days; they did, however, note an increase in progressive motility in spermatozoa subjected to a thermoresistance test after supplementation (88). An increase in the progressive motility of fresh semen might have been found if the dogs used in our study had started with poorer progressive motility. As noted in the stallion study, animals that have marginal fertility and those whose spermatozoa have poor tolerance to cooling and freezing would be animals most likely to benefit from supplementation (71).

In regards to the curvilinear velocity, there was a significant increase seen in control dogs versus treatment dogs. Although the sub-fertile dogs did show an increase as well, it was not significant. The seminal plasma did have a significant decrease in omega-6 fatty acids; however the true significance of this is unable to be determined. The initial seminal plasma samples were collected the day before the dogs presented for cryopreservation. However, when the dogs returned for the 8 week freezing collection, the seminal plasma was not removed from the sample prior to mixing with the Irvine Scientific refrigeration media. Therefore the second seminal plasma data is inaccurate as it is not a neat sample, and refrigeration media may contain

fatty acids. Gas chromatography was not performed using refrigeration media alone, but was performed on a 1:1 ratio of the refrigeration media and freezing media, (See table 8) as would be used in the normal semen freezing process.

Modifications during spermatozoa maturation in the epididymis occur according to the protein and lipid profile of the epididymal fluid [41]. Ramos et al found an increase in saturated fatty acids between the epididymal body and tail, but not for DHA concentration. Therefore, it can be inferred that during the spermatozoa transit between body and tail of the epididymis, DHA from the epididymal lumen was incorporated into spermatozoa cells, thus consuming the DHA concentration of the epididymal fluid and increasing the lipid concentration of the spermatozoa plasma membrane. For these reasons, in dogs, fatty acids of the epididymal fluid may act as signaling factors and regulators of the lipid secretion by the epididymal cells at head and body level, as they are later incorporated into the spermatozoa at the final stages of spermatozoa maturation (26). This may also explain why there was no elevation in the levels of the DHA in the seminal plasma in our study, as it had already been incorporated into the spermatozoa plasma membranes.

A problem is that in vitro analysis does not equal in vivo function. Ejaculated spermatozoa examined in vitro do not exhibit the characteristics they will take on as they traverse the reproductive tract of the female (4). Going forward, it would be ideal to use known sub-fertile males, that are administered the supplement and allowed to breed naturally. Fertility status and semen parameters can be monitored for improvement.

9. Conclusions

Based on the results of this study, fatty acid and antioxidant supplementation in the canine can lead to more motile spermatozoa surviving the cryopreservation process. This is thought to be a result of improved cell membrane stability, decreased activity of reactive oxygen species and increasing levels of omega 3 fatty acids, which then serves to decrease pro-inflammatory omega-6 fatty acids in the serum. It appears that sub-fertile males may benefit most from such supplementation.

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Tables:

Table 1: Total spermatozoa numbers at time 0 (initial) and after 8 weeks. The sub-fertile dogs were trending towards significance P = 0.063.

Group		Initial TSN (in millions)	8 weeks TSN (in millions)
Control dogs		624.6 ± 114.3	700.7 ± 366.6
Treated dogs	Normal	458.1 ± 204.5	609.2 ± 237.8
	Sub-fertile	634.7 ± 364.9	807.2 ± 304.1

Table 2: Progressive motility (PM) at time 0 (initial) and after 8 weeks. There was no significant difference between the control and treatment dogs.

Group		Initial PM (in percent)	8 weeks PM (in percent)
Control dogs		74.3 ± 8.1	80.0 ± 7.8
Treated dogs	Normal	77.0 ± 9.9	85.5 ± 3.3
	Sub-fertile	73.3 ± 18.6	78.5 ± 6.6

Table 3: Curvilinear velocity of the dogs at time 0 (initial) and after 8 weeks

Group		Initial (um/sec)	8 weeks (um/sec)
Control dogs		130.7 ± 23.05	140.95 ± 15.78
Treated dogs	Normal	142.05 ± 19.96	139.93 ± 20.54
	Sub-fertile	133.33 ± 20.58	140.63 ± 17.63

Table 4: Normal morphology of the dogs at time 0 (initial) and after 8 weeks, morphology slides were made on the same days as the cryopreservation was performed.

Group		Initial normal morphology (% normal)	8 weeks normal morphology (% normal)
Control dogs		56.8 ± 8.5	53.3 ± 19.1
Treated dogs	Normal	56.8 ± 12.3	59.5 ± 20.8
	Sub-fertile	64.5 ± 15.9	74.3 ± 6.7

Table 5: Gas chromatography of the serum fatty acids at time 0 (initial) and after 8 weeks. The * denotes significance. The fatty acids are expressed as an area percentage of the total fatty acids.

Fatty acids	Time	Control dogs	Treated dogs
Omega-6 Linoleic acid	Initial	23.8	27.14
	8 weeks	25.14	22.42 *
Omega-6 Arachidonic acid	Initial	14.53	15.60
	8 weeks	14.47	13.97
Omega-3 Alpha-linolenic acid	Initial	0.35	0.21
	8 weeks	0.77	0.39
Omega-3 DHA	Initial	3.17	2.99
	8 weeks	2.82	2.72
Omega-3 EPA	Initial	3.23	3.38
	8 weeks	2.55	3.27
Omega-9 Oleic acid	Initial	10.80	9.04
	8 weeks	13.56	8.02

Table 6: Gas chromatography of the frozen thawed semen fatty acids at time 0 (initial) and after 8 weeks. There was no significant difference found between the control and treatment dogs. The fatty acids are expressed as an area percentage of the total fatty acids.

Fatty acids	Time	Control dogs	Treated dogs
Omega-6 Linoleic acid	Initial	20.05	19.78
	8 weeks	20.74	20.64
Omega-6 Arachidonic acid	Initial	2.27	2.49
	8 weeks	2.35	2.43
Omega-3 Alpha-linolenic acid	Initial	0.39	0.39
	8 weeks	0.38	0.37
Omega-3 DHA	Initial	0.60	0.64
	8 weeks	0.60	0.62
Omega-3 EPA	Initial	0.00	0.00
	8 weeks	0.00	0.00
Omega-9 Oleic acid	Initial	29.95	33.83
	8 weeks	36.59	37.02

Table 7: Gas chromatography of the seminal plasma fatty acids at time 0 (initial) and after 8 weeks. The asterisk (*) denotes significance. The fatty acids are expressed as an area percentage of the total fatty acids.

Fatty acids	Time	Control dogs	Treated dogs
Omega-6 Linoleic acid	Initial	1.64	6.11
	8 weeks	7.34	0.07*
Omega-6 Arachidonic acid	Initial	8.60	2.99
	8 weeks	0.80*	0.46
Omega-3 Alpha-linolenic acid	Initial	0.00	0.02
	8 weeks	0.11	0.20
Omega-3 DHA	Initial	6.82	12.76
	8 weeks	0.70	0.37
Omega-3 EPA	Initial	0.00	1.10
	8 weeks	0.00	0.00
Omega-9 Oleic acid	Initial	10.93	5.35
	8 weeks	6.71	1.44

Table 8: Gas chromatography of a 1:1 mixture of the Irvine Scientific refrigeration and freezing media. This mixture represents the same media used in the frozen thawed semen. The fatty acids are expressed as an area percentage of the total fatty acids.

Fatty acids	Freezing media
Omega-6 Linoleic acid	19.97
Omega-6 Arachidonic acid	2.26
Omega-3 Alpha-linolenic acid	0.38
Omega-3 DHA	0.55
Omega-3 EPA	0
Omega-9 Oleic acid	37.90

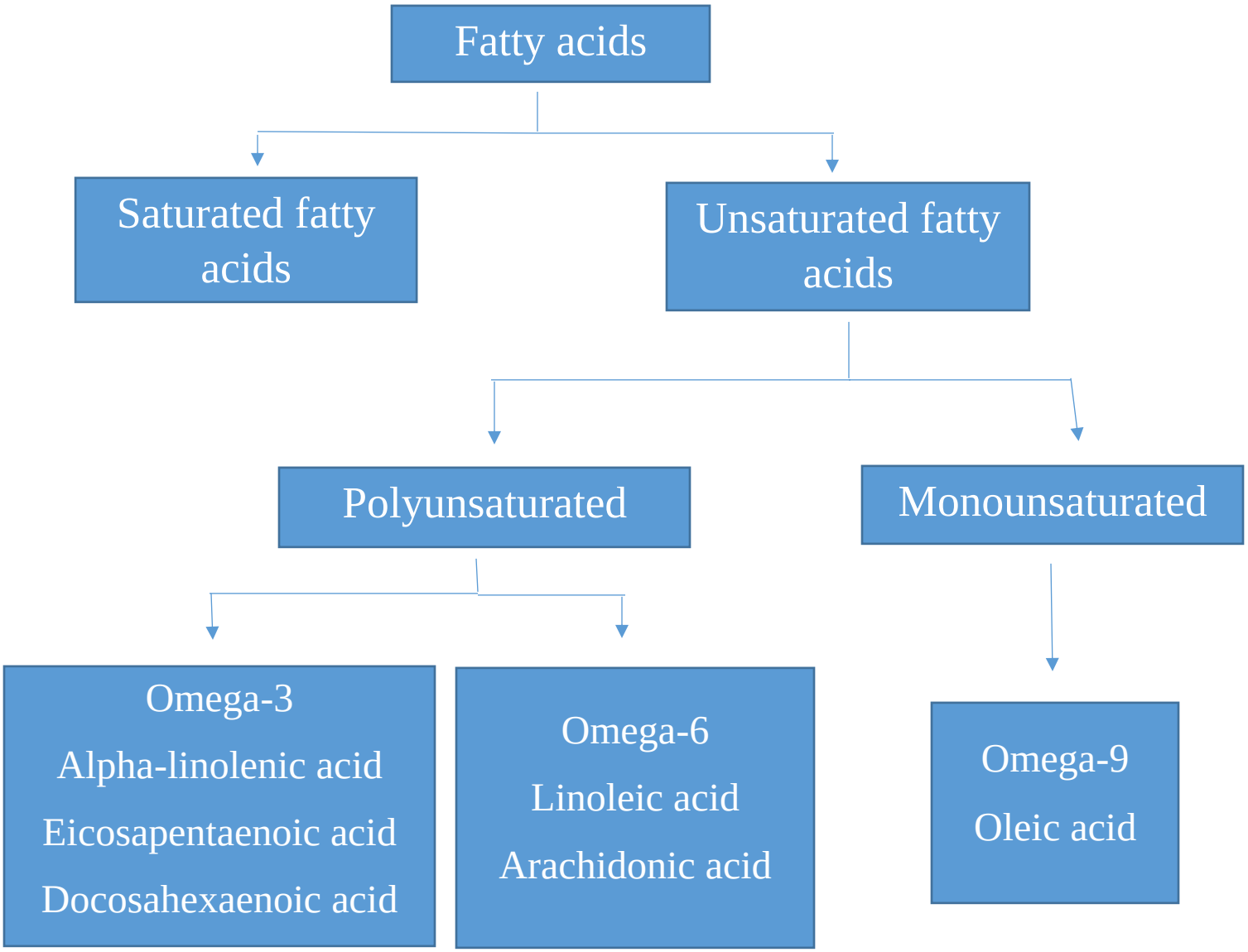

Figure 1: Schematic representation of fatty acids classifications

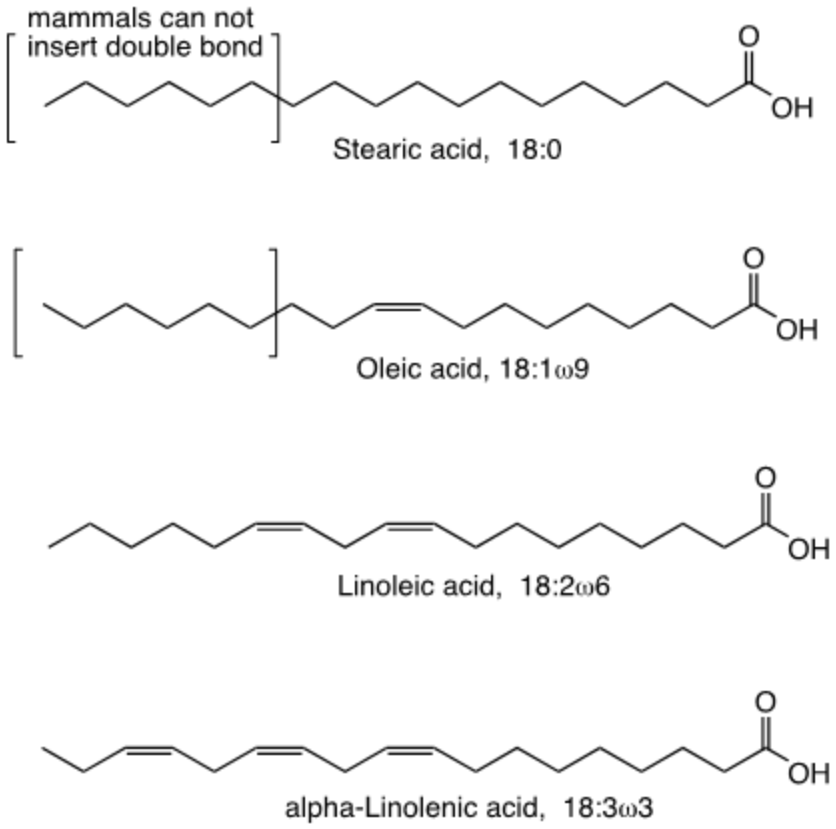

Figure 2: Schematic representation of the most common fatty acids, indicating the area where mammals are unable to insert double bonds, thus making linoleic acid and alpha-linolenic acid essential fatty acids.

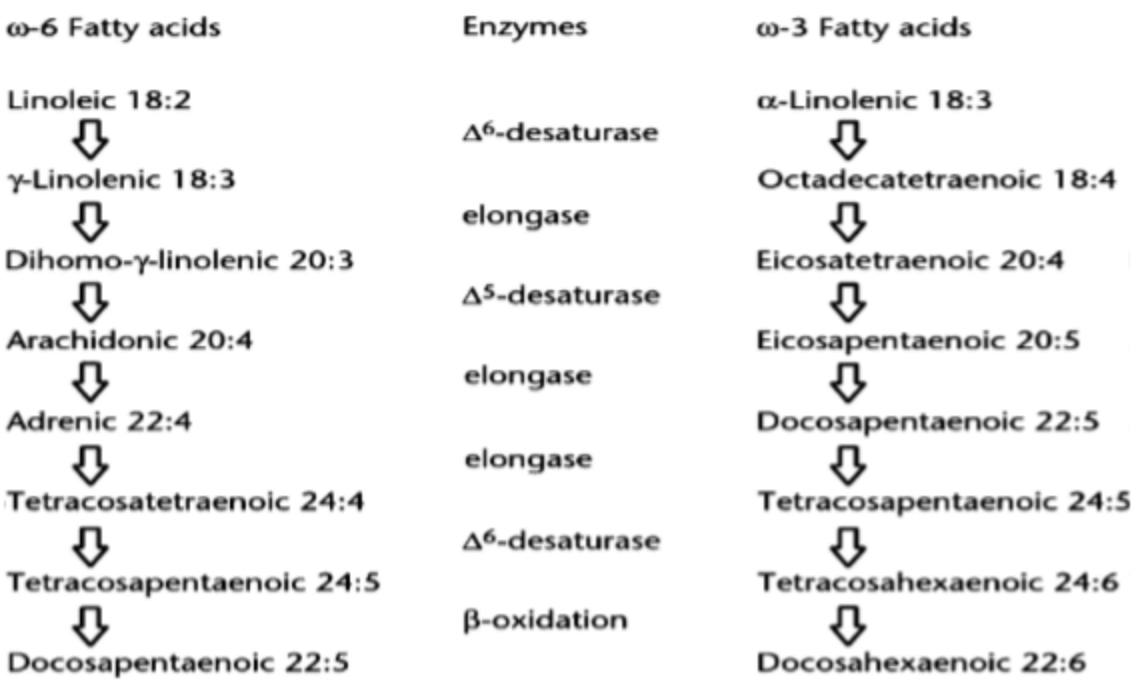

Figure 3: Synthesis of omega-3 and omega-6 polyunsaturated fatty acids. Taken from: Esmaeili V, et al. Dietary fatty acids affect semen quality: a review. Andrology. 2015;3(3):450-61 (68).

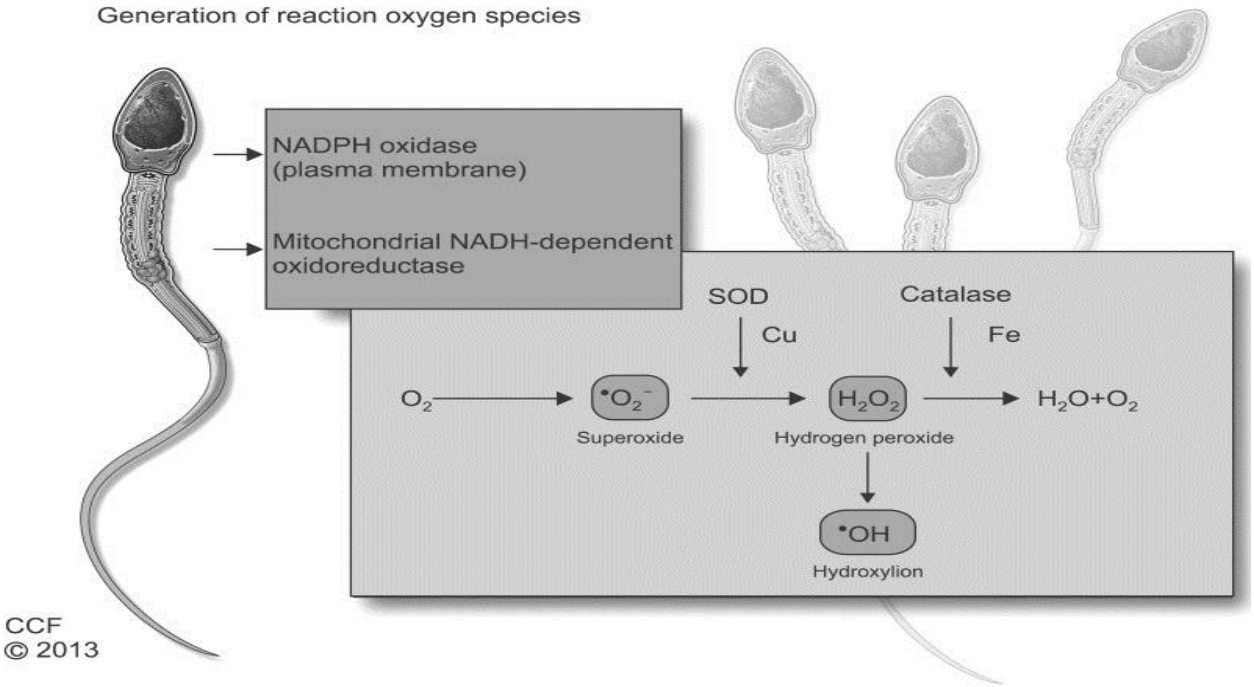

Figure 4: Depiction of the generation of reactive oxygen species. Taken from: Agarwal A, et al. Effect of oxidative stress on male reproduction. The world journal of men's health. 2014;32(1):1-17. (43).

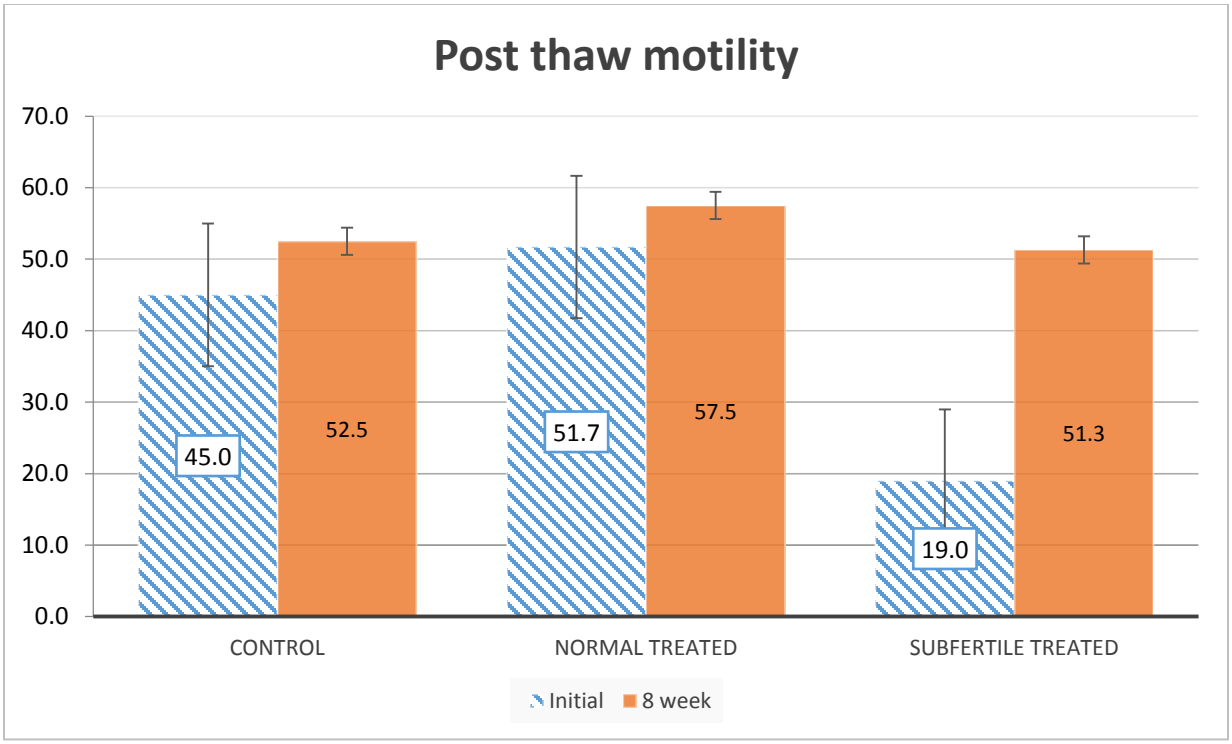

Figure 5 – Post thaw motility at time 0 (initial), shown in stripes, and after 8 weeks, shown in solid.

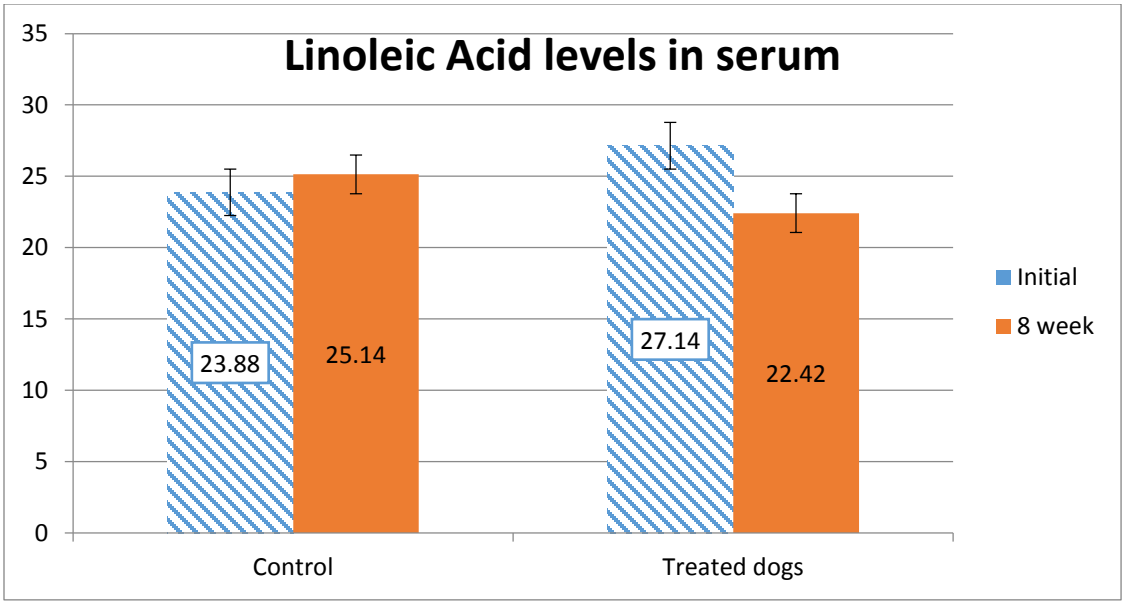

Figure 6 – Linoleic acid concentrations in serum of control and treatment dogs. Initial levels at time 0 (initial), shown in strips and after 8 weeks, shown in solid.

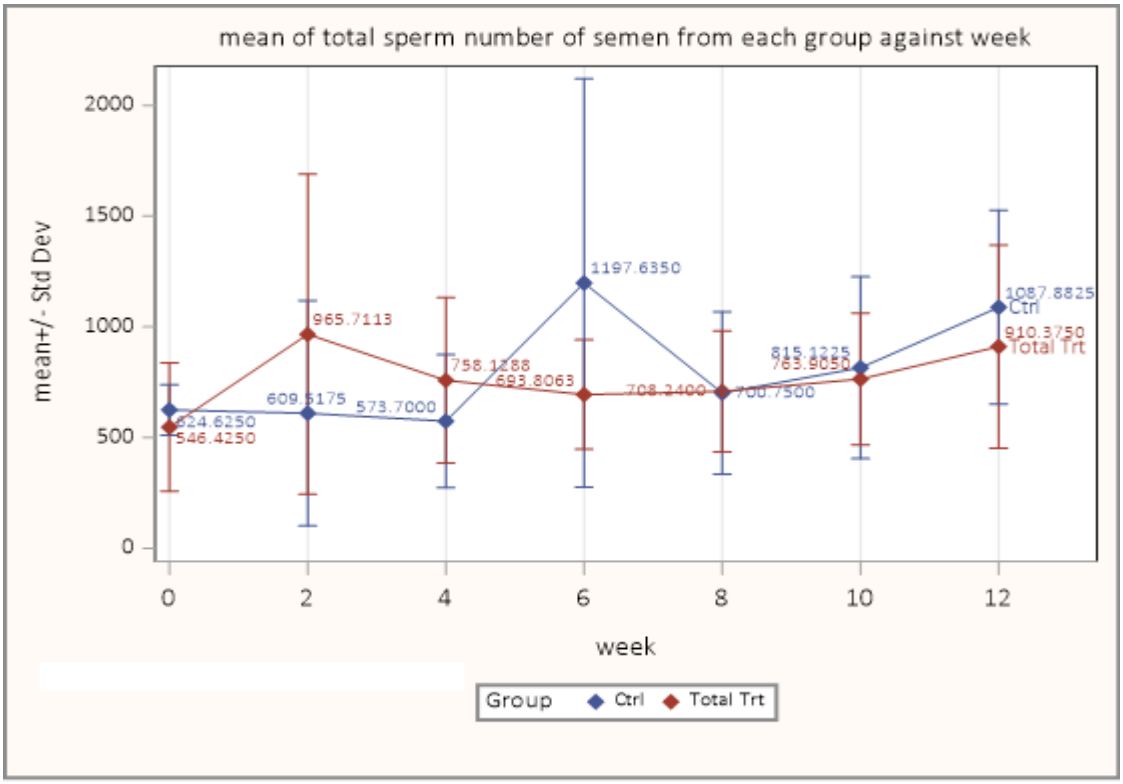

Figure 7 – Mean total spermatozoa number (in millions) measured every 2 weeks

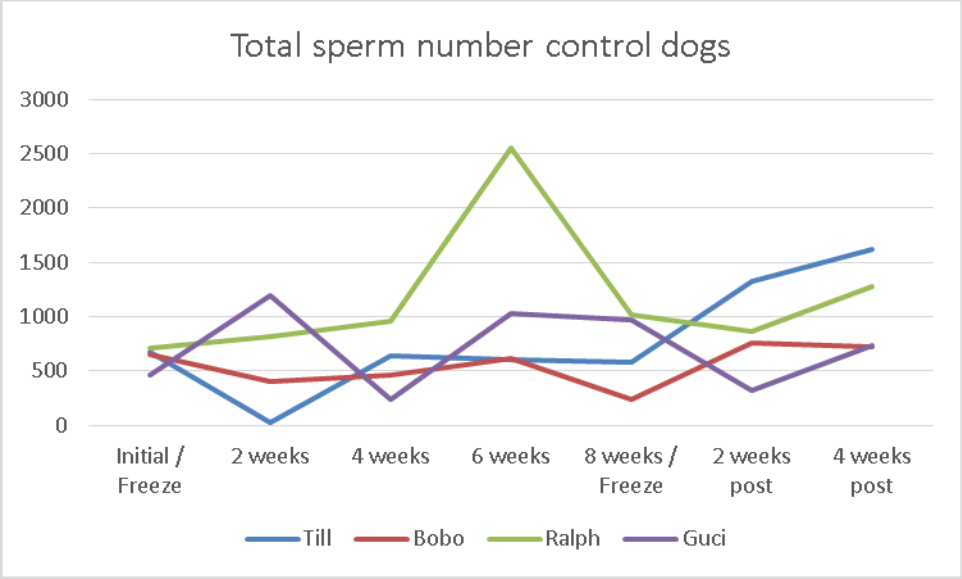

Figure 8 – TSN (in millions) for each dog every 2 weeks in the control group

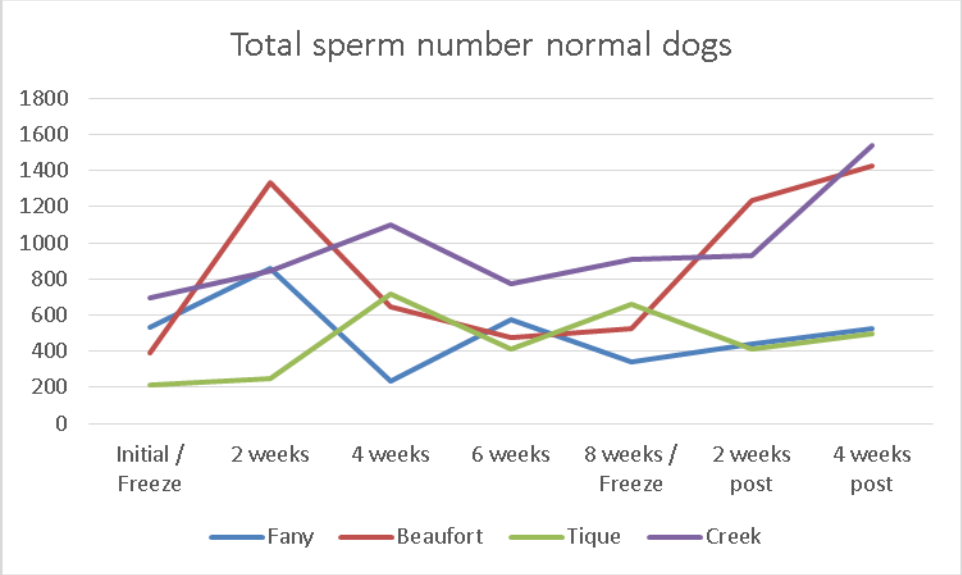

Figure 9 – TSN (in millions) for each dog every 2 weeks in the normal treatment group

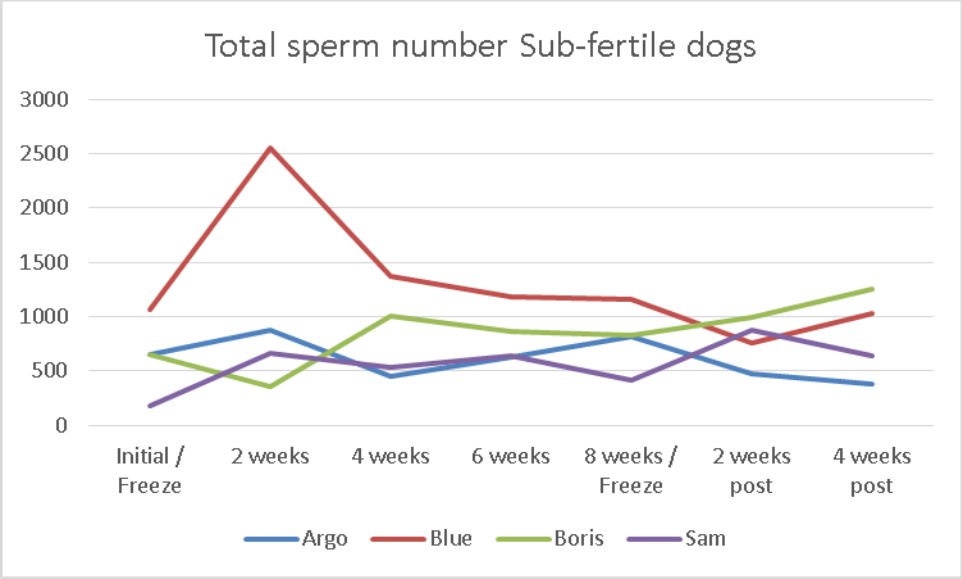

Figure 10 – TSN (in millions) for each dog every 2 weeks in the sub-fertile treatment group

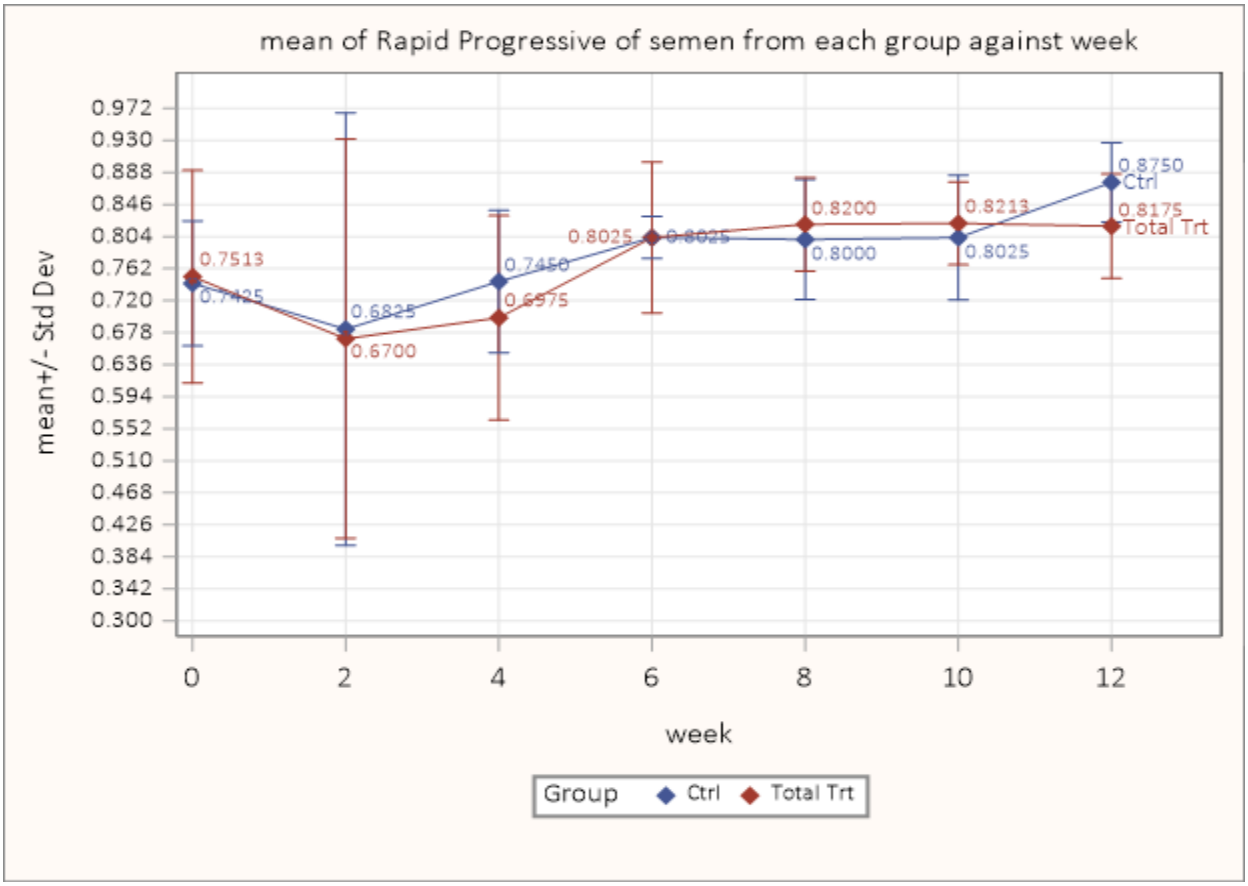

Figure 11 – Mean progressive motility (in percent) every 2 weeks for control and treatment dogs

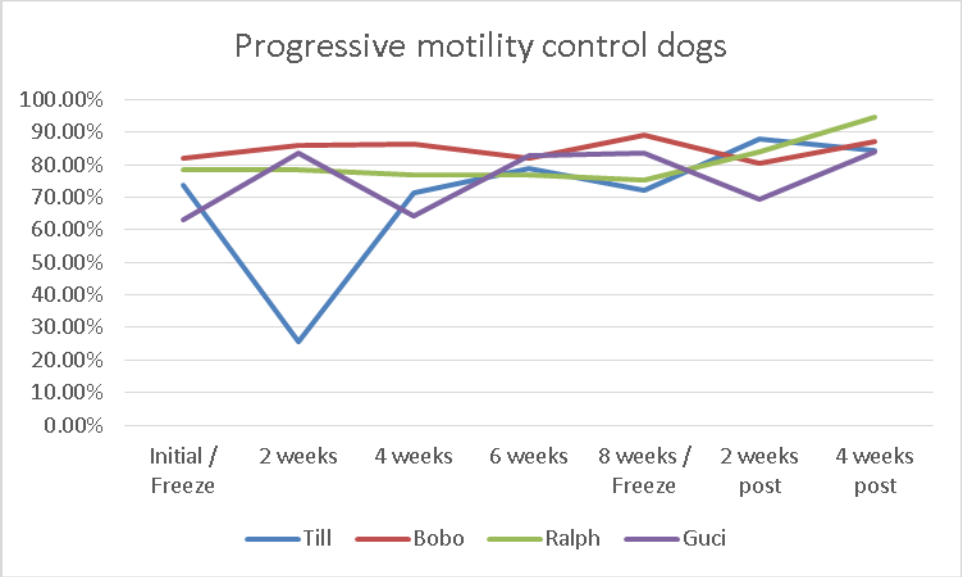

Figure 12 – Progressive motility (in percent) for each dog every 2 weeks for control dogs

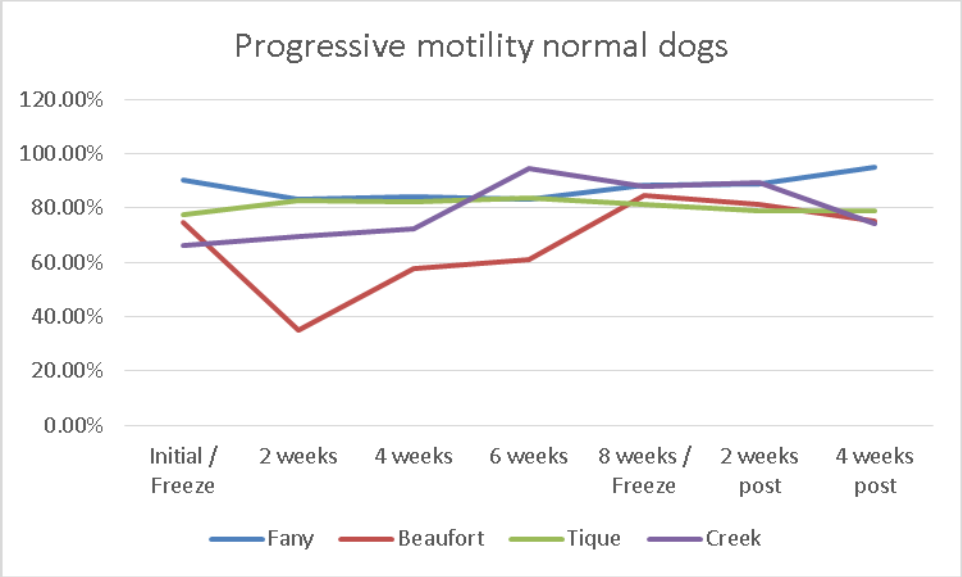

Figure 13 – Progressive motility (in percent) for each dog every 2 weeks for normal treatment dogs

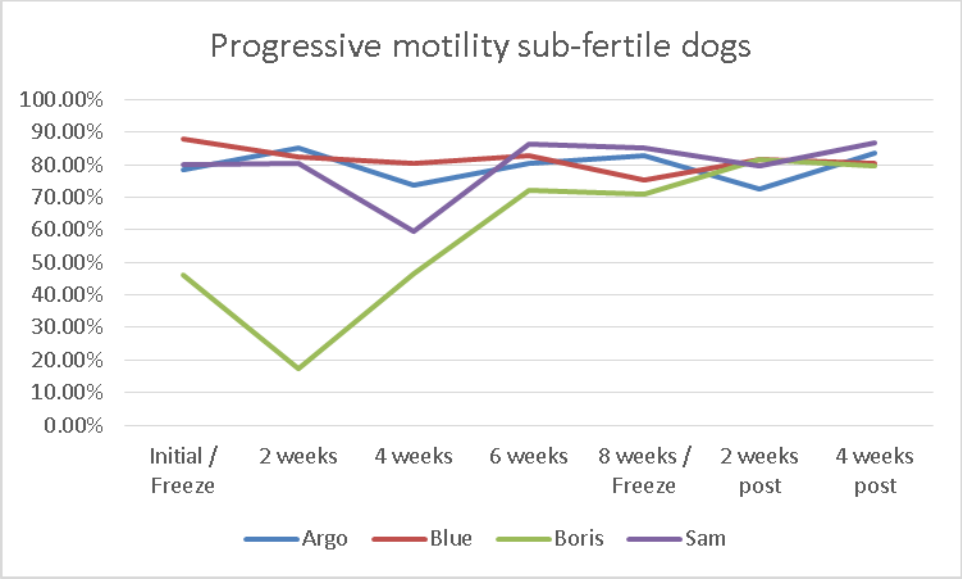

Figure 14 – Progressive motility (in percent) for each dog every 2 weeks for sub-fertile treatment dogs

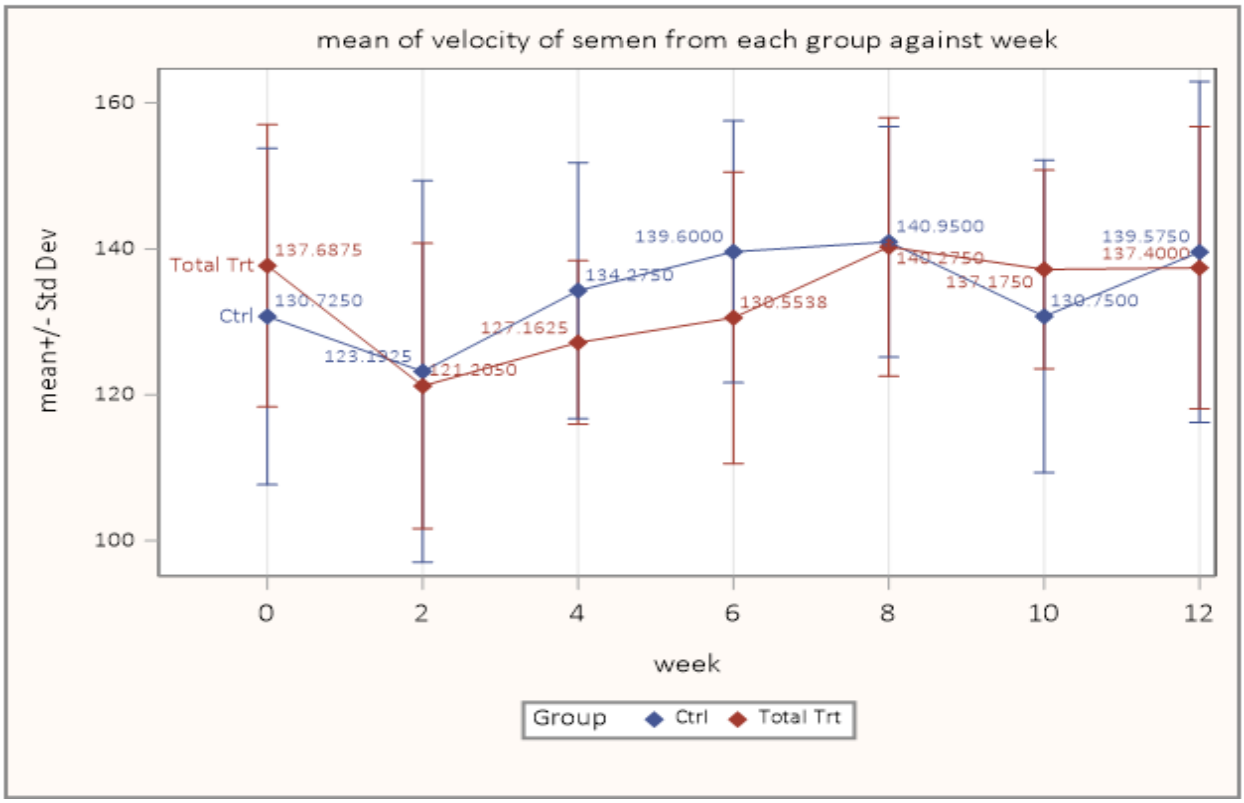

Figure 15 – Mean velocity (in um/sec) every 2 weeks for treatment and control dogs

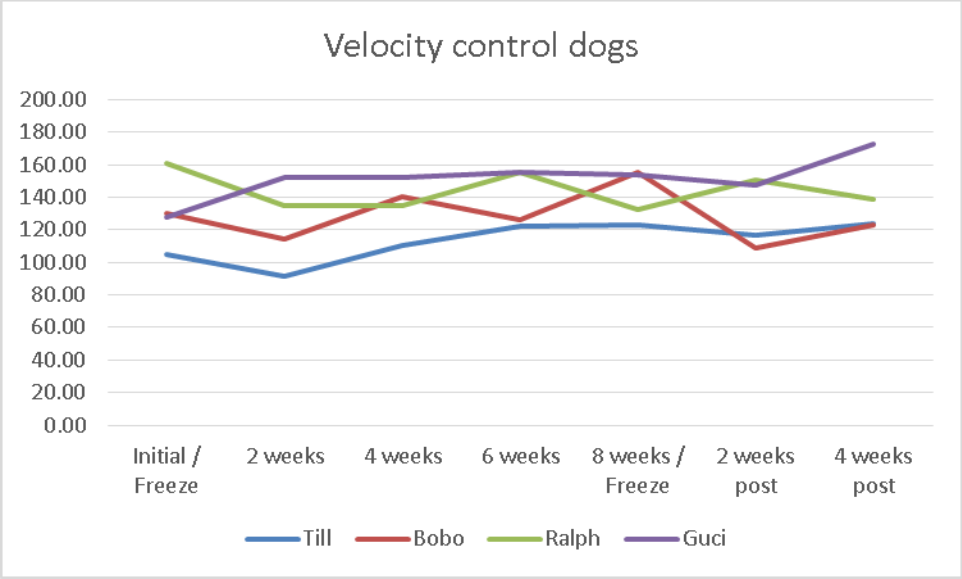

Figure 16 – Velocity (um/sec) every 2 weeks for control dogs

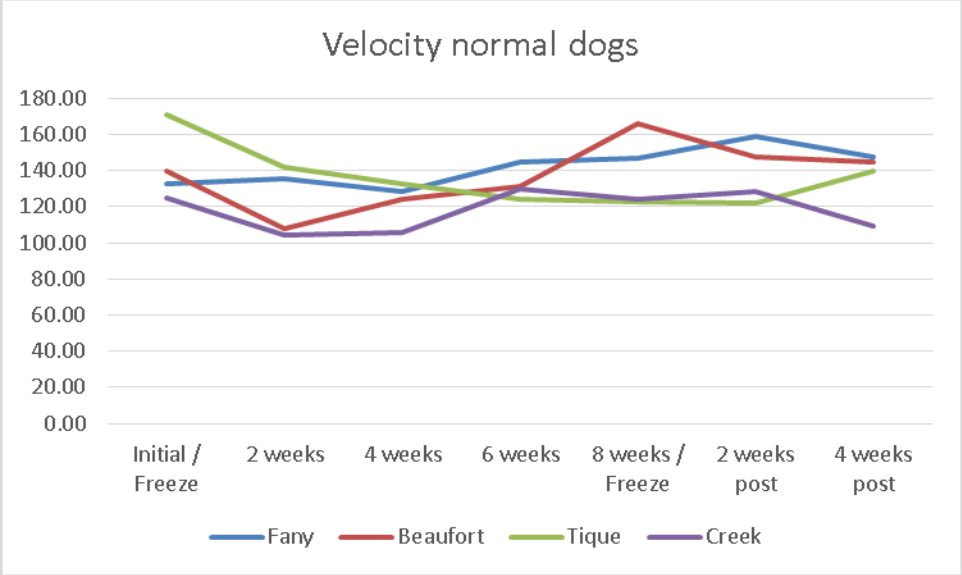

Figure 17 – Velocity (um/sec) every 2 weeks for normal treatment dogs

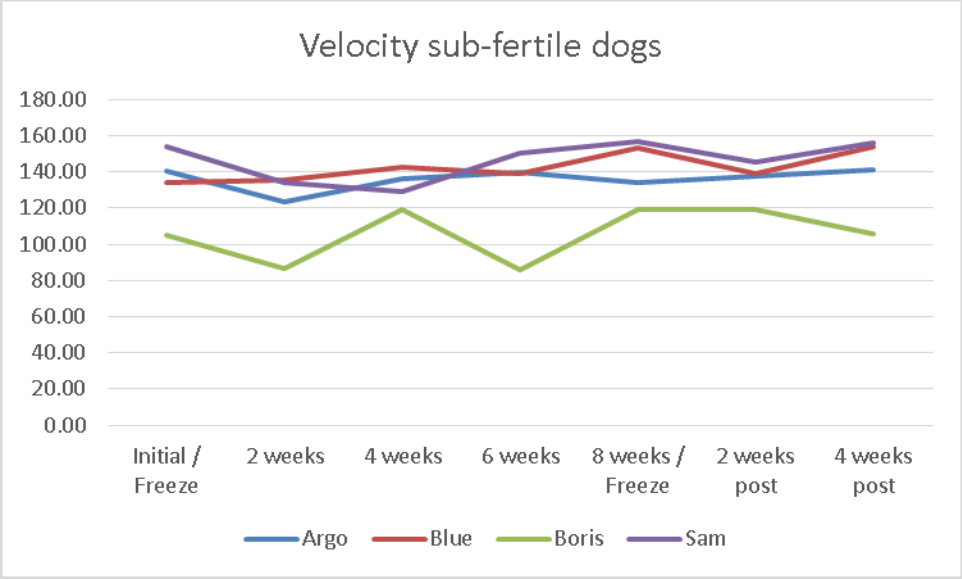

Figure 18 – Velocity (um/sec) every 2 weeks for sub-fertile treatment dogs

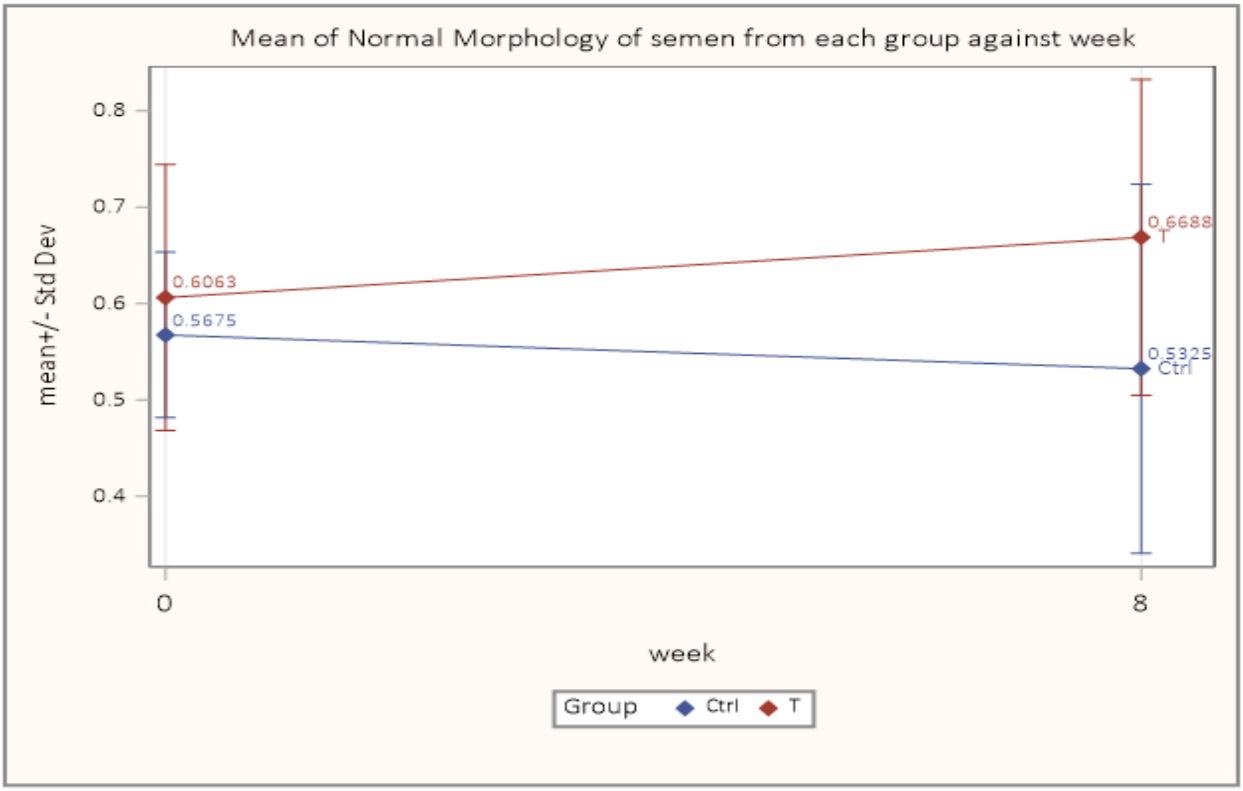

Figure 19 – Mean normal morphology (in % normal) for treatment and control dogs

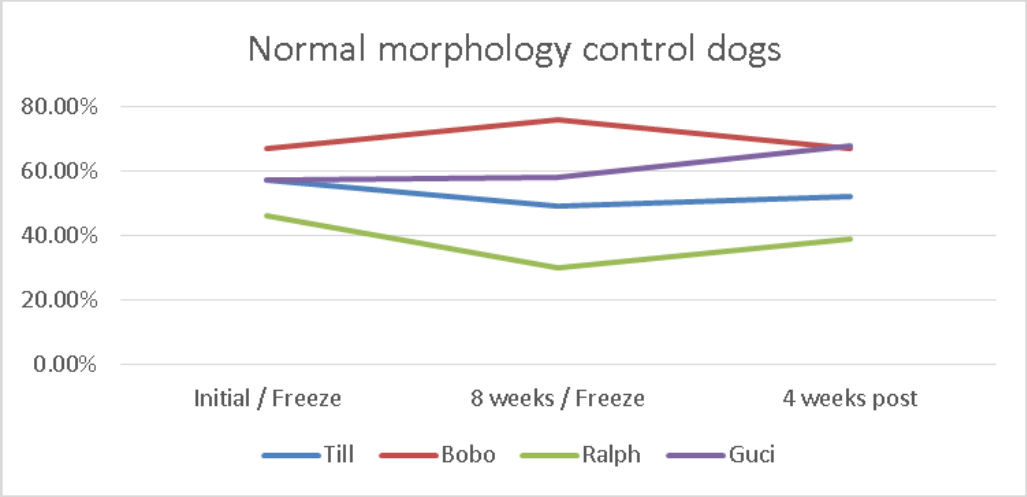

Figure 20 – Normal morphology (in % normal) for control dogs

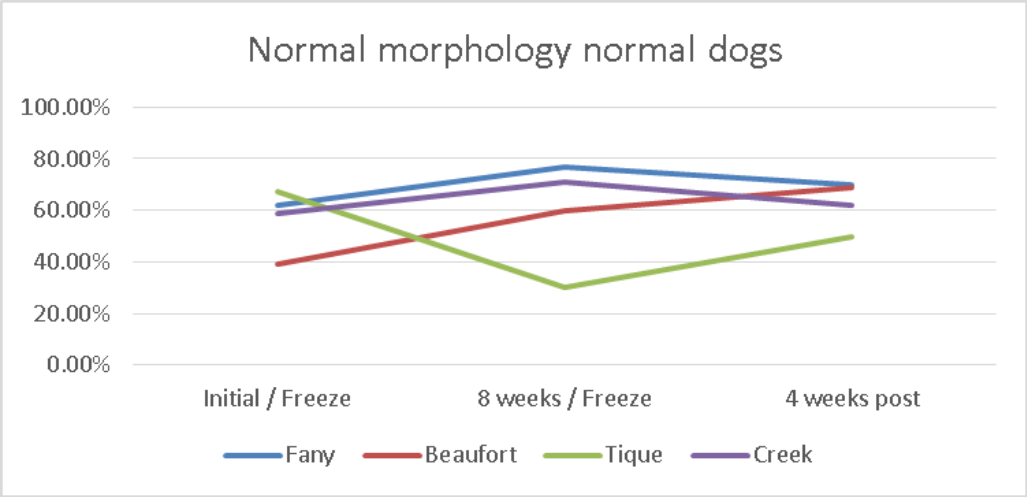

Figure 21 – Normal morphology (in % normal) for normal treatment dogs

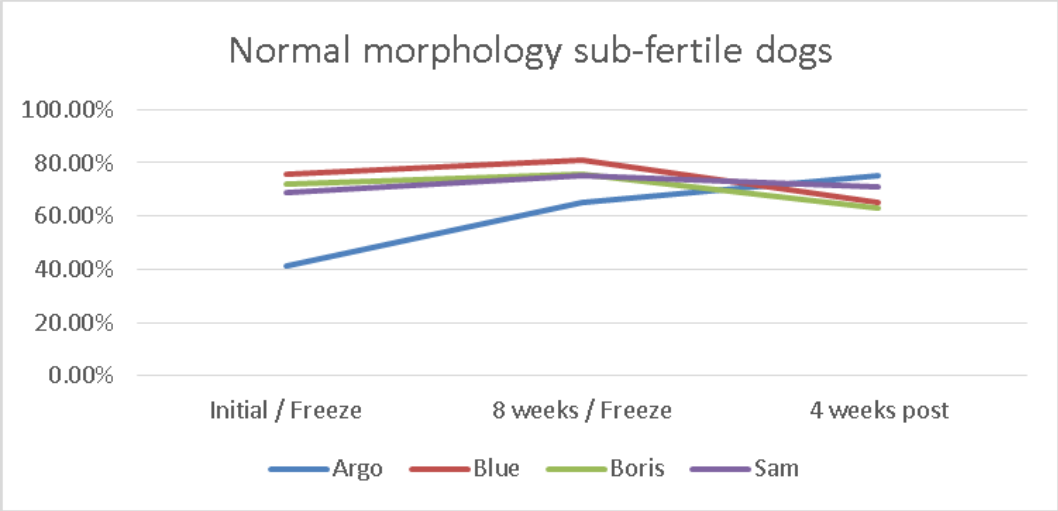

Figure 22 – Normal morphology in percent for sub-fertile treatment dogs

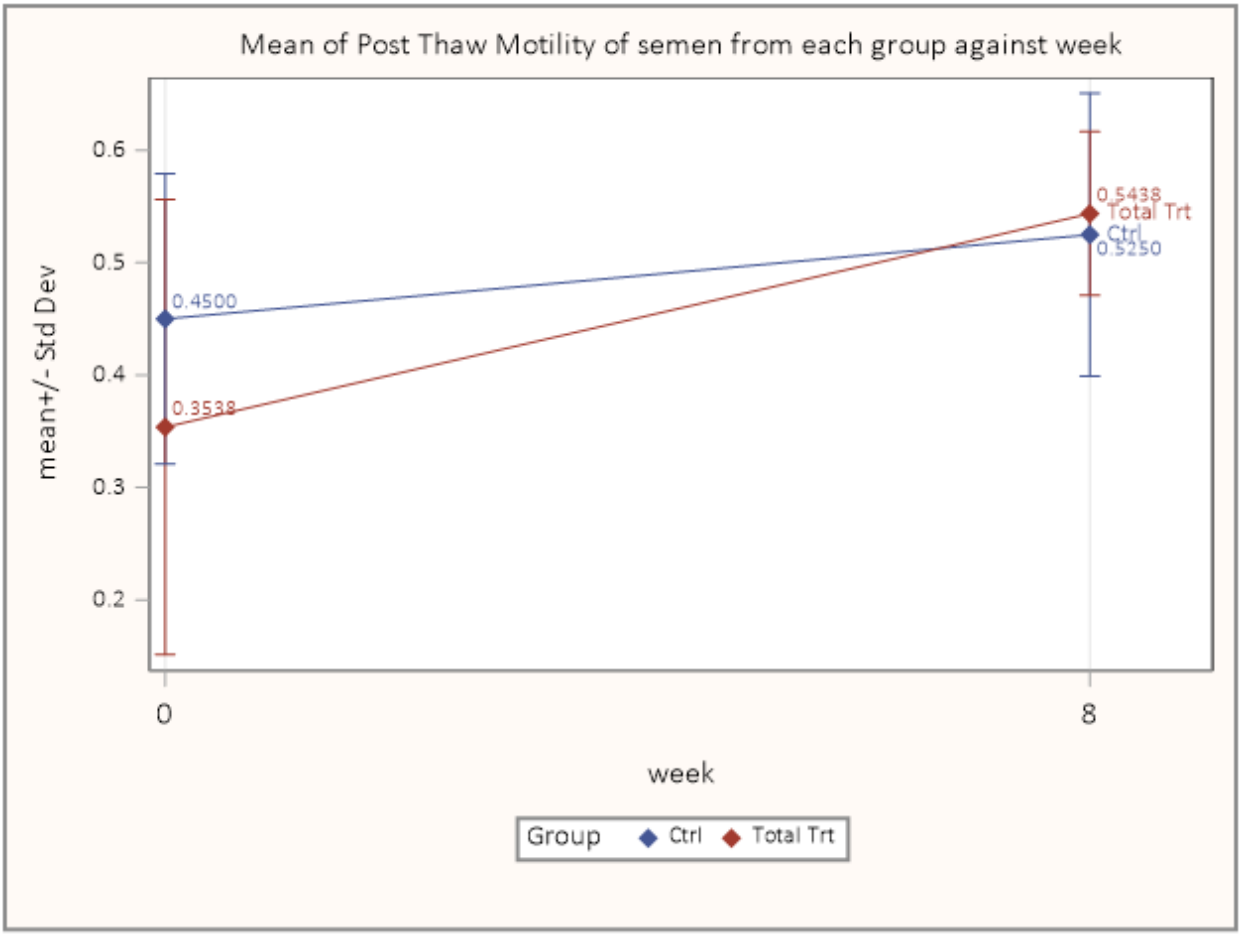

Figure 23 – Mean post thaw motility (in percentage) for treatment and control dogs

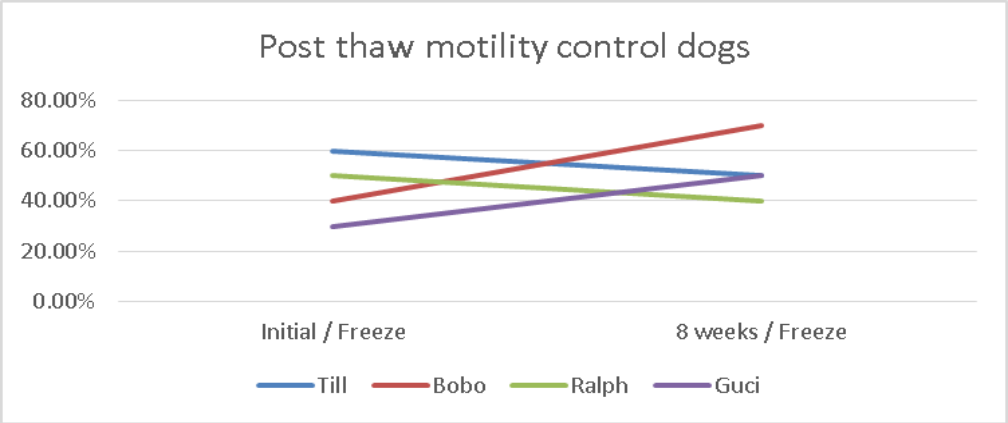

Figure 24 – Post thaw motility (in percent) for each dog in control group

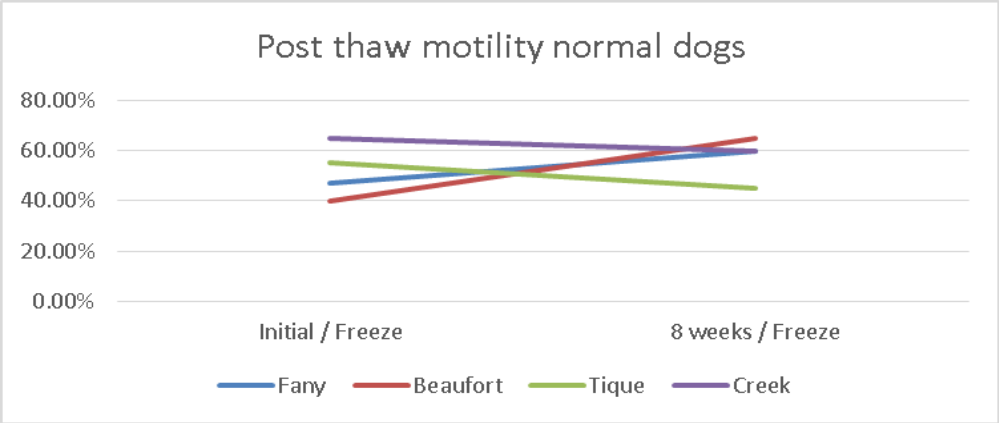

Figure 25 – Post thaw motility (in percent) for each dog in normal treatment group

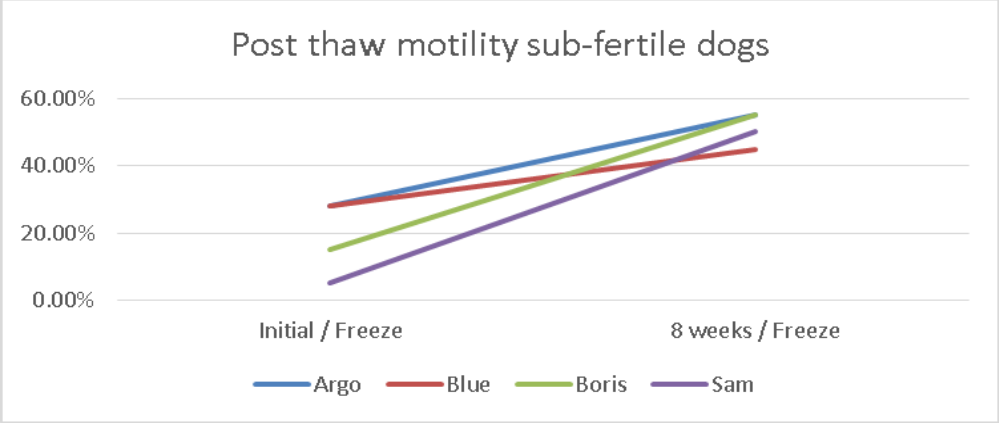

Figure 26 – Post thaw motility (in percent) for each dog in sub-fertile treatment group