Evaluation of Dietary Supplementation with Antioxidants on Fertility Parameters in Stallions

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ABSTRACT

The aim of the present study was to investigate whether dietary supplementation with antioxidants affects semen fertility parameters in New Zealand Standardbred stallions. Fourteen Standardbred stallions of varying fertility, from 3 studs located throughout New Zealand, were allocated to one of 3 treatments: minerals (Se, Cu, Zn, Mn) and vitamin E supplement, oil (canola) supplement and control (no supplement). The studs were provided with the supplements as aliquots to be added to each feed once per day. Stallions from one stud were fed a different basal diet from the other two studs. At least 3 semen samples were collected from each stallion and sent to Equibreed NZ Ltd, before, and after feeding the supplements for around 60 days. Spermatozoa fertility parameters evaluated included total motility, progressive motility, total progressive motility, morphology (normal, loose heads, head defects, mid-piece defects, tail defects), acrosome status (FITC-PNA), membrane integrity (hypo-osmotic swelling test), and concentration. These parameters were assessed at various times including 6-8h and 24h after collection of semen, and immediately and 30min after thawing, frozen semen. Blood levels of Se, Cu and Zn were measured before and after supplementation. Per cycle pregnancy data was also obtained from questionnaire responses from studs A and B at the end of the trial.

This study demonstrates that there was a statistically significant effect of feeding oil as a dietary supplement on sperm motility at 24h after collection (longevity) and also on the per cycle pregnancy rates when treatment groups were combined. Consequently, we were able to elucidate a difference in the actions of mineral and vitamin E supplementation when compared with oil supplementation on fertility parameters measured in this trial. Other beneficial effects of antioxidant supplementation on sperm parameters were suggested from the results of the fourteen stallions on two separate diets, but were not found to be statistically significant.

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LIST OF ABBREVIATIONS

AI	Artificial Insemination
NRC	National Research Council
ANOVA	Analysis of Variance
AV	Artificial Vagina
PBS	Phosphate Buffered Saline
TM	Total Motility
PM	Progressive Motility
TPM	Total Progressive Motility
HOS	Hypo-osmotic Swelling
Mosmol	Milliosmol
FITC	Fluorescein Isothiocyanate
PNA	*Arachis hypogaen* (Peanut) Agglutinin
PSA	*Pisum sativum* (Pea) Aggluthinin
EDTA	Ethylene Diaminetetracetic Acid
PVA	Polyvinylacetate
GnRH	Gonadotrophin Releasing Hormone
LH	Luteinizing Hormone
FSH	Follicle-Stimulating Hormone
SOD	Superoxidase Dismutase
Prx	Peroxiredoxins
GRx	Glutathione Peroxidases
DHA	Decosahexanoic Acid
<	Less Than
>	Greater Than
S.D.	Standard Deviation
rpm	Revolutions Per Minute
°C	Degrees Celsius
gm	Grams
mg	Milligrams
µl	Microlitre

µg	Micrograms
nmol/l	Nanomoles per litre
µmol/l	Micromoles per litre
$10^6.ml^{-1}$	Million per ml
g.	Gravitational Force
min	Minute
h	Hours
ml	Millilitre
cm	Centimetre
Kg	Kilogram
IU/kg	International Units per Kilogram

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CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Of all the domesticated species, horses have the lowest fertility rate (Baczynska et al., 2007), possibly as a result of selectively breeding horses for their performance and conformation characteristics rather than their fertility success. The aim of this study was to investigate if antioxidant supplementation increases fertility parameters in New Zealand Standardbred stallions.

Much of the artificial insemination research for stallions to date has been directed towards finding the optimum standard operating procedures for cooling, freezing and thawing semen. This research has evaluated the effects of various extenders and cryoprotectants on semen quality after ejaculation. However, this study will look at dietary options available to improve semen quality before it is even collected from a stallion.

Both before and after ejaculation, spermatozoa are exposed to oxidative stresses which limit the fertilization capacity of transported semen (Aurich, et al., 1997; Baumber, et. al., 2000). Oxidative stress is a consequence of an over production of metabolic breakdown products called reactive oxygen species (Baumber, et al., 2000). These reactive oxygen species cause sperm plasma membranes to break down and thereby reduce sperm motility, longevity and viability (Lenzi, et al., 2002). Previous studies have suggested that antioxidant dietary supplementation will help prevent damage to the sperm plasma membrane and thereby improve spermatozoa fertility parameters (Brinsko, et al., 2005; Diechsel, et al., 2008; Elhordoy, et al., 2008; Estienne, et al., 2008; Gliozzi, et al., 2009; Harris, et al., 2005; Rooke, et al., 2001).

1.2 Background on Artificial Insemination in Horses

Artificial insemination was first reported in an Arabic text from 1322 when a sheikh successfully inseminated one of his mares with stolen semen from his enemy's prized stallion (Perry (1968) as cited by (Davies Morel, 1999). However it was not until after 1777 that successful artificial insemination was documented for the first time, first in canines and later in horses by Spallanzani (Davies Morel, 1999). By removing the seminal plasma Spallanzani discovered that it was the spermatozoa that caused fertilization, not the associating fluid (seminal plasma) and also that by removing the seminal plasma the spermatozoa lived for longer (Davies Morel, 1999). Spallanzani was also the first to discover that you could 'freeze' spermatozoa with snow, causing them to become motionless and then the spermatozoa would revive when they were warmed again (Davies Morel, 1999). Subsequently, the first artificial vagina's (AVs) were developed in Russian laboratories under the direction of I.I. Ivanoff in 1930 (Davies Morel, 1999). It wasn't until the 1930s that horse AI became well established, spreading internationally (Davies Morel, 1999). By the 1940s Equine growth in equine AI was stunted due to the development and growth of the automobile industry resulting in reduced research funding for military studs. Bovine and even sheep AI developed exponentially while Equine AI growth was stunted for many years until horses became popular again for leisure pursuits.

In the 1950s the use of AI in Standardbreds became popular across USA as more than 20,000 Standardbred mares were bred using AI techniques each year (Davies Morel, 1999). The Standardbred industry was the leading equine industry in the 1970s and 1980s in the use of AI techniques using mainly raw or diluted fresh semen at breeding farms where stallions were collected from, rather than storing or transporting semen (Davies Morel, 1999). The use of artificial insemination was first approved in New Zealand at an Executive Meeting held by Harness Racing New Zealand on 28th of June, 1966. In the 1991/1992 breeding season the use of transported semen was approved. In the 1996/1997 breeding season frozen and imported semen was approved. To control any problems that may occur due to dishonesty compulsory DNA typing commenced in the 2004 breeding season (John French, pers. Comm., 2009). Most Standardbred studs worldwide exclusively use AI technique to breed from their mares today.

There are various advantages and disadvantages in the use of AI in horses as outlines below.

Advantage of AI:

- Mare pregnancy can be achieved by inseminating a low concentrated dose of semen. Therefore a single ejaculate may be split into several doses and this reduces the cost of stallion collection (Blanchard et al., 2003; Metcalf, 2007).
- It is less expensive to transport semen rather than the mare (Graham, 1996a).
- There is less stress to the mare that may be induced by transport or by adjusting to a new environment (Graham, 1996a).
- Increase availability of semen in breeds that allow it (Blanchard et al., 2003).
- Decreased risk of transmitting diseases with proper hygiene during collection and addition of antibiotics in semen extenders (Aurich et al.,1997; Blanchard et al., 2003; Graham, 1996a; Pineda and Dooley, 2003).
- Reduced risk of injuries to both the mare and stallion (Blanchard et al., 2003).
- Enables early detection of fertility problems in stallions (Blanchard et al., 2003).
- Increased availability of desirable genetics (Aurich et al., 1997).
- Increased access to new technologies, such as sex pre-selection of spermatozoa.

Disadvantage of AI:

- Transporting chilled semen is limited due the sperms short-term fertility capacity (Aurich et al., 1997). On average chilled semen is only viable for 2 days after collection and dilution (Aurich et al., 1997)
- More expensive equipment is required and increased knowledge is required in the process of semen collection and insemination to ensure greater success (Blanchard et al., 2003; Graham, 1996a).
- Increased risk of human injury (Blanchard et al., 2003).

- Success of artificial insemination varies widely between stallions (Ball and Vo, 2001)
- Lower Pregnancy rates for some stallions (Graham, 1996a).
- Number of mares at breeding facilities may decrease.
- Training and set up cost of collecting for stallions owned privately or costs in transporting stallions to specialised facilities.

It is important to continue to investigate ways to improve fertility in horses not only to increase the affordability and success of performing AI procedures, but it may also be important for further studies in the breeding of endangered species in captivity.

To investigate the effect of feeding antioxidants to Standardbred stallions, knowledge of the structure of spermatozoa, the process of spermatogenesis, fertilisation, semen collection, chilling and cryopreservation is needed to better understand the physiological role reactive oxygen species (ROS) and antioxidants play in fertilisation.

1.3 Structure of spermatozoa

Spermatozoa are sperm cells that were first identified in 1677 under a microscope by Anto van Leeuwenhock and Johan Hamm in 1677 (Davies Morel, 1999; Storey, 2008). Spermatozoa are unique multicompartmental cells (Fig 1.1) which are specially designed for fertilization and are produced during the process of spermatogenesis and modified during transit through both the male and female reproductive tract (Hammerstedt et al., 1990).

The head of the spermatozoa includes the acrosome cap which has hydrolytic enzymes required for fertilization with the oocyte and a highly condensed nucleus. Morphologically normal sperm often have an abaxial (off centre) location of the mid-piece (Dowsett et al., 1984; Graham, 1996b) . The tail of the spermatozoa includes the neck, midpiece, the principal piece and the end piece which are all surrounded by the plasma membrane (Barth & Oko, 1989). The plasma membrane of a spermatozoa is high in long-chain (C_{22}) polyunsaturated fatty acids (Aurich et al., 1997; Deichsel et al., 2008) . This high concentration of polyunsaturated fatty acids in the plasma membrane increases the

susceptibility of the membrane to oxidative damage (Aurich et al., 1997). Loss of membrane integrity results in impaired cellular function, decreased motility and decreased ability to bind to the oocyte (Aurich et al., 1997).

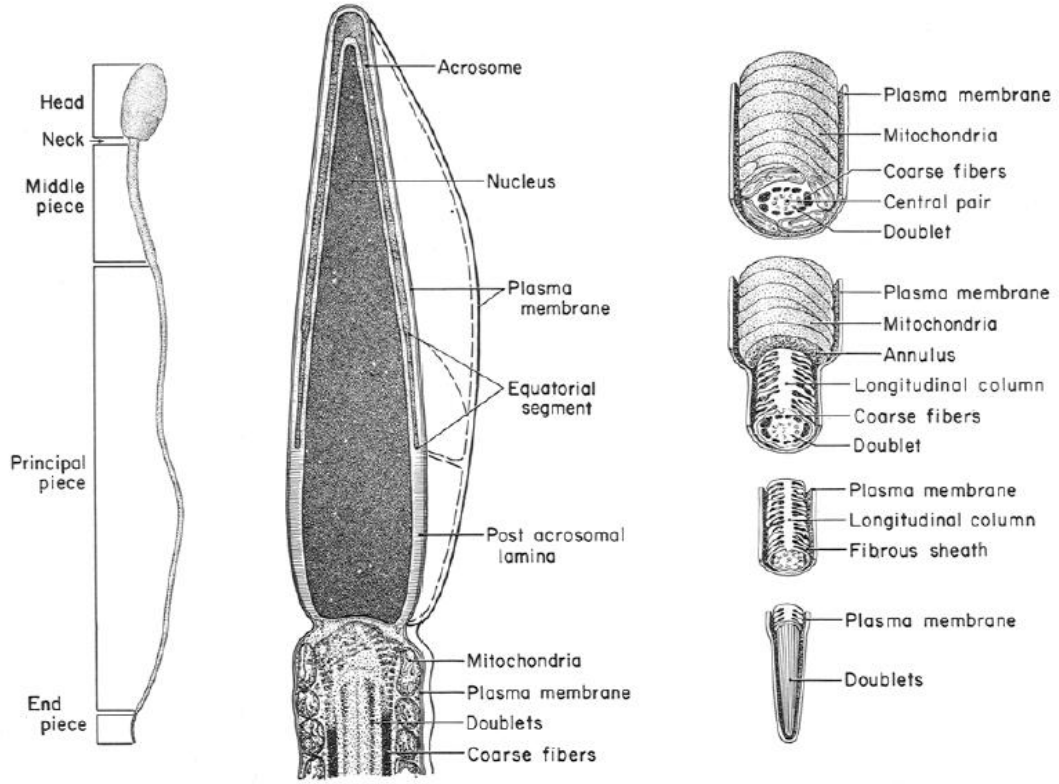


Figure 1. 1 Diagram representing a stallion spermatozoon (Image obtained from Amann & Pickett, 1987).

1.4 Process of Spermatogenesis

Spermatogenesis is the process by which spermatozoa develop within the seminiferous tubules in a complicated sequence of cellular divisions and transformations (Barth & Oko, 1989; Johnson, et al., 2000; Johnson, et al., 1997). Fig 1.2 gives a generalised image of the processes of spermatogenesis.

The process of spermatogenesis is broken down into Spermatocytogenesis, Meiosis, Spermiogenesis and Spermiation all of which occur in the seminiferous tubule (Johnson, et al., 1997). Spermatocytogensis is the mitotic division of the spermatogonia to form the primary spermatocyte (Barth & Oko, 1989; Johnson, et al., 1997;Senger, 2003). The meiosis step includes the division and differentiation of the primary spermatocyte to produce the round spermatid (Barth & Oko, 1989; Johnson, et al., 1997). Spermiogenesis is the differentiation of the round

spermatid to produce elongated spermatozoa (Barth & Oko, 1989; Johnson, et al., 1997). This step of spermatogenesis is often broken down into four steps as shown in Fig 1.3.

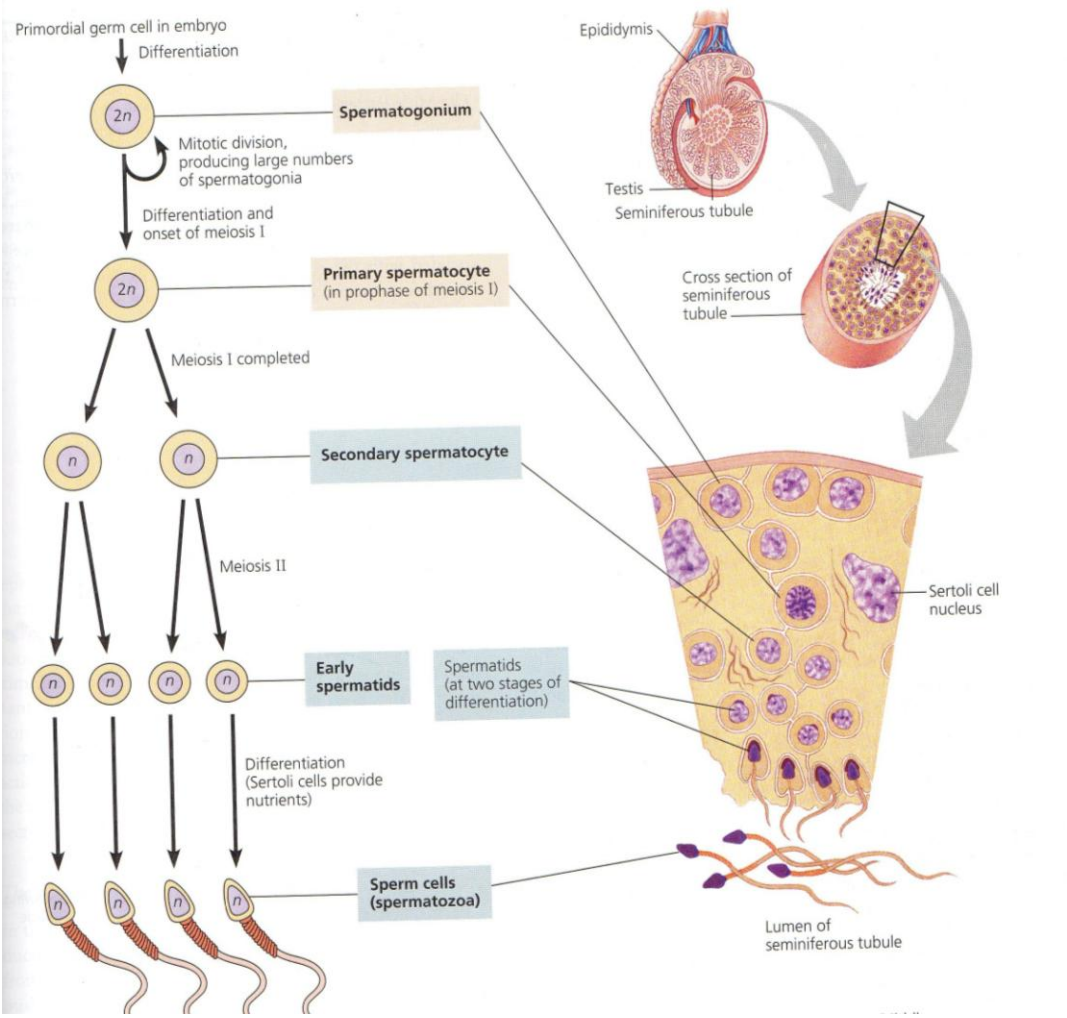


Figure 1. 2 Simple diagram to visually represent the process of spermatogenesis (Image obtained from Campbell and Reece, 2002).

The Golgi phase is where the Golgi apparatus produces the acrosome capsule that migrates to the end of the nucleus (Barth & Oko, 1989; Senger, 2003). At the same time the centriole migrates to the other side of the nucleus (Barth & Oko, 1989; Johnson, et al., 1997; Senger, 2003).

The cap phase is where the acrosome shape changes to form a cap over the nucleus while the Golgi moves away and primitive flagellum begin to form (Barth & Oko, 1989; Johnson, et al., 1997; Senger, 2003).

The acrosomal phase is when the manchette form (only found in spermatids) and the acorome continue to form a distinctive cap shape over the nucleus (Barth & Oko, 1989; Johnson, et al., 1997; Senger, 2003). During this stage of spermiogenesis the chromatin histones along with some nonhistone proteins are replaced by sperm-specific proteins (Barth & Oko, 1989). The nucleus is condensed further during transport through the epididymis when disulfide bonds form cross linkages between protamines (Barth & Oko,1989).

Maturation phase is where the manchette (composed of microtubules) connect and elongate down to become a supporting shaft for the flagella canal before it dissolves (Johnson, et al., 1997). The mitochondria then move to cluster on the flagellum at the midpiece region and membrane forms to complete the spermatozoa (Barth & Oko, 1989; Johnson, et al., 1997; Senger, 2003).

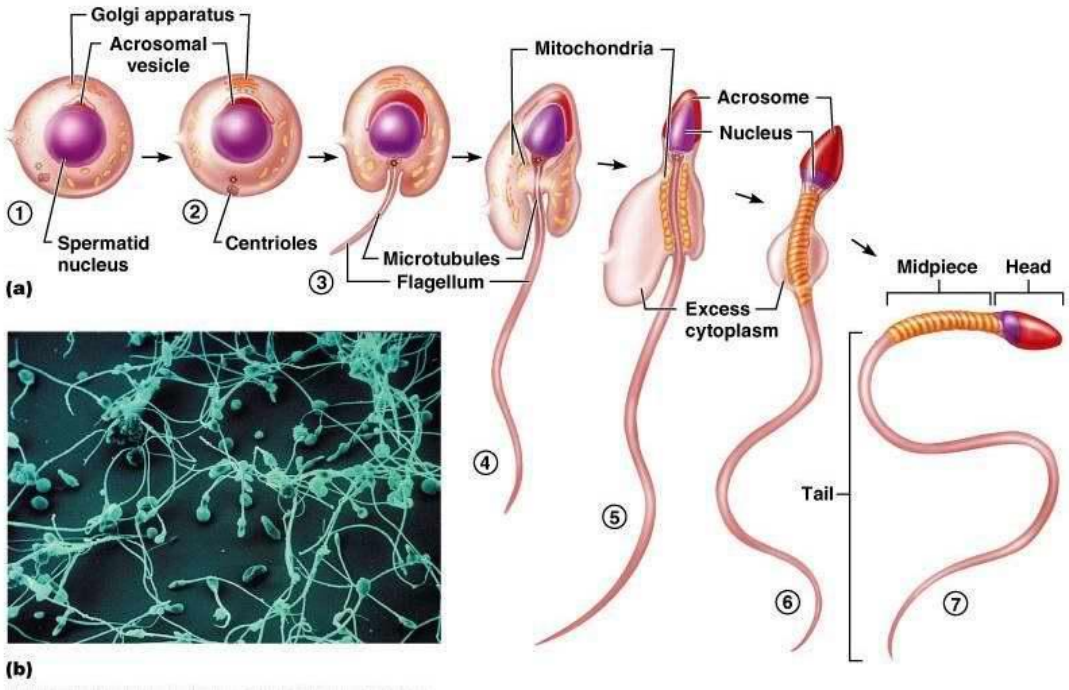


Figure 1. 3 This image visually shows the complex process of Spermiogenesis (Image retrieved from http://www.znanje.org/i/i25/05iv06/05iv0626/MARINA/Galerija.htm on 20/1/10).

Spermiation is the release of the spermatozoa from the sertoli cell into the seminiferous tubule and their transportation out of the testes (Johnson, et al, 1997; Senger, 2003). However, these spermatozoa are not fully mature as they have a cytoplasmic droplet attached to the midpiece and they are not motile at this stage

in their development (Johnson, et al, 1997). The spermatozoa go through another maturation phase in the epididymis where the necessary hormones are present to induce motility and they lose the cytoplasmic droplet as they move down the epididymis (Johnson, et al., 1997). Spermatogenesis may take 55-57 days and further maturation and transport through the epididymis it takes approximately 9 days (Deichsel et al., 2008; Johnson, et al., 1997; Pineda and Dooley, 2003) .

Spermatozoa are so specialised for fertilisation that these cells do not contain the necessary equipment for self repair and are therefore very susceptible to changes in environmental conditions (Baumber, et al., 2003; Daves Morel, 1999). Any oxidative or physical stress that may occur as a result of human interference or an abnormal occasion during the process of spermatogenesis may have detrimental effects on the spermatozoon's development and its ability to fuse with the oocyte (Davies Morel, 1999). Environmental toxicity and injury of spermatozoa is reflected in the presence of abnormal spermatozoa in the ejaculate 59-64 days later after a full spermatogenesis process has taken place (Deichsel et al., 2008; Pineda and Dooley, 2003).

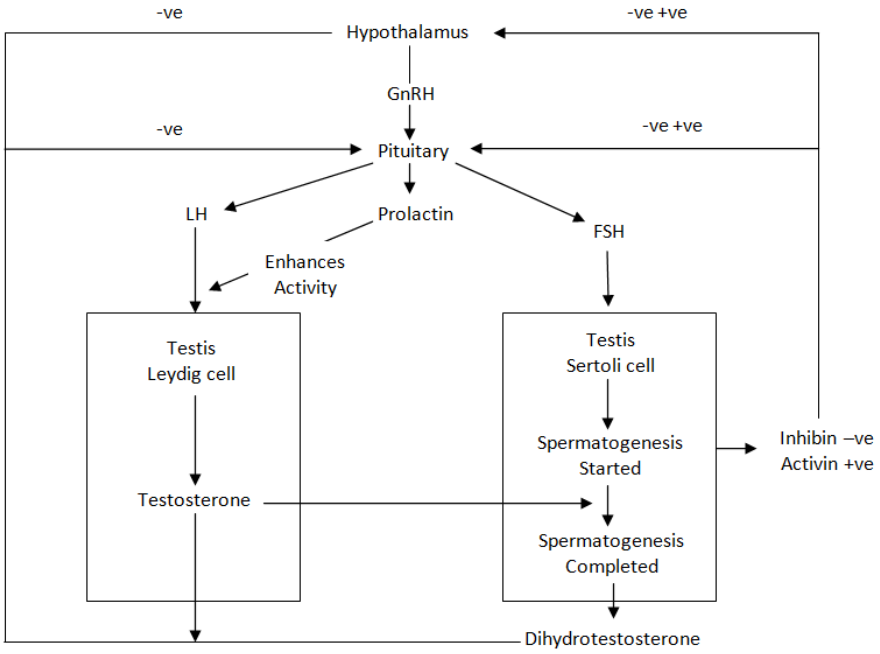


Figure 1. 4 This flow diagram shows the endocrine pathway which drives spermatogenesis (Image slightly modified from Davies Morel, 2003).

Seasonal changes can influence the photoperiod and therefore the levels of gonadotropin-releasing hormone (GnRH), Luteinizing Hormone (LH), Follicle-

Stimulating Hormone (FSH) and Testosterone (Pineda and Dooley, 2003). Fig 1.4 shows the relationship of these hormones to spermatogenesis. Seasonal changes have been shown to have variable effects on each stallion (Janett, et al., 2003). Quality and fertility of an ejaculate is believed to be optimal during the reproductive season (Deichsel et al., 2008; Pineda and Dooley, 2003), however Janett, et al. (2003) showed that semen collected during autumn had the highest quality semen and survived cryopreservation better.

1.5 Fertilization

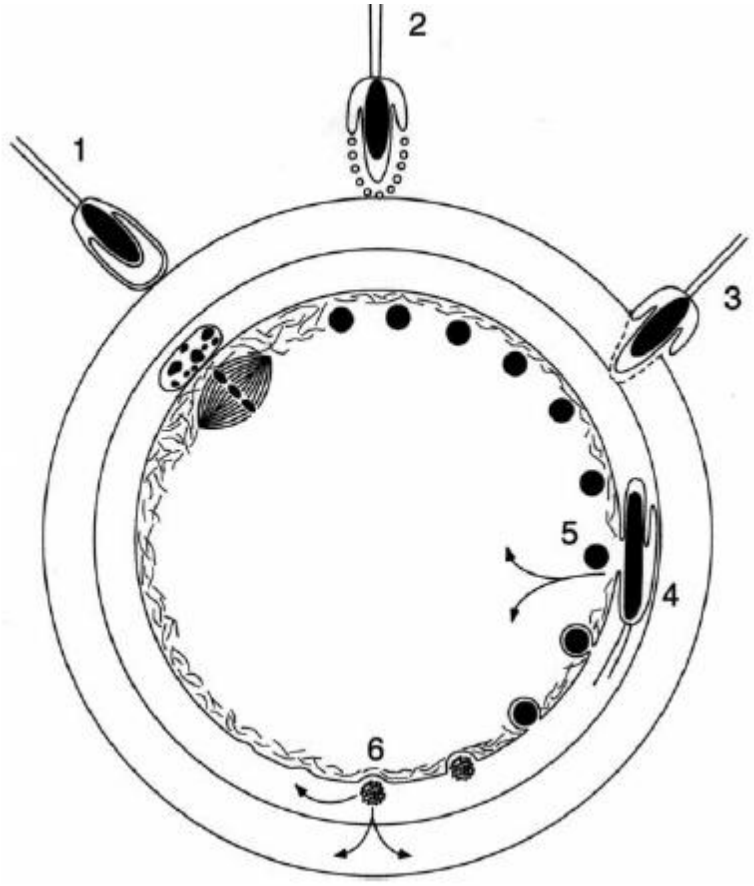


Figure 1. 5 The simple diagram shows the sequence of events that occur during fertilization. 1) Spermatozoon binding to the zona pellucida. 2) Acrosome reaction. 3) Penetration through the zona pellucida. 4) Binding and fusion of sperm with the oocyte (fertilization). 5) Activation of oocyte and initial fast unstable block of other sperm penetration (poly spermy block). 6) Cortical granule secreted causing stable and complete poly spermy block. (Image obtained from Gadella, et al., 2001).

During artificial insemination the semen is deposited into the mare's uterus by the inseminator with the use of an insemination pipette. After semen is

deposited into the mare's uterus, the immotile, damaged or dead spermatozoon are removed by IgG which label cells to be destroyed by phagocytosis (Katila, 2001a). Chilled semen should be inseminated within 12-24hrs of ovulation, while frozen semen should be inseminated 6hrs before ovulation (Pineda and Dooley, 2003). Previous studies show that per cycle pregnancy rates may be affected more by mare management (i.e. timing of insemination, site of semen disposition history of mare, etc.) than by semen concentration and the total number of progressively motile sperm (Metcalf, 2007; Watson, 2000).

Fig 1.5. visually represents of the sequence of cellular interactions involved in fertilization (Gadella, et. al., 2001).

1.5.1 Capacitation and the Acrosome Reaction

Capacitation is an important physiological process that spermatozoa undergo to enable the sperm cells to fertilise the oocyte (Colenbrander, et al., 2003; Graham, 1996c; Graham and Moc'e, 2005; Meyers, 2001; Meyers, 2005). This process includes membrane modifications, changes in lipid composition and surface properties, intercellular pH decreases, changes in fluidity of the cell and calcium ion permeability all of which hyper activates the sperm motility and enable the acrosome reaction to occur (Graham and Moc'e, 2005; Meyers, 2001; Meyers, 2005; Rathi, et al., 2001). Normally capacitation occurs in the female reproductive tract (Graham, 1996c) and therefore is difficult to understand the process of capacitation due to unreliable and complicated methods of assessing this process (Rathi, et al., 2001).

Studies have shown that the loss of cholesterol from the sperm plasma membrane and an increase in intercellular calcium which occurs during capacitation is necessary for the acrosome reaction to occur (Cross, 1998; Graham, 1996c; Meyers, 2005; Sabeur, et al., 1998). The acrosome contains hyaluronidase enzymes (Sabeur, 1997) that are released during an exocytosis process and specifically dissolves the zona pellucida allowing the sperm and oocyte membranes to fuse during fertilization (Barth & Oko, 1989; Colenbrander, et al., 2003; Rathi, et al., 2001). The equatorial segment (thinner posterior region of the acrosome) remain intact while the acrosome reacts with and penetrates the zona pellucida and then aids in the sperm-oocyte membrane fusion (Barth & Oko, 1989; Melo, et al., 2007).

1.6 Collection of Semen

Stallions are conditioned to mount a mare or more often a mounted padded phantom (Pineda and Dooley, 2003). Teaser mares (mare in oestrus) are not always required, although having one present may result in the stallion ejaculating a larger volume of semen and increased number of sperm (Pineda and Dooley, 2003). Once the stallion has an erection the stallion handler will then lead the stallion towards the phantom and allow the stallion to mount the phantom as if it were a mare. Once mounted, a second person holding the AV will step in and direct the stallion's penis into the AV. After ejaculation the stallion will dismount the phantom and the penis slides out of the AV.

There are several different AV models the most common of which are Missouri and Hanoverian models. The Hanoverian AV consists of a rigid outer casing with a bladder inside which is filled with water after an AV liner is inserted. The Missouri is a double lined soft rubber bladder which has a leather harness to help when carrying it. Water (45°C-50°C in temperature) is used to fill the bladder and semen safe lubrication is placed inside the AV. The volume of water added can be adjusted to achieve proper pressure depending on the stallion's preferences. A sterile bag and filter, which removes the gel fraction of ejaculate, is inserted into a clean plastic bottle and screwed onto the end of the AV. An insulating cover can be place over the bottle to control changes in temperature in the ejaculate as dramatic changes in temperatures can damage spermatozoa (Pineda and Dooley, 2003). There are a variety of extenders that are often added to semen directly after collection which may differ from stallion to stallion (Pineda and Dooley, 2003).

1.7 Shipping Chilled Semen

Uses of chilled-stored semen has become more popular and most equine associations have accepted registration of progeny conceived with the use of shipped chilled semen (Aurich, et. al., 1997; Samper et al., 2007). Skim milk-based extenders containing glucoses, sugars, salts, and antibiotics are added to the semen shortly after it is collected which helps keep the sperm cells viable during

chilling and transport (Pineda & Dooley, 2003; Rota, et al., 2004). Adding extender also reduces detrimental effects caused by the seminal plasma (Rota, et al., 2004). Extenders often contain milk or egg yolk components which reduce the detrimental effects of cold shock and improve motility (Rota, et al., 2004). The buffer base in some extenders regulates the metabolic pH changes and antibiotic reduce excessive growth of bacteria (Pineda & Dooley, 2003; Rota, et al., 2004). Controlled cooling rates and storage of spermatozoa at 4-6°C will ensure the best survival rates of the spermatozoa for 24-48hrs (Malmgren, 2001; Pineda and Dooley, 2003; Rota, et al., 2004). Chilled semen can either be transported in a Styrofoam box or an Equitainer (better in extreme temperature and for longer periods of transportation) (Malmgren, 1998). There is a greater chance of successful fertilisation with fresh semen than with frozen because cryopreserved spermatozoa have reduced viability and appear to be functionally impaired largely as a result of oxidative stress (Watson, 2000).

1.8 Cryopreservation of spermatozoa

Many breed associations allow foals that were conceived with the use of frozen semen to be registered and this has caused a surge in equine spermatozoa cryopreservation (Moore, et al., 2006). In 1948 glycerol was accidentally discovered as a cryoprotectant (Samper et al., 2007), but this wasn't until 1957 that the first foal was born with the use of frozen semen (Ball and Vo, 2001; Graham, 1996a). There are many advantages and disadvantages to freezing equine semen such as:

Advantages of Cryopreserved semen:

- No need to schedule semen collection and shipment to coincide with the mare's ovulation (Samper et al., 2007).
- Stallions can continue to compete without disrupting his competition behaviour. (Samper et al., 2007).
- No limitation on the delivery time of semen means semen can be marketed and sold anywhere in the world (Aurich et al., 1997; Samper et al., 2007) thus increasing the availability of semen from foreign countries that would

otherwise be unavailable due to costs and quarantine protocols (Graham, 1996a).

- Frozen semen can be an insurance that valuable genetics are not lost as a result of stallion illness or death (Graham, 1996a; Samper et al., 2007).
- Transport and grazing costs are reduced (Samper et al., 2007).
- Reduced risk of injury (Samper et al., 2007).

Disadvantages of Cryopreserved Semen:

- Expertise is necessary for freezing of the semen, handling and insemination of frozen semen (Graham, 1996a) and therefore more expensive (Pineda and Dooley, 2003; Samper, et al., 2007).
- There can be a large variation between stallions' freeze-ability and some may not freeze at all (Pineda and Dooley, 2003; Samper et al., 2007).
- Frozen semen pregnancy rates are lower than fresh or chilled pregnancy rates (Pineda and Dooley, 2003; Samper et al., 2007).
- No standard protocol for freezing semen therefore need to be adjusted to suit individual stallions (Samper et al., 2007).
- Mare management is more intensive to ensure semen is inseminated close to ovulation as frozen semen have shorter life spans (Samper et al., 2007).
- Early embryonic death is higher with frozen semen (Samper et al., 2007).

During cryopreservation there are many factors may cause damage to the sperm cells such as:

- Temperature change – rapid cooling causes membrane damage known as "Cold Shock"- irreversible reduction in sperm motility caused by rapid cooling to 0-5°C (Jones et al., 1978; Meyers, 2005; Watson, 2000). Cooling and rewarming of sperm cells may also cause them to capacitate early (Watson, 2000).
- DNA Damage such as DNA fragmentation (Baumber, et al., 2003; Watson, 2000).
- Alterations to the sperm plasma membrane (Watson, 2000). Change to the organisation of lipids in the membrane may affect movement of water and cryoprotectants in and out of the cell (Hammerstedt et al., 1990). As a

result of cryopreservation many lipids may be lost from within the membrane and therefore reduce the fertilising capacity of the frozen spermatozoa (Graham and Moc'e, 2005).

- Ice crystal formations and dissolving in the extracellular environment that result in a hyperosmotic environment (Meyers, 2005; Watson, 2000).
- Osmotic pressure – This can be reduced by adding cryoprotectants like glycerol that act by increasing the extracellular solute concentration causing water to move out of the cell and thus dehydrate it reducing the risk of ice crystals forming within the sperm cells and destroying it (Meyers, 2005; Moore, et al, 2006; Watson, 2000). However sperm cell death may still occur if spermatozoa shrink or swell beyond species-specific osmotic tolerances and if water movement in or out of the cell is too rapid (Watson, 2000).
- Toxicity of cryoprotectants depends on relative permeability of the cryoprotectant and amount used (Watson, 2000). The rapid addition and in particular the rapid removal of cryoprotectants from spermatozoa causes large osmotic gradients between the cell membrane and its' surrounding environment which may result in a decline in viability, motility and mitochondrial membrane potential as cells become damaged or lysed (Moore, et al., 2006; Watson, 2000).
- Oxidative damage (Meyers, 2005; Watson, 2000). The process of cryopreservation is also well known to induce reactive oxygen species formation which if produced in excessive amounts are detrimental to the spermatozoa fertilizing ability (Watson, 2000).

Protocols for freezing and thawing sperm cell and the choice of extenders used should be designed to contain compounds that reduce the freezing point, reduce ice crystal formations, and to minimize changes to the membrane structure as much as possible (Graham and Moc'e, 2005; Hammerstedt et al., 1990; Pineda and Dooley, 2003). However regardless of current optimal semen freezing protocols, 40-50% of the semen population do not survive cryopreservation (Watson, 2000). Much research is still studying motility, morphology, membrane integrity and function, and energy metabolism of sperm cells to improve cryopreservation protocols (Metcalf, 2007; Moore, et al., 2006).

1.9 Assessment of spermatozoa

Studies describe various assays that maybe performed on spermatozoa to assess their viability and stallion fertility (Graham and Moc'e, 2005). Laboratory assays are also important because they help to eliminate poor semen samples from being used for AI and to determine what spermatozoa parameter defects are present to explain why some males may exhibit low fertility (Graham and Moc'e, 2005).

The best assessment of stallion fertility is pregnancy itself (Samper, et al., 2007). However fertility is a binomial variable which means that a large number of mares would need to be inseminated to use conception rates as an assessment of stallion fertility. Over 100 mares would need to be inseminated to reduce the statistical variable to ± 7% (Graham and Moc'e, 2005). This is extremely time consuming, expensive and often unrealistic. Also mare pregnancy rates can be strongly influenced by the inseminated mare's age and history, and by breeding management (Colenbrandar, et al., 2003).

Spermatozoa have a very complex multicompartmental physiology and there are many different attributes a spermatozoon requires to successfully fertilise the female gamete such as:

- Progressive motility (i.e. Forward swimming).
- Functioning mitochondria to provide the necessary energy for motility.
- An intact acrosomal membrane that has not yet been activated by capacitation to ensure the acrosome reaction occurs at the right moment.
- Antigen receptor molecules in the plasma membrane to permit the binding of the sperm to the zona pellucida and oocyte membrane.
- Plasma membrane that is able to fuse with the oocyte membrane.
- Chormatin that are then able to reorganise becoming less condense and perform genetically in ensuring the development of the zygote and embryo (Graham and Moc'e, 2005).

Because of the complexity of a spermatozoon there is no single analysis that correlates perfectly with stallion fertility or infertility, therefore it is strongly recommended that more than one assessment be performed on each semen sample (Colenbrander, et al., 2003; Graham and Moc'e, 2005; Katila, 2001b; Meyers,

2001; Samper et al., 2007). However it is also unreasonable to conduct all possible semen parameter analyses because of the time to perform them and the financial expense (Graham and Moc'e, 2005). This has directed researchers to continue to seek the most accurate, least labour intensive and most cost effective combinations of spermatozoa parameter analyses (Colenbrander, et al., 2003).

Initial assessments of semen samples are observations of the colour and appearance of the raw semen directly after collection. pH analysis can also be performed on raw semen, optimal pH is 7.7 (Samper et al., 2007).

Analysis of semen is often performed by observing the percentage of motile sperm on a glass slide, observing the morphology of the spermatozoa after they have been stained, observing the acrosomal integrity with the use of fluorescent dyes, assess in the membrane function integrity in osmotic tests and counting the semen to get concentrations (Pineda and Dooley, 2003).

1.9.1 Motility

Motility assessments are performed by placing a small drop of the extended semen sample onto a pre-warmed glass slide. A coverslip is placed on top and several fields of view are studied under a microscope to determine the percentage of motile and progressively motile sperm. The observational assessments should be performed in the middle of the cover slide as motility declines more rapidly at the edges than in the centre as a result of drying and exposure to air (Samper, et al., 2007). Raw semen samples should not be used for motility assessments due to agglutination or sticking of sperm cell to glass (Samper et al., 2007). Longevity sperm analysis are done by assess sperm motility at 6, 12, 24, and 48 hours (Samper et al., 2007).

It is considered by many that visual assessments can be subjective and that computer assisted motility assessments available are preferred (Graham, 1996b) however there is little evidence to suggest that computer assisted analysis of motility better correlates with fertility (Colenbrander, et al., 2003). Computer assisted motility equipment is very expensive and is sometimes not found in semen laboratories.

Like other semen assessments, there is little correlation between motility and fertility but because it is a very quick semen assessment it is often used in

semen laboratories (Graham, 1996b; Katila, 2001a; Rota, et al., 2004). It is possible that motility does not correlate with fertility because myometrical contractions in the female reproductive tract induced by oxytocin and prostaglandins in seminal plasma are involved in sperm transit towards the oviduct (Katila, 2001a).

1.9.2 Morphology

Morphology assessments are performed by gently mixing a drop of semen sample with a drop of stain (e.g. eosin-nigrosin) then making a smear on a labelled frosted tip slide. Once this slide is dried a drop of oil, cover slide, and then other drop of oil is placed onto an area where there is a high concentration of spermatozoa found under a microscope. Under x1000 magnificent, 100 spermatozoa are observed and categorised according to their morphological defects that are present closest to the head end of the spermatozoa (e.g. head defects, mid-piece defects, etc.). Harsh mixing or smearing will increase the number of detached heads. Smears should also be made on warm slides and dried quickly to minimize the exposure time of live cells to the stain. Eosin-nigrosin stain can also be used to assess the percentage of live and dead spermatozoa in a sample because live cells will exclude the dye whereas dead cell allow passive diffusion of the dye across their non-functional membrane and appear pink (Long, et al., 1990; Samper et al., 2007;).

Morphological defects are either the result of malfunctions that occur during spermatogenesis (such as two head or tails, absence of midpiece or the tail or extremely coiled tails) or sperm maturation in the epididymis (proximal cytoplasmic droplets) (Davies Merol, 2003). Damage to the spermatozoa which occurred during ejaculation (bent, coiled, kinks or swelling in the tail, loose heads or distal droplets) or as a result of incorrect handling after semen is collected (loss of the acrosome, fraying/thickness of the midpiece or the bursting of sperm head) (Davies Merol, 2003). Stallions with high fertility will often have >60% normal sperm and <5% abnormalities of acrosome and midpiece (Samper et al., 2007).

Although morphology does not appear to correlate perfectly with fertility in stallions, unlike with other species such as bulls, boars and rams (Long, et al.,

1990), it is still considered an important sperm assessment as it may indicate particular fertility problems in a stallion (Dowsett et al., 1984).

1.9.3 Acrosome Integrity

Acrosome analyses are useful assessments to perform along with motility and morphology analysis for diagnosing and treating of factors causing infertility mainly in humans (Meyer, 2001).

Acrosome integrity is often analysed by using types of lectins which specifically bind to the acrosome (Gadella, et al., 2001) because these reagents are more accessible. Lectins bind to glycoconjugates located either within the acrosomal matrix or on the acrosomal membrane (Cheng et al., 1996; Cross & Meizel, 1989; Rathi, et al., 2001). PSA (*pisum sativum* agglutinin from an edible pea) and PNA (*Arachis hypogaen*, Peanut agglutinin) are common, non-toxic lectins used (Cross & Meizel, 1989; Katila, 2001b). Although PNA is considered to be better as it stains the acrosome more specifically and brightly (Graham, 1996b) and can also be used to assess acrosome integrity in living spermatozoa (Rathi, et al., 2001).

This analysis is performed by taking a smear semen sample, placing a droplet of either FITC-PSA (Meyer, 2001; Overstreet, et al., 1995; Sabeur, et al., 1998) or FITC-PNA (Cheng et al., 1996; Rathi,et al., 2001) onto the dried sperm smear. The slide is incubated before being rinsed and dried. Fluorescent enhancers or anti-fade solutions are often added before the slide is observed under a fluorescent microscope (Meyers, 2001). Intact acrosomes will fluorescence brightly over the sperm head to the equatorial region while partly reacted acrosomes appear patchy or only fluorescence at the equatorial segment and reacted acrosomes do not fluorescence (Cheng, et al., 1996; Cross & Meizel, 1989)

1.9.4 Hypo-osmotic Swelling (HOS) Test

Plasma membrane functional integrity is important for sperm metabolism and for successful fertilization (Jeyendran, et al., 1984). Hypo-osmotic swelling (HOS) tests are often used to assess the functionality and integrity of sperm

plasma membranes (Aurich, 2005; Colenbrander, et al., 2003; Jeyendran, et al., 1984; Nie & Wenzel, 2001).

HOS analysis is performed by placing a sample of spermatozoa in a hypo-osmotic solution where fluid is transported across the plasma membrane as the spermatozoa attempts to reach an osmotic equilibrium. Functionally intact membrane cells swell, putting tension on the tail fibres causing them to bend and coil which can be observed under a microscope (Jeyendran, et al., 1984; Katila, 2001b; Samper et al., 2007). Spermatozoa with damaged membranes are recognised by their straight tails during microscope observations (Fig 1.6).

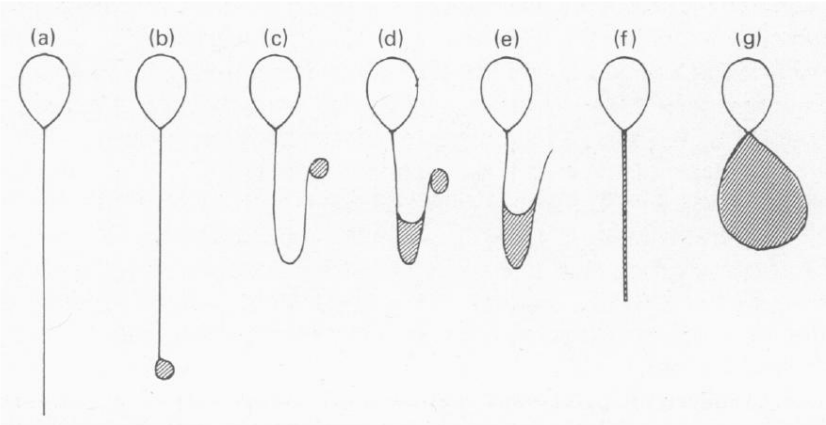


Figure 1. 6 This diagram is a basic representation of the various degrees of swelling observed when performing the HOS analysis (Image obtained from Jeyendran, et. al., 1984). (a) Is an uncoiled tail spermatozoa which represents a damaged sperm membrane, (b-g) Are various degrees of tail coiling indicating that the membrane are intact.

100µl of stallion semen added to 1.0mls of pre-warmed 100mosmol sucrose solution has been observed to produce the optimal plasma membrane swelling in a study by Nie and Wenzel (2001). They also found that the incubation temperature and time, along with the more than 100 sperm cells observed made little difference.

1.9.5 Concentration

1 gram of testes will produce 19 million spermatozoa per day (this reduces to 15 million during the non-breeding season) (Samper et al., 2007). There are computer assisted technologies to assess the number to spermatozoa per ml of a sample, like ARS densimeters. However haemocytometers are thought to be more

accurate when assessing extended semen samples (Samper et al., 2007). Fig 1.7 demonstrates how a haemocytometer is used.

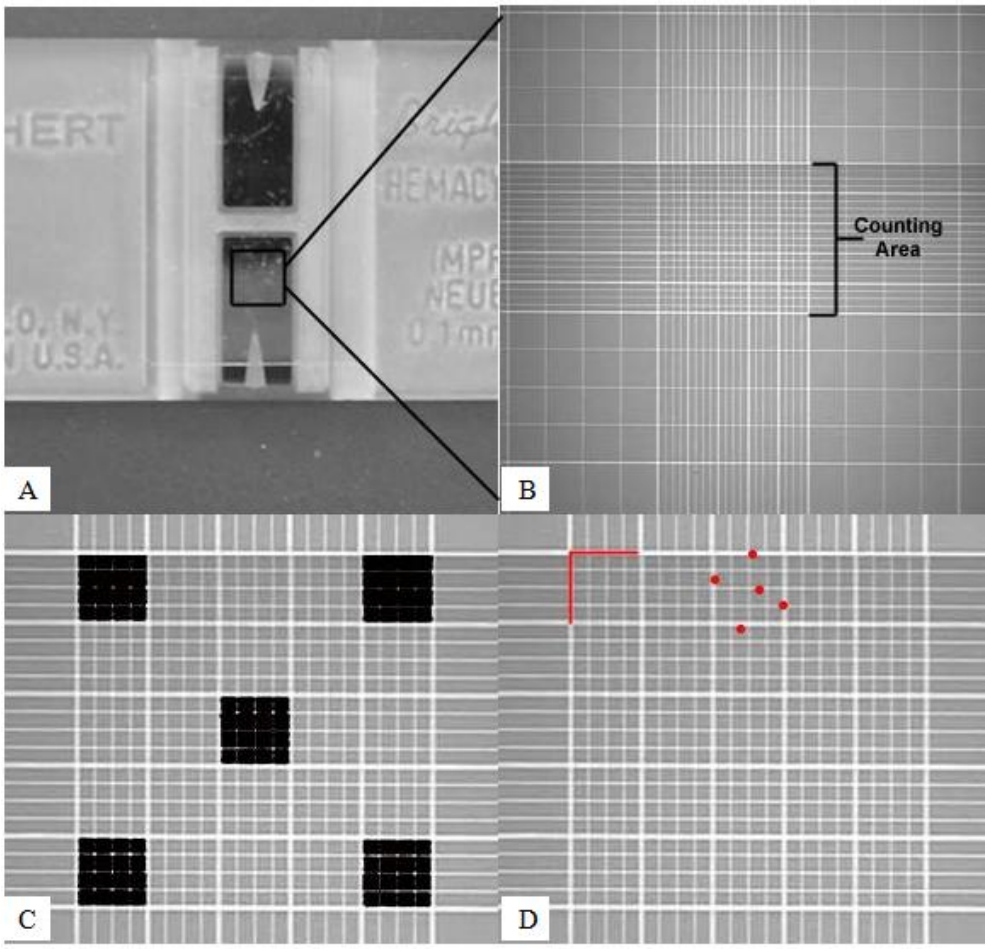


Figure 1. 7 Brief demonstration how a haemocytometer is used. A) Sample is gently pipette under the cover slide and allowed to stand for 3 min. B) Under x100 magnification the counting grid is located. C) Under X400 magnification five squares are counted. D) Shows how the sperm cells are counted. The side of the square with the red lines are not counted therefore in the dotted square only three dots (cells) would be counted. (Image retrieved from http://www.vivo.colostate.edu/hbooks/pathphys/reprod/semeneval/hemacytometer.html on the 26/1/10).

1.9.6 Per cycle pregnancy rate

Studies have shown that stallion per cycle pregnancy rates tend to be lower than other domestic animals (43-60%) and may vary greatly between individual stallions (35-90%) (Colenbrander, et al., 2003). This is calculated by recording the number of oestrous cycles that a mare is covered or inseminated by a stallion before they get pregnant. As mentioned before 100 mares should be inseminated

before using conception rates to assess stallion fertility (Graham and Moc'e, 2005).

1.10 Reactive Oxygen Species (ROS)

1.10.1 What are ROSs?

Reactive Oxygen species (ROSs) are free radicals (compounds with one or more unpaired elections) and various peroxides (NRC, 2007; Sharma & Agarwal, 1996; Sikka, et al., 1995). The ROS's are produced by normal cellular metabolism, leukocyte-induced inflammatory responses (respiratory bursts), and exposure to environmental oxidising agents (UV radiation, pollution, chemical agents, tobacco smoke) (NRC, 2007). They play a vital role in intracellular signalling, redox regulation of transcription factor activity and are used as a defence against infections by activated phagocytes producing ROSs to kill bacteria (Nordberg & Arner, 2001). ROSs are used by several growth factors, neotransmitters, hormones and cytokines as a secondary messenger in intracellular signalling (Nordberg & Arner, 2001; Sharma & Agarwal, 1996). Hydrogen peroxide and superoxide (less reactive than some ROSs) have a similar role to

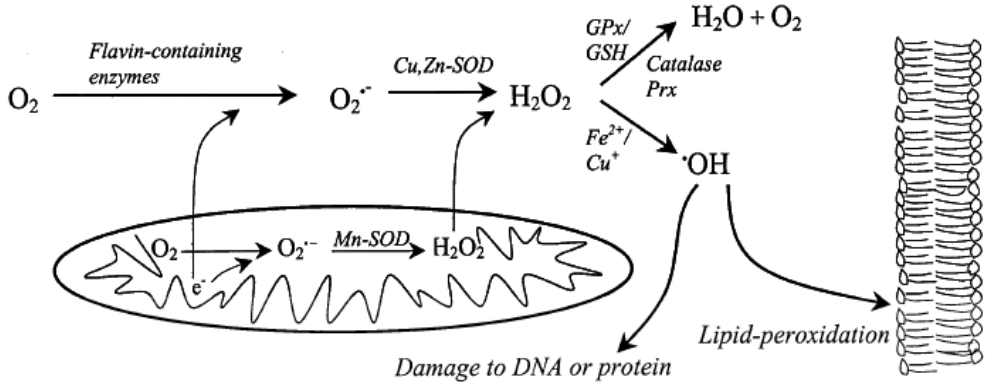


Figure 1. 8 A diagrammatic representation of common oxidative and antioxidative processes in cells. Super oxide ($O_2^{\cdot -}$) lack the ability of hydrogen peroxide (H_2O_2) to penetrate the membrane, even though it is a free radical it is not very reactive. Hydrogen peroxide is not a free radical and is very important in producing more reactive ROSs such as hypochlorous acid (HOCL) and Hydroxyl radical ($^{\cdot}OH$), the later can cause more damage than any of the other ROS (Obtained from Nordberg & Arner, 2001)

nitric oxide in regulating transcription factor activities and other gene expression processes (Nordberg & Arner, 2001). ROS's are often thought of as a by product of oxidative metabolism in mitochondria (Fig 1.8).

Spermatozoa produce highly reactive oxygen species (ROS) which play a physiological role in intercellular signalling cascades mediating sperm capacitation; acrosome reactions; hyperactivation; and sperm-oocyte fusion (Ball, et al., 2001a; Baumber, et. al, 2003; Nordberg and Arner, 2001; Samper et al., 2007). It has been indentified that ROS production is increased during capacitation and acrosome reactions and has been suggested to be important in controlling tyrosine phosphorylation during these processes (Ball, et al., 2001a). Superoxide has been shown to be important in regulating the initiation of capacitation and hyper-activation of sperm (Ball, et al., 2001a). The reduction of capacitation after the addition of catalase (main enzyme that regulates hydrogen peroxide oxidative cell damage, Table 1.1) suggests that hydrogen peroxide is essential in the regulation of capacitation (Ball, et al., 2001a).

However, not all ROS production is beneficial. Chain reactions occur when ROSs react with the biomolecules (Nordberg & Arner, 2001) and may damage lipids, carbohydrates, proteins and DNA (NRC, 2007). Continued production of ROSs due to chain reactions are believed to be integral to the pathogenesis of carcinogenesis, aging ostroarthritis, cardiovascular, and other degenerative diseases (NRC, 2007). In horses excessive ROS is correlated with pathogenesis of joint disease and recurrent airway obstruction (NRC, 2007). Excessive production of ROSs is likely to come from damaged spermatozoa, infiltrating leukocytes or spermatozoa that have residual cytoplasm (Aurich, 2005; Neild, et al., 2005; Pagl, et al., 2006; Sikka, et a., 1995) ROS's are also produced in mitochondria as a by product of spermatozoa flagella activity (Pagl, et a., 2006). In spermatozoa, excessive production of ROS cause plasma membranes to break down thereby reducing sperm motility, metabolic activity, longevity, and viability, and increased morphologically abnormal spermatozoa also known as oxidative stress (Aitkens & Baker, 2006; Aurich, 2005; Ball, et al., 2001a; Baumber, et al., 2000; Jones et al., 1978; Lenzi, et al., 2002; Nordberg and Arner, 2001; Samper et al., 2007; Sikka, et al., 1995). Infertility in human males has been shown to be largely contributed to by oxidative stress (Aitkens & Baker, 2006). ROS related oxidative stress has also been shown to cause significant DNA

(mitochondrial and nuclear genome) damage in spermatozoa (Aitkens & Baker, 2006; Baumber, et al., 2002). A certain level of sperm DNA damage can be repaired after fertilization but spermatozoa cannot repair themselves (Baumber, et. al., 2003). Significant DNA damage is associated with impaired implantation of the embryo and embryonic development and offspring are affected by genetic diseases, cancer and infertility (Aitkens & Baker, 2006).

Table 1. 1 Common ROSs found in cells, main sources they are produced from, the enzymatic defence systems against them and the products that are produced after enzymes have broken down the ROS molecule.

ROS molecule	Main sources	Enzymatic defense systems	Product(s)
Superoxide ($O_2^{\bullet-}$)	'Leakage' of electrons from the electron transport chain Activated phagocytes Xanthine oxidase Flavoenzymes	Superoxide dismutase (SOD) Superoxide reductase (in some bacteria)	$H_2O_2 + O_2$ H_2O_2
Hydrogen peroxide (H_2O_2)	From $O_2^{\bullet-}$ via superoxide dismutase (SOD) NADPH-oxidase (neutrophils) Glucose oxidase Xanthine oxidase	Glutathione peroxidase Catalases Peroxiredoxins (Prx)	$H_2O + GSSG$ $H_2O + O_2$ H_2O
Hydroxyl radical ($^{\bullet}OH$)	From $O_2^{\bullet-}$ and H_2O_2 via transition metals (Fe or Cu)		
Nitric oxide (NO)	Nitric oxide synthases	Glutathione/TrxR	GSNO

Common ROS's that are association with reduced fertility include superoxide ($O_2^{\cdot-}$) anion, hydrogen peroxide (H_2O_2), peroyl ($ROO^{\cdot}$) radicals and the reactive hydroxyl ($^{\cdot}OH$) radicals (Sikka, et al., 1995). While superoxide is more common in spermatozoa, hydrogen peroxide is responsible for more sperm damage as it is more stable and able to cross the sperm membrane more readily (Aitkens & Baker, 2006; Ball, et al., 2001a, Baumber, et. al., 2003). Hydrogen peroxide is also largely responsible for DNA damage in equine cells (Baumber, et al., 2003). Initiation of the lipid peroxidation cascade occurs when hydrogen peroxide attacks polyunsaturated fatty acids which cause the plasma membrane to lose the fluidity and structural integrity required for fusion with the oocyte membrane during fertilisation, and is directly correlated with loss of motility and DNA damage (Aitkens & Baker, 2006; Baumber, et. al., 2003; Jones et al., 1978). Endogenous preoxidation or addition of peroxidised fatty acids have been shown to result in structural damage, leakage of vital intercellular components, loss of motility and a decline in sperm metabolism processes in ram spermatozoa (Jones, et al., 1978).

Equine spermatozoa are particularly susceptible to oxidative attack due to high concentrations of polyunsaturated fatty acids in the plasma membrane and limited repair mechanisms (Aurich, 2005; Baumber, et al., 2003; Pagl, et al., 2006; Samper, et al., 2007). Free radicals target polyunsaturated fatty acids especially because of their double bonds (Nordberg & Arner, 2001). Oxidative damage occurs more in the mid-piece of stallion spermatozoa than in the sperm head piece similar to bovine sperm (Neild, et. al., 2002). This is a result of ROS produced by mitochondria located at the midpiece during flagella activity (Pagl, et al., 2006). As a result of oxidative stresses, the fertility capacity of transported semen is limited (Aurich, et al., 1997; Baumber, et al., 2000).

Further negative effects on equine spermatozoa are caused by transportation and cryopreservation. Hydrogen peroxide generation has been shown to increase 5-fold in frozen/thawed spermatozoa (Ball, et al., 2001a). High levels of ROS are produced by repeated centrifugation (Aitken and Clarkson, 1988), which is part of the preparation for cryopreservation. Irreversible damage can occur to spermatozoa when cooled to 5ºC and a significant loss of fertility capacity when semen is stored for longer than 24h, however antioxidant added to semen extenders can increase viability of chilled semen to 48h (Dennistion, et al., 2000).

1.11 Antioxidants

1.11.1 What are Antioxidants?

To stop a potential damaging ROS chain reaction a free radical must react with another free radical, enzymatic defence systems (Table 1.1), or with a free radical scavenger such as an antioxidant (Nordberg & Arner, 2001). Antioxidants (individually or as part of a larger defence system) are able to convert ROS molecules into less reactive compounds or inactivate them thereby protecting the cells from ROS damage (NRC, 2007). Nitric acid is considered to be a free radical scavenger more than an ROS because it reacts more readily with free radicals (Nordberg & Arner, 2001).

1.11.2 Enzymatic Antioxidants

Enzymatic antioxidants are superoxide dismutase (SOD), catalase, thioredoxin peroxidises (Prx), glutathione preoxidases (GPx) and other glutathione related systems (Nordberg & Arner, 2001; Pagl, et al., 2001). SOD reduces superoxide to hydrogen peroxide (Nordberg & Arner, 2001; Sikka, et al., 1995). Superoxides are produced in high concentrations in the mitochondria due to loss of electrons during respiration chain reactions (Nordberg & Arner, 2001; Sikka, et al., 1995). SOD must be critical in regulating superoxide production as there are no diseases found to be associated to SOD deficiency and in knockout mice experiments (that have no mitochondrial SOD) the young die shortly after birth (Nordberg & Arner, 2001).

Catalase converts hydrogen peroxide into water and oxygen (Atikens & Baker, 2006; Nordberg & Arner, 2001) thereby lowers the risk of highly reactive hydroxyl radical forming from hydrogen peroxide (Nordberg & Arner, 2001). Catalase can also bind to Nicotinamide adenine dinucleotide phosphate (NADPH) to inhibit the enzyme from being broken down so it works for longer (Nordberg & Arner, 2001).

Peroxiredoxins (Prx) works directly to reduce peroxides, while there are four types of GPx (Nordberg & Arner, 2001). GPx 4 has been found to have three functions in relation to sperm cells; 1) insoluble enzymatic action in spermatids 2) structural protein in mature spermatozoa 3) aids in chromatin condensation in spermatozoa.

Glutathione is the most abundant intracellular thiol-based antioxidant that is significantly present in seminal plasma as an enzymatic ROS scavenger (Lenzi, et al., 2002; Nordberg & Arner, 2001). Its main function in cells is as a sulfhydyl buffer and also detoxifies compounds through conjugation reactions catalyzed by glutathione S-transferases or directly in GPx catalyzed reaction (Nordberg & Arner, 2001). Phospholipid hydroperoxide glutathione preoxidose (PHGPx) is highly specific in reducing phospholipid hydroperoxides, produced by ROSs, into safer alcohols, and thereby interrupts radical chain reactions that may lead to greater membrane damage (Lenzi, et al., 2002)

“Semen itself contains a variety of antioxidants compounds and defence mechanisms including uric acid, taurine, tiols, ascorbic acid (Vit C), tocopherols

(Vit E) catalase, SOD, and glutathione-peroxidase-reductase to counteract the harmful effects" (Castellini, et al., 2007). Proteins like lactoferin and glutathione peroxidise found in the epididymis have been suggested to prevent oxidative damage in spermatozoa (Samper et al., 2007). Researchers have also found enzymatic antioxidants (such as catalase) in seminal plasma (Koskinen et al., 2002). Their study discovered that the fraction of semen with the highest concentrations of catalase also had the best motility (Koskinen et al., 2002). Semen collection and processing may remove many of these scavengers that defend the membrane integrity against ROS damage (Ball, et. al., 2002).

Studies have also shown that decosahexanoic acid (DHA) content in the plasma membrane correlated to sperm concentration and motility in humans, boars and chickens (Gliozzi, et al., 2009) Reduced amount of fatty acids in sperm lipid correlates with reduced human sperm concentration, progressively motility and normal morphology, and is associated with aging in chickens and bulls (Rooke, et al., 2001).

1.11.3 Minerals and Vitamins

Antioxidants are used in horse feeds as preservative compounds to inhibit poly unsaturated fats and vitamins oxidative reactions which result from exposure to ROS oxidation (NRC, 2007). Oxidisation of fats cause "feed discolouration, deterioration, and fat rancidity and ultimately reduces feed palatablilty and quality" (NRC, 2007). Synthetic antioxidants added to feed include: butylated hydroxyanisole, butylated hydroxytoluene and tertieru butyl hydroquinone (NRC, 2007). Mineral content in feed may vary depending on the region, plant type, soil, the phase of the vegetation growth and time of the vegetation is harvested (Biricik, 2005).

The principle micronutrient antioxidants are vitamin E, selenium, vitamin C, zinc, copper, and manganese. The body cannot manufacture these micronutrients so they must be supplied in the diet. Horses get many of these micronutrient antioxidants from plants in their normal diet. A number of essential nutrients play a vital role as metabolic antioxidant that protect against endogenous and exogenous ROSs (NRC, 2007). There are many trace minerals that are components of antioxidant enzymes, such as glutathione peroxidise (selenium),

superoxides diemutase (copper, zinc, manganese), and catalases (iron) (NRC, 2007). "Other non-enzymatic mineral-dependant antioxidants include ceruloplasmin (copper) and ferritin (iron)." Fat soluble Vitamin A and E are part of all biological functions as cellular antioxidants (NRC, 2007).

There are also low molecular weight antioxidants such as vitamin E (α-tocopherol) and C (ascorbic acid), selenium compounds, lipoic acid and ubiquinone which all interact with mammalian thioredoxin systems (Nordberg & Arner, 2001).

1.11.3.1 Vitamins

Vitamin E (α-, β-, γ-, and δ-tocopherols, and α-, β-, γ-, and δ-tocotrienols) is considered the most powerful, lipid soluble, chain-breaking antioxidant and is a major part of membrane protection (Castellini, et al., 2007; Deichsel, et al., 2008; Gliozzi, et al., 2009). Its antioxidant function is to break lipid peroxidation chain reactions and to bind with peroxyl and alkoxyl radicals (Castellini, et al., 2007). It contains hydroxyl groups which cause it to react with and reduce unpaired electrons present in free radicals (Nordberg & Arner, 2001; NRC, 1989). Therefore naturally occurring forms of vitamin E are often unstable (Nordberg & Arner, 2001; NRC, 1989). Vitamin E regulates similar biological processes as many ROSs such as cellular signalling, immune function, gene expression activity and induction of apoptosis (Castellini, et al., 2007). Addition of low to moderate levels of vitamin E in goat diets has been shown to increase the activity of SOD and antioxidant competence, while decreasing nitric oxide within the testis (Hong, et al., 2010). Vitamin E supplementation has also increased SOD activity in bovine sperm (Hong, et al., 2010).

Vitamin C (ascorbic acid) has been shown to increase membrane integrity and decreases progressive motility (Atiken & Baker, 2006) because it scavenges free radicals and interacts positively with vitamin E to regulate ROS oxidative damage (Deichsel, et al., 2008).Vitamin E and C or a combination of the two vitamins supplemented in drinking water has been shown to reduce ROS production and thereby increasing fertility parameters in rabbits (Gliozzi, et al., 2009). Vitamin C dietary supplementation has also been shown to protect human spermatozoa DNA from endogenous oxidative damage (Fraga, et al., 1991).

It is recommended that 50IU/kg of vitamin E should be fed each day (NRC, 2007). Beneficial effects of vitamin E supplementation are inconclusive in horses but studies have shown that it is essential during growth and deficiencies in weanling rabbits cause muscle dystrophy and neurological lesions in rats (Castellini, et al., 2007; NRC, 2007). However, feeding low levels of vitamin E along with 100,00IU of vitamin A daily has been shown to benefit reproduction in barren mares (NRC, 2007). The same results were not found when feeding vitamin E and A to mares that had been bred previously (NRC, 2007).

Vitamin E supplementation has been shown to improve semen quality in rams, pigs and rabbits whereas, in stallions, libido and semen parameters were not affected when 5,000 IU of vitamin E was added to their daily diet in comparison with unsupplemented diets of grain mix and grass hay (Castellini, et al., 2007; NRC, 2007). However, another study has shown small improvements in post thaw sperm motility when feeding vitamin E and C supplements (Sikka, et al., 1995). Vitamin E has also been suggested to be an effective antioxidant at high concentration that does not affect motility, but does significantly enhance sperm-oocyte fusion (Aitken and Clarkson, 1988).

1.11.3.2 Selenium

Selenium is usually taken up by the body in the form of selenium acids from plants and cereals (Sánches-Gutiérrez, et al., 2008). Selenium minerals commonly range from 0.05-0.3pp and are present normally in feed as selenocystine, selenocysteine, selenomethionine (organic) (NRC, 1989). Estimated amount of selenium required in a horse diet is 0.1mg/kg of diet but only 77% of selenium in the diet is absorbed (NRC, 1989). 140ng of se/ml of plasma /serum is adequate to prevent problems associated with selenium deficiency (NRC, 1989). Selenium dietary concentrations have been suggested to not correlate with Se blood concentrations (NRC, 2007) even though selenium blood samples have been used when studying selenium supplementation effects in horses (Richardson, et al., 2006).

Selenium is an essential component in selenium-dependant glutathione peroxidise which helps regulate hydrogen peroxide damage to cell membranes

that are toxic to cell membranes (NRC, 2007). Selenium also helps to control thyroid hormone metabolism.

Selenium deficiency results in weakness, impaired locomotion, difficulty in suckling and swallowing, respiratory distress and impaired cardiac function (NRC, 2007). On the other hand hair loss and changes in hooves are also often associated with very high Selenium doses. Excessive amounts of Selenium can cause acute toxicity resulting in perspiration, blindness, abnormal pain, head pressing, diarrhoea, colic, lethargy, and increased heart rate and respiration rates (NRC, 2007). Chronic selenium toxicity results in allopecia, especially about mane and tail and cracking of the hooves around the coronary band (NRC, 2007).

Selenium is required for the biosynthesis of testosterone and for normal spermatogenesis, and either high or low levels of selenium can be detrimental (Shanlini & Bansal, 2006). Selenium deficiency induces oxidative stress because reduced fertility was associated with higher levels of lipid preoxidation (Sánches-Gutiérrez, et al., 2008). Selenium deficiency has been observed to impair spermatogenesis in pigs, rats and mice by a reduction in motility, and an increase in tail and midpiece defects (Sánches-Gutiérrez, et al., 2008; Shanlini & Bansal, 2006). Mice deficient in selenium expressed reduced numbers of germ cells, differentiation and reduced fertility (67%) (Sánches-Gutiérrez, et al., 2008; Shanlini & Bansal, 2006).

1.11.3.3 Copper

Copper is necessary in several copper-dependent enzymes essential in the maintenance and syntheses of elastic connective tissue, detoxificating superoxide, movement of iron stores, melanin synthesis and preserving the integrity of the mitochondria (NRC, 1989). Copper interacts with many other minerals which can influence copper metabolism (NRC, 1989). Copper absorption ranges from 24-48% and interacts with many other minerals including molybdenum, sulphur, zinc, Se, sliver, cagmiun, iron and lead (NRC, 2007). Copper deficiency causes lameness in foals (NRC, 2007)

1.11.3.4 Manganese

Manganese has been found to be necessary for the metabolism of lipids and carbohydrates and chondroitin synthesis which is essential in cartilage formation (NRC, 1989). Manganese is one of the least toxic trace elements (NRC, 2007). Large amounts of manganese interfere with phosphorus absorption which is important in energy transfer reactions and synthesis of phospholipids, nucleic acids and phosphoproteins (NRC, 2007). Manganese deficiency has been shown to result in abnormal cartilage development (NRC, 2007).

1.11.3.5 Zinc

Zinc is included in more than 100 enzymes in the body such as carboxypeptidase, alkaline phosphatise and carbonic anhydrase (NRC, 2007). High concentrations of zinc are found in the iris and charoid of the eyes and the prostate gland. It is recommended to feed 15-40mg of zinc/kg to horses, however only 5-10% of zinc is absorbed. Appropriate sources of supplemental zinc include zinc sulphate, zinc oxide, zinc chloride, zinc carbonate and various zinc chelates. (NRC, 1989) Ratio of zinc and copper is important as it is believed they use same transporter mechanism (NRC, 2007). Zinc serum and plasma level are not good indicators of zinc status, and serum zinc concentration varies with respect to age (NRC, 2007). Zinc deficiency decreases semen quality, stallion libido, and delays ovulation in mares (Biricik, 2005)

Excessive amounts of Zinc, Manganese and Copper can cause a decrease in the ability to utilise iron in the diet (NRC, 1989).

1.11.3.6 Blood Mineral Levels

There appear to be no studies that have investigated an association between blood mineral levels and spermatozoa parameters or fertility. This study uses blood mineral levels in its assessment to investigate if there are any possible changes in the blood mineral levels as a result of dietary supplementation that correlate with possible changes in spermatozoa parameters. If so, then monitoring mineral levels in the blood may be developed to use as an indicator of dietary requirements for optimal sperm quality.

A previous study performed by Zahn, et al. (2006) showed that the freezability of semen is positively correlated to blood serum protein bands B3, B6, B7, B12, and B13. Hormone concentrations in blood plasma were also studied and found to be independent of seminal plasma except testosterone levels (Audet, et al., 2009)

1.11.3.7 Omega-3 Oil

Omega-3 is derived from fresh grass, certain plants and flaxseed (Platinum Performance, 2004). It stems from alpha-linolenic acid while omega-6 stems from linolenic acid (Platinum Performance, 2004). The correct balance of omega-3 and omega-6 is important in horse diets. However grain and vegetable oils in many horse diets are high in omega-6 as opposed to natural forage diets that are higher in omega-3. Studies have found that omega-3 significantly increases sperm motility, percentage of morphological normal spermatozoa and increases sperm concentration (Pagan, 2008). Studies have also shown feeding omega-3 have various other benefits such as enhancing the transfer of immunity from mares to their foals, reduced joint inflammation in young exercised horses and arthritic older horses, and increased red blood cell membrane flexibility (Pagan, 2008; Platinum Performance, 2004). Omega-3 oil such as fish oil has also been found to increase pregnancy rates and embryonic survival in cattle (Pagan, 2008).

Little is known about the natural concentration of omega-3 fatty acids in horses (King, et al., 2008). Addition of omega-3 to diets can result in vitamin E deficiency in chicks, ducks, dogs, swine, calves and lambs and their study suggests supplementation with moderate levels of omega-3 to avoid any complications resulting from Vitamin E deficiency (Siciliano & Wood, 1993).

1.11.4 Where are Antioxidants Most Likely to Influence Fertility?

Because of the complexity of spermatogenesis it is difficult to determine where antioxidants are likely to be most effective. It is likely that antioxidants are important in the regulation of ROSs throughout all the stages of spermatogenesis to ensure that sperm cell develop correctly. Studies have suggested that germ cells are more susceptible to ROSs due to higher concentration of polyunsaturated fatty

acids in the membrane and low concentrations of cytoplasmic antioxidants. (Sánches-Gutiérrez, et al., 2008)

Antioxidant would be important during storage in the epididymis because sperm cells become motile and lose their cytoplasmic droplet in the presences of essential hormones. Motility is a result of mitochondria producing the necessary energy required for flagella movement and ROS by products would also be produced.

Antioxidants in seminal plasma would also be important after ejaculation to reduce the effects of the immune response in the mare's uterus producing massive concentrations of ROSs (Neild, et al., 2005). This has been supported in other studies that have found a reduction of semen parameters when seminal plasma is removed and in the addition of antioxidants in semen extenders.

Addition of antioxidants to semen extenders have be suggested to maintain the membrane integrity better and thereby increase total motility, and progressive motility (xanthurienic acid – most effective in protecting spermatozoa) (Aurich, 2005; Dennistion, et al., 2000). Egg yolk in extenders has been shown to be very effective as it contains antioxidants lipids and miroelements (Maldjian, et al., 2005). Pyruvate (2mM) added to skim milk based extender was helped maintain sperm motility and help maintain fertility in spermatozoa chilled stored for 48h (Bruemmer, et al., 2008). Different antioxidant extender additives have been found to inhibit lipid peroxidation at different stages in the preoxidation cascade, therefore previous studies have suggested that a combination of different antioxidant may have better results in protecting spermatozoa from oxidative damage (Dennistion, et al., 2000).

Addition of vitamin E (α-tocopherol) to extenders has been observed to benefit chicken, tuckey, bull, and horse semen (Castellini, et al., 2007). Addition of vitamin E or C before storing semen at 5ºC with or without seminal plasma protects plasma membranes and maintains progressive motility (Neild, et al., 2005). However another study showed that addition of enzyme scavengers, catalase or a variety of lipid or water soluble antioxidants had not beneficial effects on equine sperm motility while stored at 5ºC (Ball, et al., 2001b).

1.11.5 What are the effects of diet on fertility?

Diet can have a significant impact of fertility. Stallions that are under weight or are obese have reduced fertility (Warren, 2005). As already mentioned, deficiencies in any of the essential micronutrients also has larger impacts on all components of fertility including for example decreased libido and semen quality in the case of zinc deficiency. One of the earliest published studies on dietary composition effects on semen quality was performed by Berliner, et al., in 1939. They looked at changing stallion diet to improve semen quality and found a mixture of grain (oats, wheat bran and wheat or corn) was required to stimulate and maintain higher rates of sperm production.

Previous studies have suggested that antioxidant dietary supplementation may help prevent damage to the sperm plasma membrane thereby improving semen fertility parameters in stallions. Various studies in stallions have already shown beneficial effects of feeding antioxidants (usually omega-3 oils) in increasing motility, spermatozoa concentrations, and reductions in morphologically abnormal spermatozoa, but not all in the same study (Brinsko, et al., 2005; Diechsel, et al., 2008; Elhordoy, et al., 2008; Harris, et al, 2005; Squires, 2008) and not all studies have found improvement in stallion semen parameters (Grady, et al., 2009).

Effects of dietary supplementation in reproductive processes have also been shown in other species. In a study performed by Aitkens and Clarkson (1998) they found that the addition of vitamin E supplementation to human diet significantly increased sperm-oocyte fusion. Antioxidant supplementation also reduced DNA fragmentation in humans particularly in men with high levels of DNA fragmentation though the action of dietary antioxidants has not been established (Zini, et al., 2009).

It is believed that feeding nutriceutical compounds rich in omega-3 fatty acids improves various semen parameters in some stallions possibly be due to alternation in the cholesterol: phospholipid composition ratio of the spermatozoa plasma membrane, making it more resistant to oxidative stress (Metcalf, 2007). Some antioxidants have been shown to suppress preoxidation in spermatozoa (Jones, et al., 1978).

Stallions have similar polyunsaturated fatty acids content in spermatozoa plasma membrane as boar spermatozoa. Rooke, et al. (2001) found an increase in fatty acid composition in pig semen after feeding tuna oil for 5-6 weeks and increases in progressive motility, normal acrosomes and reduced sperm abnormalities. Another study in boars revealed that omega-3 supplementation increases sperm concentrations in ejaculates therefore increasing the number to AI doses per ejaculate and increasing profit (Estienne, et al., 2008) This study found no increases in motility but did find a reduction in the percentages of morphological tail defects (Estienne, et al., 2008). This is possibly a result of increased DHA content in spermatozoa plasma membrane that was shown in another omega-3 supplementation study in boars (Maldjian, et al., 2005). It has been suggested vitamin supplementation influences boar reproductive performance, especially during periods of intensive semen collection (Audet, et al., 2009). A nonclinical study showed that higher sperm concentrations and motility rates were associated in human males that had more antioxidants in their diet (Eskenazi, et al., 2005). However, omega-3 and Vitamin E supplementation in turkeys did not increase motility and viability, or reduce the percentage of dead spermatozoa (Zaniboni & Cerolini, 2009).

In other domesticated livestock species, severe deficiencies of vitamin A and E, and selenium have been closely associated with reduced spermatogenesis and this has been suggested also be the case in stallions (Davies Merol, 2003).

1.11.6 Why should antioxidants have any effect on fertility?

Antioxidants supplied in the diet such as vitamins, minerals and omega-3 reduce the detrimental effect caused by ROS excessive production. Vitamins bind to, scavenge and break down ROS chain reactions, while dietary minerals are essential in antioxidant enzymes and omega-3 oils increase DHA levels in the spermatozoa plasma membrane all of which reduces detrimental effects to plasma membranes of spermatozoa caused by ROSs. As membrane integrity is critical for normal sperm cell function and oocyte fusion, dietary antioxidants should also reduce the detrimental effects that ROSs cause to sperm plasma membrane and thereby increase fertility parameters.

The present study was designed to investigate whether vitamin E and minerals or oil antioxidant dietary supplementation affects semen fertility parameters in New Zealand Standardbred Stallions. This is the first stallion fertility study to investigate both minerals and vitamin E compared to oil antioxidant supplementation and to include per cycle pregnancy rates in assessing the effect antioxidant supplements may have on stallion fertility. It is the first stallion fertility study to freeze semen 6-8h after collection as an extreme oxidative stress test and is the first study of its kind to be performed in New Zealand.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Ethics

Ethical consent was obtained from the Animal Ethics Committee (Protocol # 727) and the Human Ethics Committee (Protocol # 92) from the School of Science and Engineering at the University of Waikato before this study began.

2.2 Subjects

A letter was sent out to introduce this study and ask for stallions from standardbred studs all over New Zealand to participate (Appendix Fig 4A.1). All studs were contacted individually and 18 stallions were volunteered to participate in this study from three different studs (one in the North Island and two in the South Island). The studs were asked to fill out a research consent form and questionnaire for all of the stallions included in this study (Appendix Fig A4.2 & A4.3). The questionnaire provided information about the stallions' pregnancy rates from the previous breeding season (Appendix Table A4.1) which was used to sort the stallions into even groups containing the same number of high, medium and low fertility stallions.

A questionnaire was also sent out in January 2009 to gather information about the stallions' feeding behaviour while being feed the supplemented diets (Appendix Fig A4.5 & A4.6). This was designed to ensure that the studs were feeding the supplements and that the stallions were eating all of the supplements as personal supervision was not possible due to the locations of the studs. Subsequent phone interviews and visits to the studs assured that stallions were fed the supplements with no issues.

2.3 Feed Allocation

The 18 stallions were allocated into three treatment groups according to their previous breeding season's pregnancy rate; control, minerals and vitamin E (Min & Vit), or oil. One group was fed a mixture of minerals and vitamin E (Nutritech supplement), the second group was supplemented with omega-3 oil (canola oil) and the third group was not feed any supplement (control group). The composition of these supplements is detailed in Appendix 3 (Table A3.1). During collection of the first three pre-treatment semen samples and before starting the supplemented diets, four stallions (15-18) were withdrawn from the study for reasons unrelated to the study. Therefore after pre-treatment analysis, eight of the stallions were fed a basic modified Dunstans feed which met the NRC 1989 recommended nutrition levels for a breeding stallion, while the remaining six stallions were fed a NRM feed as their basal diet. To ensure that the levels of minerals, vitamins and oil in each of the treatment groups were the same, these six NRM fed stallions were only given half the amount of supplement to match the stallions on the Dunstan supplied diet (Table 2.1). The treatment groups on the NRM diet are subsequently referred to as ½ control, ½ minerals + vitamin E, and ½ oil. Refer to Table A3.2, A3.3 & A3.4 in the Appendix for details of mineral levels in minerals and vitamin E supplemented diets, NRC nutrient recommended levels and nutrient details of the two diets.

The mineral and vitamin E mix, and Oil were pre-measured into labelled pottels to eliminate variation in the amount of supplement measured and fed by different people employed at the different studs. Two of the studs started feeding their stallions the supplemented diet in the middle of November 2008 and the third stud started their stallions on the supplemented diet on the 11th of December 2008. All of the stallions remained on this diet until the end of the fertility trial in the middle of March 2009.

Table 2. 1 Base diet, treatment group and amount of supplement given to each of the 18 stallions from the different studs during the treatment period of this trial. Complete details of contents of the supplements included in the different diets refer to Appendix 3, Table A3.3 & A3.4.

Standardbred Stud	Stallion ID	Base diet	Treatment Group	Supplement Amounts
A	1	Modified Dunstans	Control	0
B	2	Modified Dunstans	Control	0
B	3	Modified Dunstans	Control	0
A	4	Modified Dunstans	Minerals & Vitamin E	30gm
B	5	Modified Dunstans	Minerals & Vitamin E	30gm
B	6	Modified Dunstans	Minerals & Vitamin E	30gm
B	7	Modified Dunstans	Oil	30mls
B	8	Modified Dunstans	Oil	30mls
C	9	NRM	Control	0
C	10	NRM	Control	0
C	11	NRM	Minerals & Vitamin E	15gm
C	12	NRM	Minerals & Vitamin E	15gm
C	13	NRM	Oil	15mls
C	14	NRM	Oil	15mls
A	15*	Modified Dunstans	Oil	30mls
B	16*	Modified Dunstans	Oil	30mls
B	17*	Modified Dunstans	Control	0
B	18*	Modified Dunstans	Minerals & Vitamin E	30gm

* Stallions that were withdrawn from the trial for reasons unrelated to this study.

The last three semen samples replicates were intended to be collected after the stallions had been feed the supplemented for at least 60 days. In the case of the stallions that were started on the supplemented diet late, they had only been feed the diet for 35 days rather than the 60 days as desired. However, semen samples were collected over 35-70 days of being fed the supplemented diet.

2.4 Semen samples

Each semen sample collected was equivalent to one insemination dose and was comprised 25mls of semen extended 1:4 in either INRA 96 (IMV, France) or Equipro (Minitube, Australia) and was sent to Equibreed NZ Ltd to be assessed. The first three semen replicates were collected from each stallion between the 15th September and 8th of November 2008 prior to starting the antioxidant supplement. After the supplement treatment period, three more semen replicates were collected from each stallion between 19th of January and 9th of March 2009.

2.5 Analysis of Semen

Because semen was collected at the individual studs, raw semen analysis was not performed. Motility and morphology analyses were performed at 6-8 hours after collection and again 24 hours after collection. Three straws of each of the semen samples were frozen at 6-8 hours after collection as described below. After thawing, analysis of spermatozoa included motility, morphology, acrosome status, hypo-osmotic swelling test (HOS), and concentration.

2.5.1 Motility

Samples were relabelled with codes to ensure that the motility assessment of the semen samples were "blind". 10µl of each sample was placed on a warmed glass slide and a warmed cover-slip placed over the top. Samples were visually assessed under a microscope (WILD M20; Heerbrugg, Switzerland) on a 37°C heated stage. At least five fields of view were observed before determining an average percentage of live, motile sperm (total motility). The percentage of progressive (forward swimming) motile sperm (progressive motility) was determined by observing the average number of live motile sperm that were swimming in a straight line in several fields of view. The total progressive motility (TPM) was worked out by using the following equation:

Total Motility (TM) x Progressive Motility (PM) x 100 = Total Progressive Motility

(E.g. if TM = 80% and PM = 60% then TPM = 0.8 x 0.6 x 100 = 48%).

2.5.2 Morphology

A 10 µl drop of each sample and a 10 µl drop of eosin-nigrosin stain was mixed on a glass slide with the end of another slide then gently smeared across a pre-labelled slide at a 45° angle to create a smear and allowed to air dry quickly on a heated stage. 100 spermatozoa were visually assessed under a microscope (Olympus BHB) set at 1000x magnification with an oil immersion lens. The proportion of morphologically normal sperm, loose heads, head defects, mid-piece defects and tail defects was determined (Fig 2.1). All morphology slides were assessed together in assessment period groups (e.g. 6-8 hour per and post-

treatment all assessed together, etc.) at the end of the experimental period to avoid variation in the assessment.

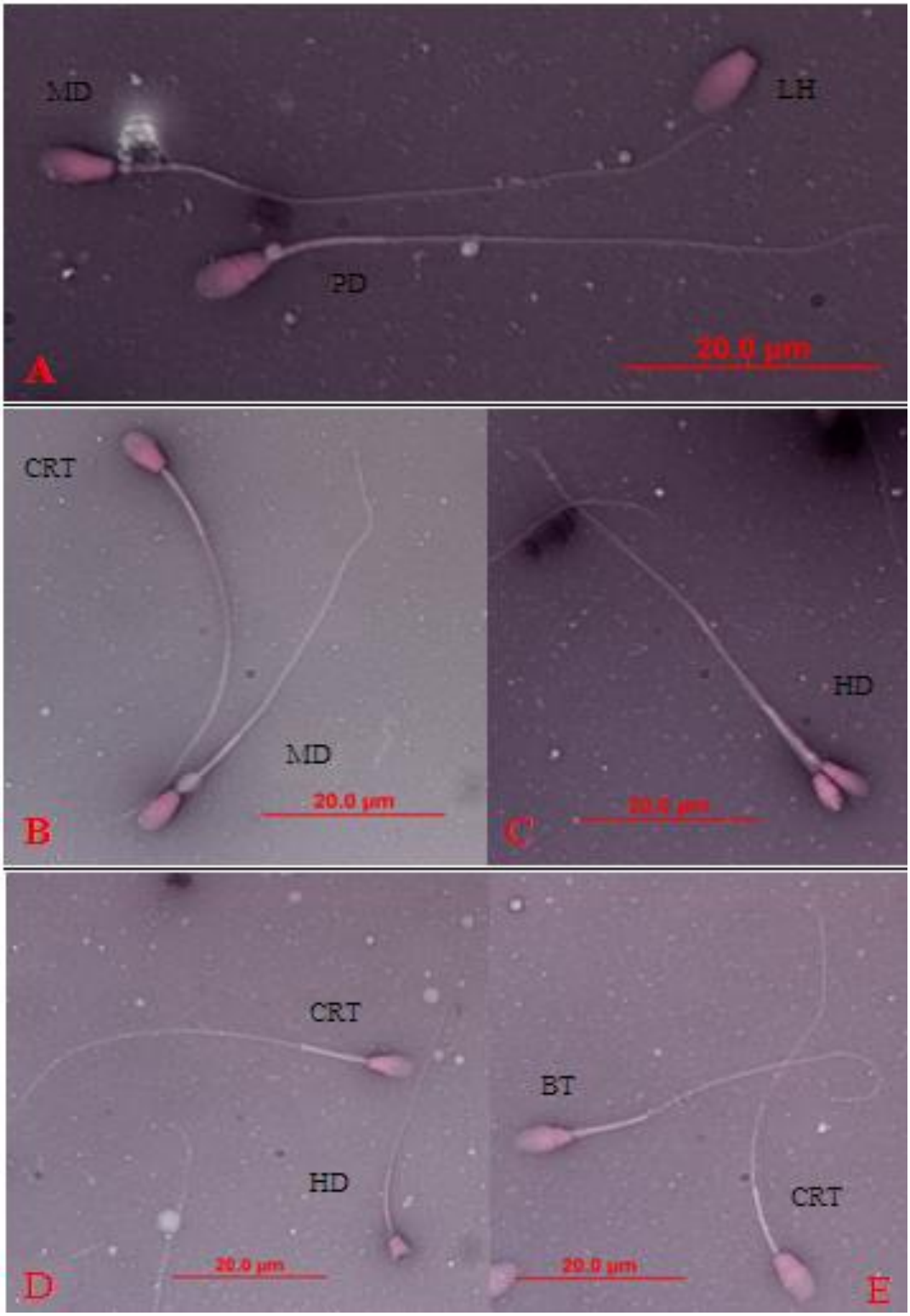
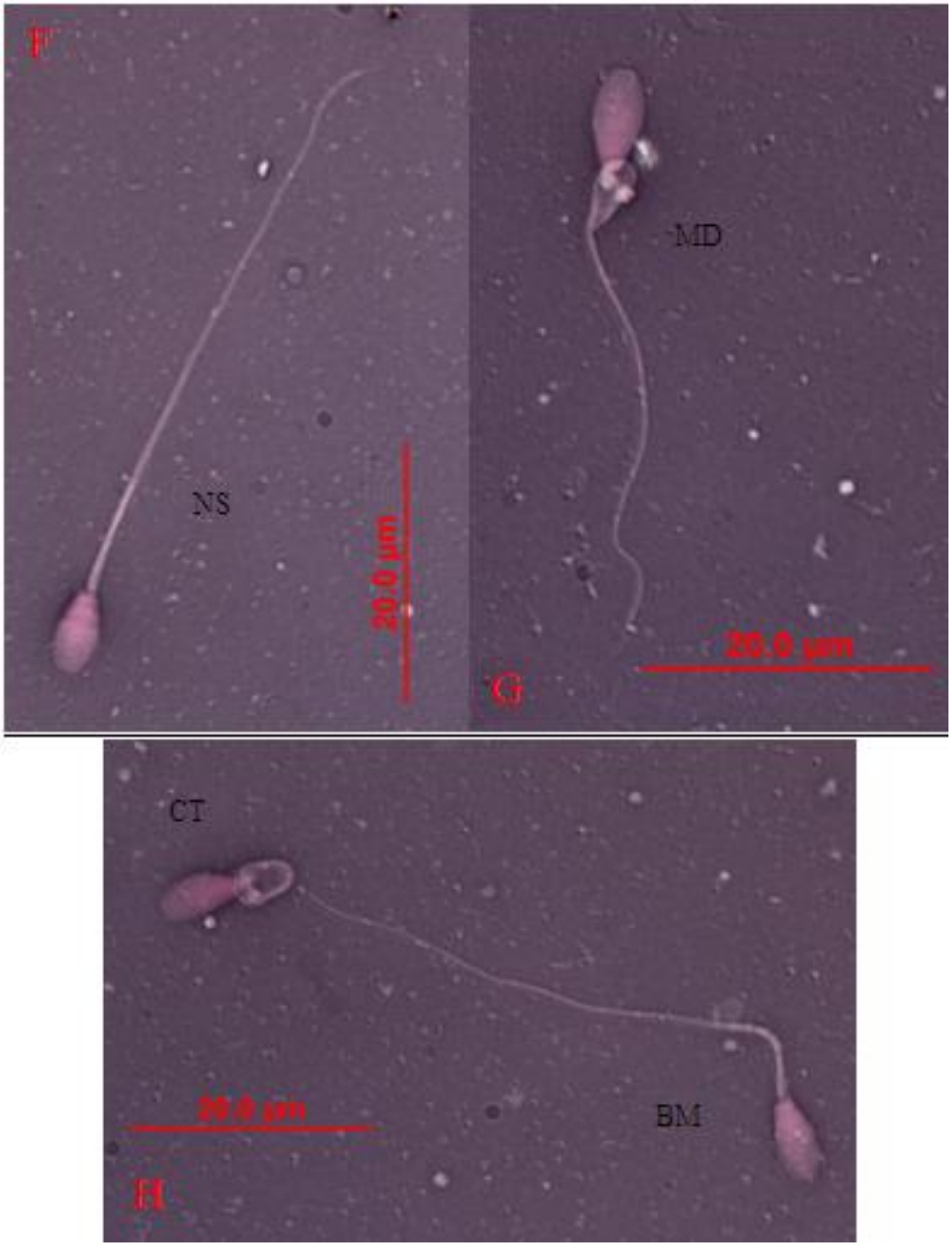
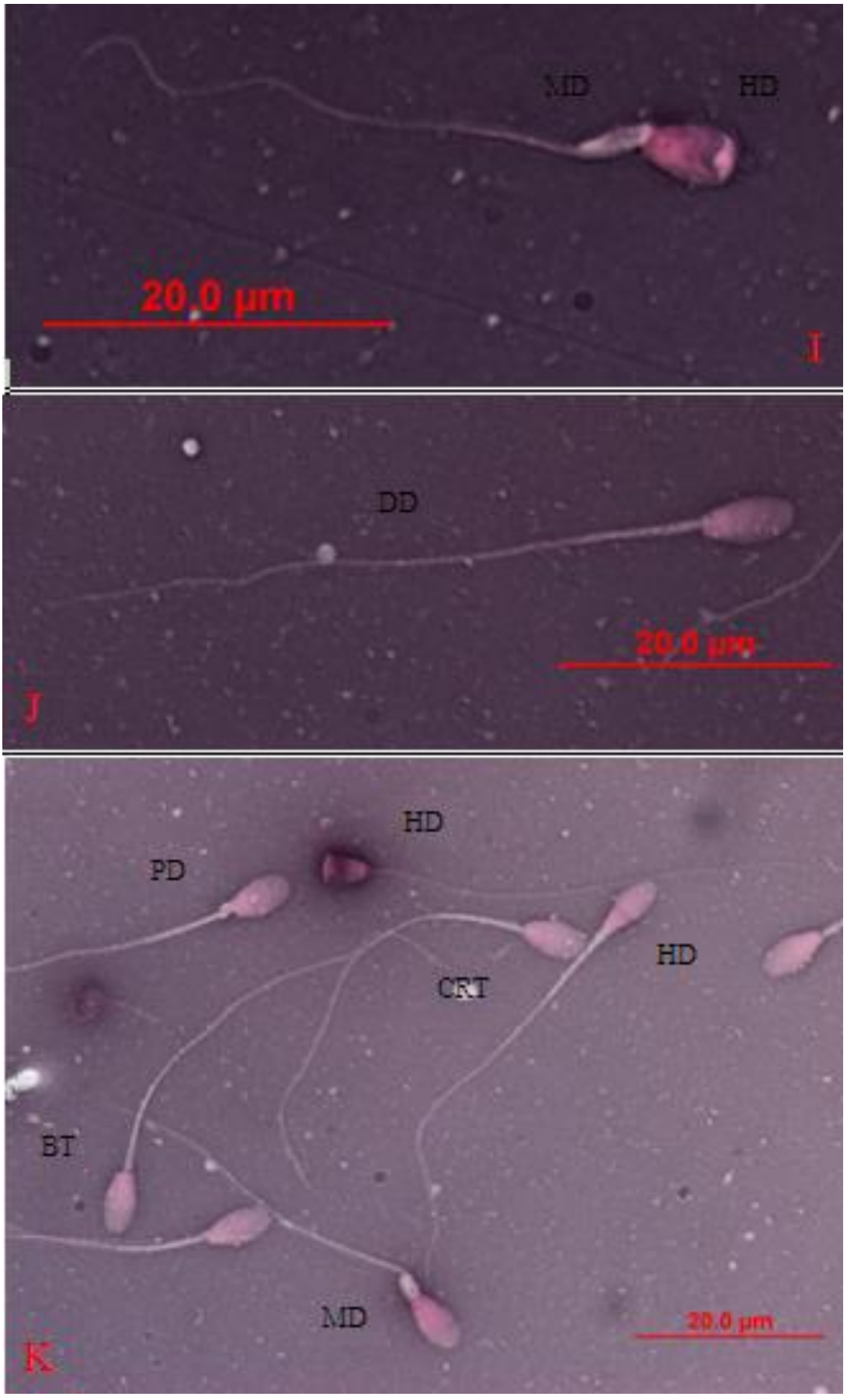


Figure 2. 1 This series of images (A-K) demonstrates the various morphology classifications of spermatozoa. MD, Mid-piece Defect; PD, Proximal Droplet; LH, Loose Head; CRT, Curved Tail; HD, Head Defect; BT, Bent Tail; NS, Normal Straight Spermatozoa; CT, Coiled Tail; DD, Distal Droplet.


F
NS
20.0 µm
MD
20.0 µm
G
CT
20.0 µm
BM
H


MD
HD
20.0 µm
I
DD
20.0 µm
J
HD
PD
HD
CRT
BT
MD
20.0 µm
K

2.5.3 Acrosome

Acrosome status was assessed after freezing and thawing. A 10 µl drop of each semen sample was placed on a coded labelled glass slide and smeared with the use of another glass slide before being air dried and fixed with 70% ethanol (Cheng, et. al., 1996). After being stored for two months acrosome assessments were completed at the University of Waikato. 20 µl of FITC-PNA (Sigma product L7381) was distributed over the sample slide and placed on top of a dampened paper towel and incubated for 30 minutes at 39°C. The slides were then rinsed in Phosphate Buffer Saline solution and air dried. Two drops of UCD mounting medium was placed onto the slide and a cover slip placed over the top. The acrosomes of 100 spermatozoa were visually assessed under a 400x magnification fluorescent microscope (Leica DMRE with a 50W Hg vapour burner, Olympus D70 camera, Ex filter BP450-490). The acrosome were categorised according to their fluorescence pattern (Fig 2.2.). Some of these slide samples were repeated until the correct protocol was established to ensure assessments were correct and repeatable.

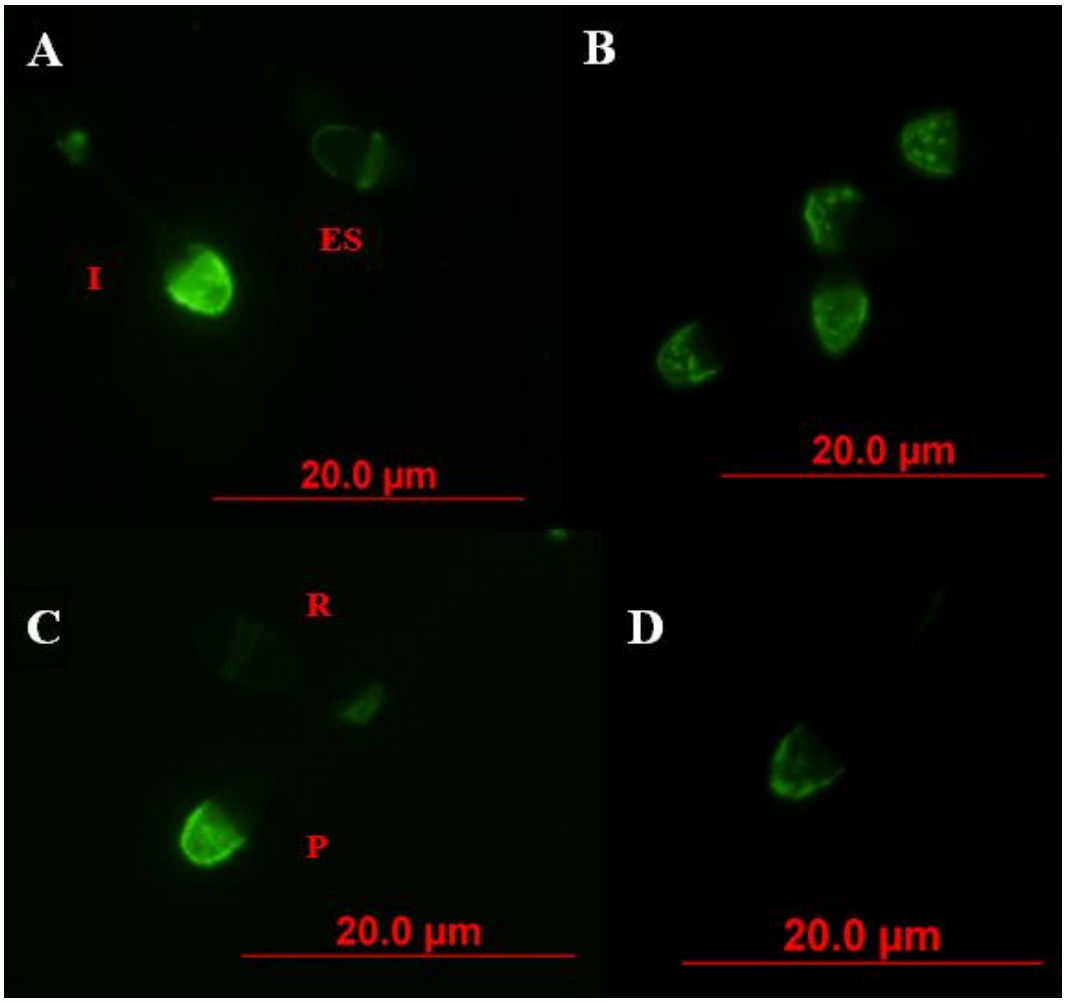


Figure 2. 2 This series of images demonstrates that various classifications of acrosomes. A demonstrates an intact acrosome (I) and an equatorial segment acrosome (ES). B demonstrates the various patchy or partly (P) reacted acrosomes. C demonstrates a reacted acrosome (R) and patchy acrosome. D demonstrates a 75% reacted acrosome.

2.5.4 HOS

The HOS test assessed the integrity of the spermatozoa membrane after freezing and thawing. 100 μL of the post-thaw semen sample was added to 1.0 mL of pre-warmed HOS media (100 mosmol sucrose solution – 1.712 gm of sucrose in 50 mL of sterile water) in a separate test tube labelled with the same code. After 60 min incubation at 32°C, a 10 μL sample was analysed on a haemocytometer and the percentage of coiled tailed spermatozoon were counted out of 100 spermatozoa observed.

2.5.5 Concentration

The sperm concentration was assessed in post-thaw semen samples. This was done at the same time as the HOS analysis by counting the number of spermatozoa in the five standard squares on the haemocytometer. If less than five squares were required to obtain the percentage of coiled tailed spermatozoa for the HOS analysis, then this data would be recorded before continuing to count the remaining five squares to obtain the concentration. If more than five squares were required to obtain the percentage of coiled tailed spermatozoa for the HOS analysis then this data was recorded for the concentration before starting to count the sixth square. The concentration was calculated using the following equation:

Concentration 10^3 = C x 4000/S x D

C = number of cells given in count

S = number of small squares counted

D = Dilution factor 1:11

2.6 Freezing of Semen

Semen samples were frozen upon arrival at Equibreed NZ Ltd in the following steps:

1. 15 mls of the semen sample was centrifuged for 15 mins at 300g. (1200rpm).
2. The supernatant containing the seminal plasma was aspirated with the use of a sterile disposable 3 ml pipette.

3. Freezing extender (EDTA Freezing extender plus 2% glycerol) was added to the semen rich pellet to make up 2 ml and mixed gently with a clean disposable 3 ml pipette.
4. The semen sample was then loaded into pre-labelled 0.25 ml straws and sealed with PVA powder seal and water.
5. The semen straws were then placed onto a rack and placed into a fridge to cool for 45 min.
6. The semen straw rack was then placed in a styrofoam box 3cm above the liquid Nitrogen in the vapour for 10 min before the straws were plunged directly into the liquid Nitrogen and transferred to a storage tank at -196ºC.

2.7 Thawing of Semen Samples

1. Semen sample straws were selected at random from the various storage canisters and placed into a 37 °C water bath for 30 seconds.
2. The straw ends were cut and the contents poured into 1 ml pre-warmed test tubes labelled with a number code to ensure blind assessment of the semen parameters as the first two steps were performed by individuals that had no previous knowledge of the stallions involved in the trial.
3. A 10 µL drop of the sample was used for a morphology assessment.
4. A 10 µL drop of the sample was used for an acrosome integrity assessment.
5. Another 100 µL of semen sample was added to 1.0 mL of pre-warmed HOS media (100 mosmol sucrose solution – 1.712 gm of sucrose in 50 mL of sterile water) in a separate test tube labelled with the same code. After 60 min incubation at 32°C, a 10 µL sample was analysed on a haemocytometer to obtain both the concentration and a percentage of 100 observed spermatozoa with coiled tails (Jeyendran et al., 1984; Nie and Wenzel, 2001).
6. The remainder of the sample was diluted in extender at a 1:1 ratio.
7. A motility assessment (as described above) was then performed at 0 min and 30 min after thawing.

2.8 Blood

Blood samples were taken from all of the stallions by the studs' veterinarians before and after being fed the supplementary diets for 60 days. The samples were sent to Equibreed NZ Ltd. and the levels of Copper, Selenium and Zinc were analysed in the blood serum by Gribbles Veterinary in Hamilton, NZ. Manganese was unable to be assessed in the blood.

2.9 Per cycle pregnancy data

The per cycle pregnancy data was obtained from a questionnaire that was provided at the end of the trial (Appendix 4, Fig A4.7 & A4.8). This was calculated as the proportion of pregnancies obtained out of the total number of oestrous cycles in which the mares were inseminated. For mares inseminated with semen from the stallions in the trial, each treatment group was examined both before and after the stallions had been supplemented with antioxidants. The control group of stallions had no supplement and their results enabled the effects of the breeding season and the effects of time to be accounted for. The results were analysed by Chi-square analysis.

2.10 Statistical Analysis

Statistical analysis was performed using a Minitab and Genstat statistical computer programs. The results obtained were analysed to compare the differences between the means for each of the treatment groups and for each stallion before and after treatment. The initial analysis was a one-way ANOVA to analyse differences (post-treatment-pre-treatment) semen parameters for each stallion (averaged over the multiple samples collected) for each assessment time. The two base diets (Dunstans and NRM) were treated as different and thus there were six treatment groups; Control, mineral and vitamin E, and Oil, for each diet. In the case of an overall significant result, a Dunnett's test was carried out to identify if any of the treatments groups differed from the control groups.

A subsequent one-way ANOVA analysis was also performed by Genstat regression after combining the two base diets results so there were only three

groups; control, minerals and vitamin E, and oil. This analysis tested effects of supplement addition adjusted for possible diet effects.

The effect of each treatment group on the per cycle pregnancy rate was analysed by a Chi-square test.

CHAPTER THREE: RESULTS

3.1 The Effects of Dietary Supplementation on Stallion Semen Parameters

Semen parameters that were analysed include motility (total motility, progressive motility, total progressive motility), morphology (normal, head defects, loose heads, bent midpieces, midpiece defects, proximal droplets, distal droplets, bent tails, and coiled tails) HOS, acrosome reaction (intact, patchy, 75% reacted, reacted and equatorial segment), and concentration of spermatozoa. All of these semen parameters were looked at separately to investigate if there were any changes that may have been caused by the supplementary diets.

Blood mineral levels were also analysed to assess any possible correlations between blood mineral levels and the semen parameters.

As mentioned in the methods section four stallions were withdrawn from the study for reasons unrelated to the trial. Fourteen stallions completed this study (Table 2.1.). There were three animals in the control and mineral and vitamin E groups and two animals in the oil, ½ control, ½ mineral + vitamin E and ½ oil treatments groups. Three semen samples were collected before the stallions were fed the supplemented diet. After 5-8 weeks of feeding the supplemented diets three semen samples were collected again from each of the stallions except stallion 9 (two samples), and four post-treatment semen samples were provided from stallions 6, 8, 11, 12 and 14.

3.2 Motility of Spermatozoa

3.2.1 Total Motility of Spermatozoa

Total motility (TM) of spermatozoa was recorded at 6-8h, 24h, post-thaw and 30 min after thawing. The averages of the semen samples collected before and after treatment for each stallion at each assessment time are shown in Table 3.1. The differences between the pre and post-treatment means (post-treatment - pre-

treatment) are also included in Table 3.1 and have been graphed (Fig 3.1). A possible treatment effect was suggested by the increased total motility in stallion 5 post-thaw (15%) and post-thaw + 30 min (18%), and in stallion 14 6-8h (14%) and 24h after collection (16%). However upon statistical analysis of the treatment groups no significant differences were found in response to dietary supplementation (6-8h P=0.405, 24h P=0.111, post-thaw P=0.966, and post-thaw +30 min P=0.672).

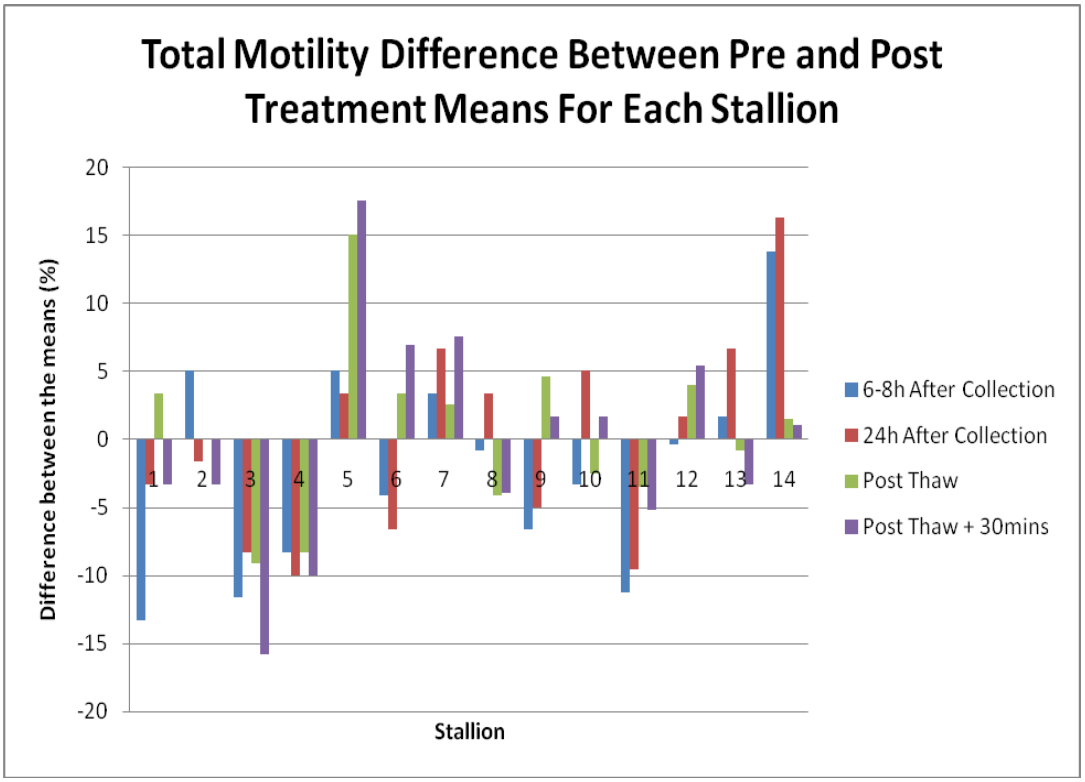


Figure 3. 1 The difference (post-treatment % - pre-treatment %) between the means of spermatozoa total motility at 6-8h, 24h, post-thaw and post-thaw +30 min before and after supplementation of individual stallions (1-14). Stallions 1, 2, 3 (Control); 4, 5, 6 (Minerals and vitamin E); 7, 8 (Oil); 9, 10 (1/2 Control); 11, 12 (1/2 Minerals and vitamin E); 13, 14 (1/2 Oil).

Table 3. 1 Total motility of spermatozoa 6-8h, 24h, post-thaw and post-thaw +30 min before and after supplementation of stallions (1-14) with minerals and vitamin E or oil compared with no supplementation (control). Stallions on the NRM diet received 1/2 the amount of supplement. The values (%) are the means ± s.d. of the semen samples collected before and after supplementation. Differences between the means = post treatment – per-treatment (%).

Treatment Group	Control			Minerals and vitamin E			Oil		1/2 Control		1/2 Minerals and vitamin E		1/2 Oil	
Stallion	1	2	3	4	5	6	7	8	9	10	11	12	13	14
6-8h After Collection														
Pre- Treatment Mean	70 ± 10	67 ± 12	82 ± 3	80 ± 10	65 ± 22	77 ± 12	64 ± 10	64 ± 6	77 ± 8	72 ± 3	85 ± 5	67 ± 6	60 ± 10	40 ± 10
Post-Treatment Mean	57 ± 15	72 ± 8	70 ± 10	72 ± 3	70 ± 10	73 ± 19	63 ± 6	63 ± 13	70 ± 0	68 ± 3	74 ± 11	66 ± 8	62 ± 13	54 ± 5
Difference Between Means	-13	5	-12	-8	5	-4	3	-1	-7	-4	-11	-1	2	14
24h After Collection														
Pre- Treatment Mean	58 ± 18	53 ± 6	63 ± 6	73 ± 12	52 ± 20	67 ± 6	50 ± 17	57 ± 6	70 ± 10	47 ± 6	78 ± 3	58 ± 18	47 ± 15	35 ± 5
Post-Treatment Mean	55 ± 18	52 ± 3	55 ± 5	63 ± 6	55 ± 5	60 ± 14	57 ± 3	60 ± 8	65 ± 7	52 ± 3	69 ± 13	60 ± 0	53 ± 6	51 ± 3
Difference Between Means	-3	-1	-8	-10	3	-7	7	3	-5	5	-9	2	6	16
Post-thaw														
Pre- Treatment Mean	28 ± 5	28 ± 3	43 ± 3	38 ± 6	25 ± 7	39 ± 11	35 ± 3	39 ± 3	39 ± 1	33 ± 10	44 ± 8	34 ± 8	29 ± 14	29 ± 8
Post-Treatment Mean	32 ± 3	28 ± 8	33 ± 10	30 ± 11	40 ± 5	43 ± 5	38 ± 7	35 ± 8	44 ± 9	31 ± 9	41 ± 3	38 ± 2	28 ± 6	31 ± 6
Difference Between Means	4	0	-10	-8	15	4	3	-4	5	-2	-3	4	-1	2
Post-thaw + 30mins														
Pre- Treatment Mean	27 ± 4	27 ± 4	48 ± 8	41 ± 1	23 ± 10	38 ± 7	31 ± 10	36 ± 7	38 ± 5	33 ± 6	48 ± 8	31 ± 11	33 ± 12	27 ± 5
Post-Treatment Mean	23 ±12	23 ± 15	33 ± 15	31 ± 1	41 ± 9	44 ± 4	38 ± 5	32 ± 4	40 ± 14	35 ± 14	43 ± 5	36 ± 5	29 ± 10	28 ± 10
Difference Between Means	-4	-4	-15	-10	18	6	7	-4	2	2	-5	5	-4	1

3.2.2 Progressive Motility of Spermatozoa

Progressive motility (PM) of spermatozoa was recorded at 6-8h, 24h, post-thaw and 30 min after thawing. The averages of the semen samples collected before and after treatment for each stallion at each assessment time are shown in Table 3.2. The differences between the pre and post-treatment means (post-treatment - pre-treatment) are also included in Table 3.2 and have been graphed (Fig 3.2). A possible treatment effect was suggested by the substantial increase in PM at 24hr in stallion 5 (27%), and a substantial decrease in PM at 6-8h shown in stallion 12 (39%). However, a substantial decrease is also suggested in stallion 9 (48%) in the ½ control group. However upon statistical analysis of the treatment groups no significant differences were found in response to dietary supplementation (6-8h P=0.942, 24h P=0.798, post-thaw P=0.950 and post-thaw + 30 min P=0.836).

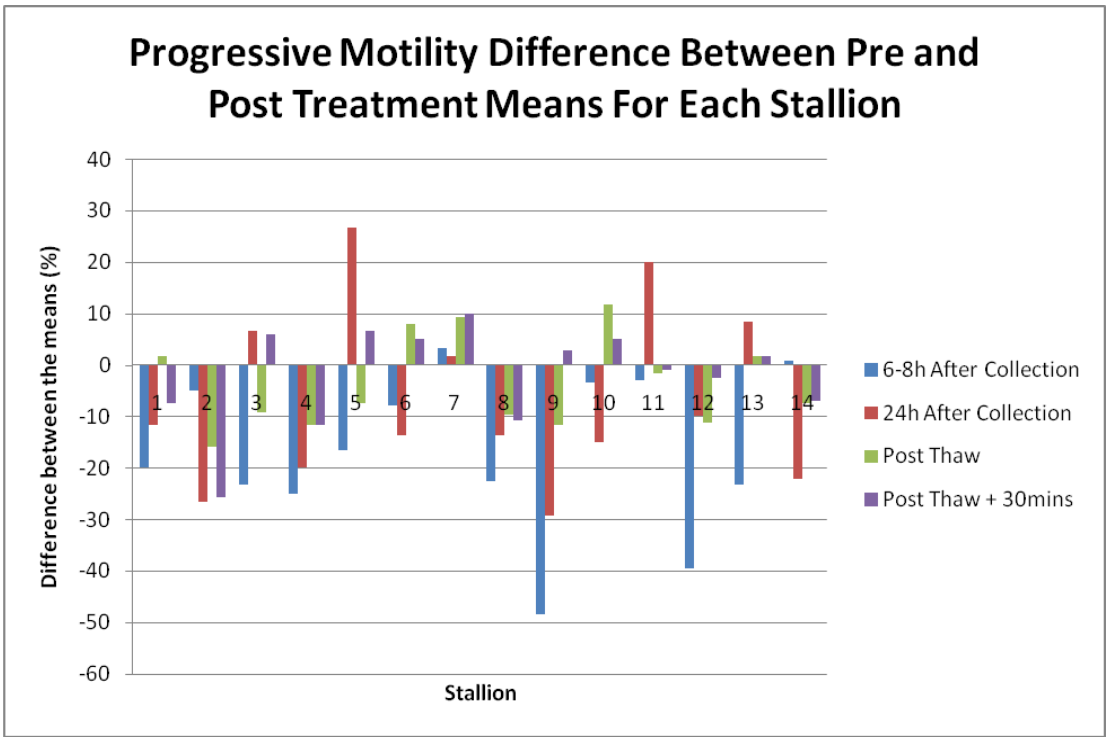


Figure 3. 2 The difference (post-treatment % - pre-treatment %) between the means of spermatozoa progressive motility at 6-8h, 24h, post-thaw and post-thaw +30 min before and after supplementation of individual stallions (1-14). Stallions 1, 2, 3 (Control); 4, 5, 6 (Minerals); 7, 8 (Oil); 9, 10 (1/2 Control); 11, 12 (1/2 Minerals and vitamin E); 13, 14 (1/2 Oil).

Table 3. 2 Progressive motility of spermatozoa 6-8h, 24h, post-thaw and post-thaw +30 min before and after supplementation of stallions (1-14) with minerals and vitamin E or oil compared with no supplementation (control). Stallions on the NRM diet received 1/2 the amount of supplement. The values (%) are the means ± s.d. of the semen samples collected before and after supplementation. Differences between the means = post-treatment – pre-treatment (%).

Treatment Group	Control			Minerals and vitamin E			Oil		1/2 Control		1/2 Minerals and vitamin E		1/2 Oil	
Stallion	1	2	3	4	5	6	7	8	9	10	11	12	13	14
6-8h After Collection														
Pre- Treatment Mean	50 ± 20	63 ± 21	50 ± 0	80 ± 6	87 ± 23	57 ± 23	23 ± 27	60 ± 27	88 ± 8	67 ± 23	77 ± 12	73 ± 12	73 ± 12	52 ± 33
Post-Treatment Mean	30 ± 10	58 ± 36	27 15	55 ± 13	70 ± 0	49 ± 16	27 ± 15	38 ± 10	40 ± 14	63 ± 18	74 ± 13	34 ± 17	50 ± 35	53 ± 17
Difference Between Means	-20	-5	-23	-25	-17	-8	4	-22	-48	-4	-3	-39	-23	1
24h After Collection														
Pre- Treatment Mean	37 ± 23	47 ± 23	18 ± 19	53 ± 35	33 ± 32	32 ± 24	10 ± 10	35 ± 33	47 ± 26	43 ± 6	40 ± 10	25 ± 23	35 ± 28	53 ± 29
Post-Treatment Mean	25 ± 23	20 ± 10	25 ± 5	33 ± 21	60 ± 0	18 ± 12	12 ± 8	21 ± 13	18 ± 18	28 ± 28	60 ± 14	15 ± 17	43 ± 29	31 ±23
Difference Between Means	-12	-27	7	-20	27	-14	2	-14	-29	-15	20	-10	8	-22
Post-thaw														
Pre- Treatment Mean	63 ± 5	72 ± 10	71 ± 8	72 ± 6	74 ± 5	68 ± 16	53 ± 18	73 ± 4	59 ± 4	69 ± 4	77 ± 3	65 ± 3	63 ± 9	55 ±9
Post-Treatment Mean	64 ± 4	56 ± 22	62 ± 10	60 ± 21	67 ± 1	76 ± 8	62 ± 3	63 ± 11	48 ± 4	81 ± 10	75 ± 6	54 ± 13	64 ± 10	48 12
Difference Between Means	1	-16	-9	-12	-7	8	9	-10	-11	12	-2	-11	1	-7
Post-thaw + 30mins														
Pre- Treatment Mean	62 ± 9	70 ± 10	58 ± 3	73 ± 9	61 ± 20	68 ± 15	53 ± 12	71 ± 1	48 ± 3	68 ± 10	74 ± 4	60 ± 5	68 ± 3	52 ± 20
Post-Treatment Mean	54 ± 13	44 ± 22	64 ± 25	61 ± 4	68 ± 7	73 ± 5	63 ± 1	60 ± 8	51 ± 2	73 ± 25	73 ± 8	58 ± 19	70 ± 10	45 ± 20
Difference Between Means	-8	-26	6	-12	7	5	10	-11	3	5	-1	-2	2	-7

3.2.3 Total Progressive Motility of Spermatozoa

Total progressive motility (TPM) of spermatozoa was recorded at 6-8h, 24h, post-thaw and 30 min after thawing. The averages of the semen samples collected before and after treatment for each stallion at each assessment time are shown in Table 3.3. The differences (%) between the pre and post-treatment (post-treatment – pre-treatment) means are also included in Table 3.3 and have been graphed (Fig 3.3). There was a possible treatment effect was suggested by the large decrease in TPM at 6-8h in stallions 4 (25%), and 12 (26%) and the large decrease at 25 h after collection observed in stallion 4 (21%). However there were also large decreases in TPM in the control groups stallions 3 (23%), 9(40%) at 6-8h and a large decrease in control group stallion 9 (20%).

Upon statistical analysis of the treatment groups no significant differences was found in response to dietary supplementation (6-8h P=0.745, 24h P=0.789, post-thaw P=0.941and post-thaw + 30 min P=0.750).

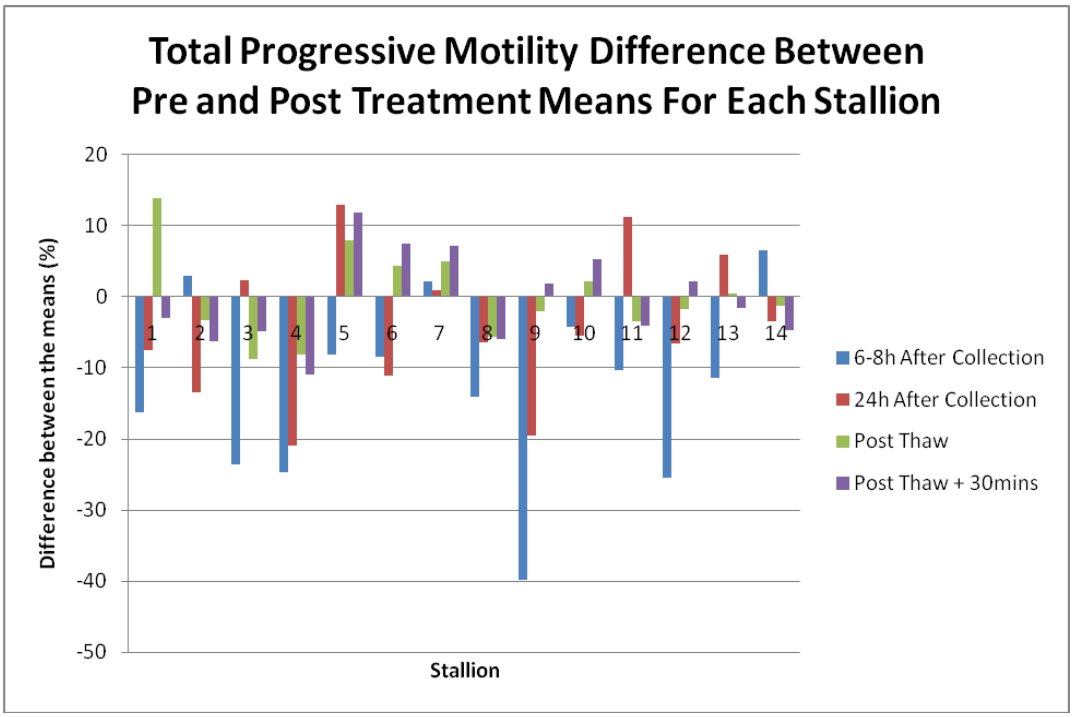


Figure 3. 3 The difference (post-treatment % - pre-treatment %) between the means of spermatozoa total progressive motility at 6-8h, 24h, post-thaw and post-thaw +30 min before and after supplementation of individual stallions (1-14). Stallions 1, 2, 3 (Control); 4, 5, 6 (Minerals and vitamin E); 7, 8 (Oil); 9, 10 (1/2 Control); 11, 12 (1/2 Minerals and vitamin E); 13, 14 (1/2 Oil).

Table 3. 3 Total progressive motility of spermatozoa 6-8h, 24h, post-thaw and post-thaw +30 min before and after supplementation of stallions (1-14) with minerals and vitamin E or oil compared with no supplementation (control). Stallions on the NRM diet received 1/2 the amount of supplement. The values (%) are the means ± s.d. of the semen samples collected before and after supplementation. Differences between the means = post-treatment – pre-treatment (%).

Treatment Group	Control			Minerals and vitamin E			Oil		1/2 Control		1/2 Minerals and vitamin E		1/2 Oil	
Stallion	1	2	3	4	5	6	7	8	9	10	11	12	13	14
6-8h After Collection														
Pre- Treatment Mean	34 ± 12	41 ± 8	41 ± 23	64 ± 8	57 ± 22	44 ± 21	15 ± 17	38 ± 18	68 ± 11	47 ± 15	65 ± 10	49 ± 7	45 ± 13	22 18
Post-Treatment Mean	18 ± 10	44 ± 30	18 ± 7	39 ± 9	49 ± 7	36 ± 17	17 ± 11	24 ± 11	28 ± 10	43 ± 11	55 ± 15	23 ± 13	33 ± 25	28 ± 9
Difference Between Means	-16	3	-23	-25	-8	-8	2	-14	-40	-4	-10	-26	-12	6
24h After Collection														
Pre- Treatment Mean	24 ± 18	24 ± 10	11 ± 11	41 ± 30	20 ± 25	22 ± 17	6 ± 6	20 ± 20	32 ± 17	20 ± 0	31 ± 8	16 ± 14	18 ± 15	20 ± 12
Post-Treatment Mean	16 ± 17	11 ± 6	14 ± 2	20 ± 12	33 ± 3	11 ± 7	7 ± 5	14 ± 10	12 ± 13	15 ± 14	43 ± 16	9 ± 10	24 ± 16	16 ± 11
Difference Between Means	-8	-13	3	-21	13	-11	1	-6	-20	-5	12	-7	6	-4
Post-thaw														
Pre- Treatment Mean	18 ± 4	20 ± 2	30 ± 3	28 ± 6	19 ± 6	28 ± 13	18 ± 6	28 ± 2	23 ± 1	23 ± 7	34 ± 7	22 ± 6	17 ± 6	16 ± 6
Post-Treatment Mean	32 ± 3	17 ± 11	21 ± 9	19 ± 12	27 ± 3	32 ± 2	23 ± 5	23 ± 9	21 ± 6	25 ± 9	31 ± 4	21 ± 6	18 ± 2	15 ± 6
Difference Between Means	14	-3	-9	-9	8	4	5	-6	-2	2	-3	-1	1	-1
Post-thaw + 30mins														
Pre- Treatment Mean	17 ± 5	19 ± 5	28 ± 5	30 ± 5	15 ± 12	25 ± 5	17 ± 8	25 ± 5	19 ± 4	22 ± 2	36 ± 7	19 ± 8	22 ± 7	18 ± 6
Post-Treatment Mean	14 ± 8	12 ± 12	23 ± 16	19 ± 1	27 ± 3	32 ± 4	24 ± 4	19 ± 4	20 ± 7	28 ± 17	32 ± 7	21 ± 7	20 ± 7	14 ± 8
Difference Between Means	-3	-7	-5	-11	12	7	7	-6	1	6	-4	2	-2	-4

3.3 Morphology of Spermatozoa

Sperm morphology (the percentage of normal, head defects, loose heads, bent midpiece, midpiece defects, proximal droplet, distal droplet, bent tail and coiled tail spermatozoa) was recorded for samples taken at 6-8h, 24h and post-thaw. The average percentage of morphologically normal spermatozoa in the semen samples collected before and after supplement treatment for each stallion at each assessment time are shown in Table 3.4. The differences between the pre and post-treatment means (post-treatment – pre-treatment) are also included in Table 3.4 and have been graphed (Fig 3.4). A general increase in the percentage of morphologically normal spermatozoa is indicated at 24h after collection; more so stallion 11 (33%). Statistical analysis found there was an overall significance at 24h (P=0.048). A Dunnett's test was performed to identify if either of the supplementary treatments differed from the control group. The Dunnett's test showed that the significant difference was between the two treatment groups and not between either of the treatment groups and their control, therefore this is probably a "random" event. No significant differences were found in response to diet supplementation at 6-8h (P= 0.366) or post-thaw 0 mins (P=0.974).

Table 3. 4 Percentage of morphologically normal spermatozoa at 6-8h, 24h, and post-thaw before and after supplementation of stallions (1-14) with minerals and vitamin E or oil compared with no supplementation (control). Stallions on the NRM diet received 1/2 the amount of supplement. The values are the means ± s.d. of the semen samples collected before and after supplementation. Differences between the means = post-treatment – pre-treatment (%).

Treatment Group	Control			Minerals and vitamin E			Oil		1/2 Control		1/2 Minerals and vitamin E		1/2 Oil	
Stallion	1	2	3	4	5	6	7	8	9	10	11	12	13	14
6-8h After Collection														
Pre- Treatment Mean	18 ± 2	25 ± 8	24 ± 5	20 ± 5	13 ± 5	26 ± 11	13 ± 8	18 ± 5	21 ± 4	18 ± 2	16 ± 1	11 ± 5	18 ± 7	17 ± 12
Post-Treatment Mean	16 ± 10	11 ± 3	12 ± 2	15 ± 1	16 ± 3	19 ± 7	11 ± 6	13 ± 7	11 ± 4	8 ± 3	15 ± 8	8 ± 7	8 ± 5	14 ± 10
Difference Between Means	-2	-14	-12	-5	3	-7	-2	-5	-10	-10	-1	-3	-10	-3
24h After Collection														
Pre- Treatment Mean	19 ± 6	24 ± 6	23 ± 4	21 ± 8	27 ± 1	38 ± 3	14 ± 6	17 ± 6	15 ± 7	16 ± 7	12 ± 5	9 ± 1	8 ± 3	17 ± 6
Post-Treatment Mean	42 ± 14	37 ± 19	39 ± 17	31 ± 7	37 ± 14	41 ± 18	26 ± 8	28 ± 9	28 ± 4	29 ± 8	45 ± 8	26 ± 10	29 ± 6	39 ± 7
Difference Between Means	23	13	16	10	10	3	12	11	13	13	33	17	21	22
Post-thaw														
Pre- Treatment Mean	45 ± 5	40 ± 7	42 ± 7	49 ± 11	32 ± 13	42 ± 19	25 ± 3	36 ± 11	36 ± 6	35 ± 4	41 ± 4	23 ± 12	36 ± 6	16 ± 9
Post-Treatment Mean	50 ± 6	41 ± 8	46 ± 6	41 ± 4	38 ± 14	48 ± 12	28 ± 9	37 ± 11	34 ± 11	39 ± 5	45 ± 9	28 ± 8	32 ± 6	30 ± 8
Difference Between Means	5	1	4	-8	6	6	3	1	-2	4	4	5	-4	14

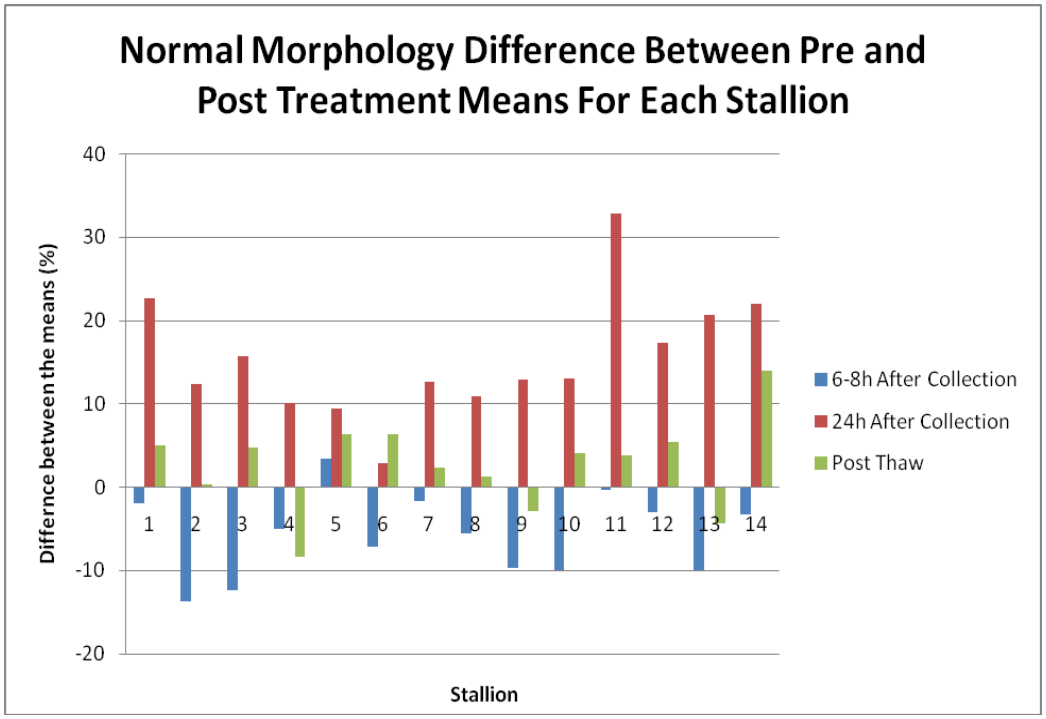


Figure 3. 4 The difference (post-treament % - pre-treatment %) between the means of morphologically normal spermatozoa at 6-8h, 24h, and post-thaw before and after supplementation of individual stallions (1-14). Stallions 1, 2, 3 (Control); 4, 5, 6 (Minerals and vitamin E); 7, 8 (Oil); 9, 10 (1/2 Control); 11, 12 (1/2 Minerals and vitamin E); 13, 14 (1/2 Oil).

The percentage of spermatozoa with morphological head defects was recorded and the averages of the semen samples collected before and after treatment for each stallion at each assessment time are shown in Table 3.5. The differences between the pre and post-treatment means (post-treatment – pre-treatment) are also included in Table 3.5 and are graphed (Fig 3.5). A possible treatment effect was suggested by the small increase in the percentage of head defects at 6-8h for stallions 5 (15%), 8 (13%), 12 (15%) and 13 (12%). However small increases were also observed in the control groups stallions 3 (15%), 9 (13%), and 10 (12%). Stallion 14 shows a small increase in head defects across the board; 6-8h (15%), 24h (9%) and post-thaw (11%). Small decreases in head defects were observed in stallions 1 (11%) and 13(10%) at 24h and post-thaw in stallion 1 (11%).

Table 3. 5 Percentage of head defects in spermatozoa morphology at 6-8h, 24h, and post-thaw before and after supplementation of stallions (1-14) with minerals and vitamin E or oil compared with no supplementation (control). Stallions on the NRM diet received 1/2 the amount of supplement. The values are the means ± s.d. of the semen samples collected before and after supplementation. Differences between the means = post-treatment – pre-treatment (%).

Treatment Group	Control			Minerals and vitamin E			Oil		1/2 Control		1/2 Minerals and vitamin E		1/2 Oil	
Stallion	1	2	3	4	5	6	7	8	9	10	11	12	13	14
6-8h After Collection														
Pre- Treatment Mean	24 ± 3	11 ± 2	7 ± 4	12 ± 4	4 ± 1	7 ± 3	21 ± 4	13 ± 3	9 ± 6	10 ± 6	5 ± 3	13 ± 4	14 ± 3	11 ± 4
Post-Treatment Mean	24 ± 5	16 ± 12	22 ± 8	12 ± 5	19 ± 17	16 ± 11	19 ± 14	26 ± 15	23 ± 2	22 ± 9	11 ± 6	28 ± 10	26 ± 6	26 ± 6
Difference Between Means	0	5	15	0	15	9	-2	13	14	12	6	15	12	15
24h After Collection														
Pre- Treatment Mean	26 ± 6	6 ± 2	6 ± 1	13 ± 7	7 ± 3	4 ± 2	22 ± 6	17 ± 6	16 ± 4	12 ± 3	9 ± 4	18 ± 3	28 ± 10	16 ± 3
Post-Treatment Mean	15 ± 4	9 ± 6	6 ± 3	12 ± 1	12 ± 10	10 ± 5	20 ± 1	16 ± 7	13 ± 1	8 ± 4	9 ± 2	16 ± 2	18 ± 4	25 ± 7
Difference Between Means	-11	3	0	-1	5	6	-2	-1	-3	-4	0	-2	-10	9
Post-thaw														
Pre- Treatment Mean	22 ± 6	10 ± 8	4 ± 1	12 ± 8	12 ± 5	10 ± 6	12 ± 7	11 ± 1	14 ± 2	13 ± 6	12 ± 2	19 ± 6	14 ± 0	8 ± 2
Post-Treatment Mean	11 ± 2	7 ± 6	10 ± 5	9 ± 2	9 ± 1	6 ± 1	21 ± 4	9 ± 1	13 ± 7	9 ± 1	6 ± 3	14 ± 5	16 ± 6	19 ± 6
Difference Between Means	-11	-3	6	-3	-3	-4	9	-2	-1	-4	-6	-5	2	11

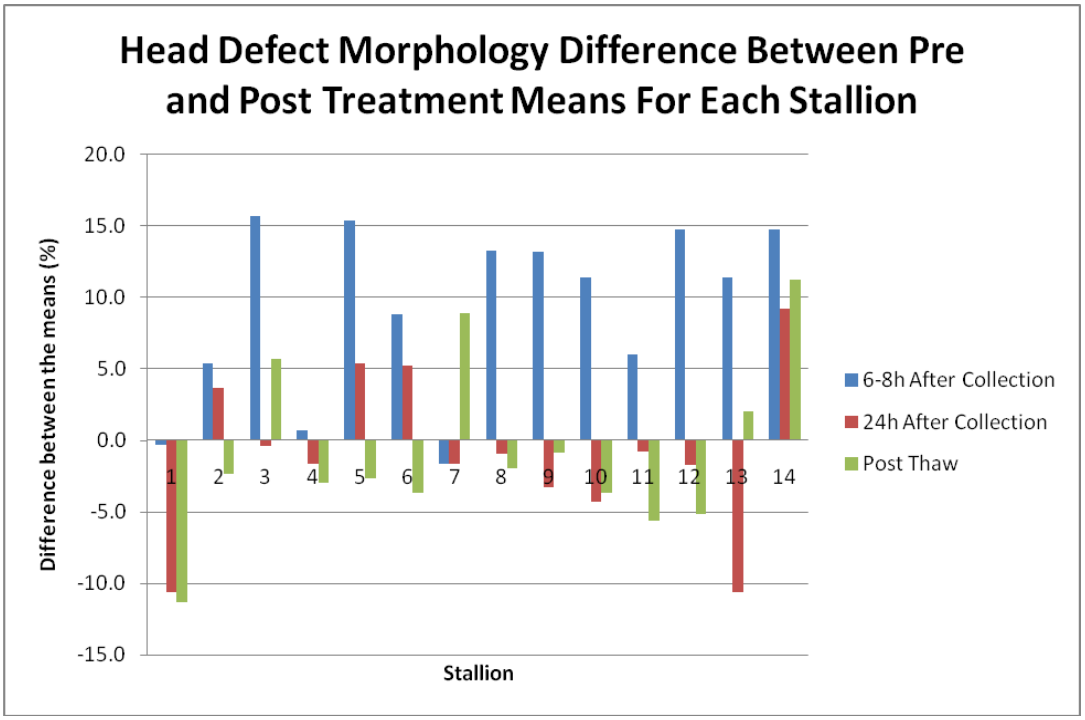


Figure 3. 5 The difference (post-treatment % - pre-treatment %) between the means of head defects in spermatozoa morphology at 6-8h, 24h, and post-thaw before and after supplementation of individual stallions (1-14). Stallions 1, 2, 3 (Control); 4, 5, 6 (Minerals and vitamin E); 7, 8 (Oil); 9, 10 (1/2 Control); 11, 12 (1/2 Minerals and vitamin E); 13, 14 (1/2 Oil).

The percentage of loose sperm head defects were recorded and the averages of the semen samples collected before and after treatment for each stallion at each assessment time are shown in Table 3.6. The differences between the means are also included in table 3.6 and are graphed (Fig 3.6). A possible treatment effect was suggested by a small decrease (15%) in the percentage of loose heads at 6-8h and a very large decrease (59%) in loose heads post-thaw in stallion 14.

Table 3. 6 Percentage of loose head defects in spermatozoa morphology at 6-8h, 24h, and post-thaw before and after supplementation of stallions (1-14) with minerals and vitamin E or oil compared with no supplementation (control). Stallions on the NRM diet received 1/2 the amount of supplement. The values are the means ± s.d. of the semen samples collected before and after supplementation. Differences between the means = post-treatment – pre-treatment (%).

Treatment Group	Control			Minerals and vitamin E			Oil		1/2 Control		1/2 Minerals and vitamin E		1/2 Oil	
Stallion	1	2	3	4	5	6	7	8	9	10	11	12	13	14
6-8h After Collection														
Pre- Treatment Mean	2 ± 1	4 ± 1	2 ± 8	2 ± 2	3 ± 4	1 ± 8	3 ± 5	3 ± 7	9 ± 4	2 ± 4	3 ± 4	3 ± 3	4 ± 4	20 ± 3
Post-Treatment Mean	1 ± 4	1 ± 6	1 ± 11	1 ± 1	5 ± 7	1 ± 6	10 ± 3	1 ± 7	6 ± 6	1 ± 8	1 ± 2	5 ± 9	4 ± 4	5 ± 5
Difference Between Means	-1	-2	-1	-1	2	0	7	-2	-3	-1	-2	2	0	-15
24h After Collection														
Pre- Treatment Mean	1 ± 3	4 ± 6	6 ± 4	1 ± 2	0 ± 12	2 ± 6	1 ± 2	3 ± 9	2 ± 7	6 ± 2	3 ± 9	2 ± 7	3 ± 4	8 ± 5
Post-Treatment Mean	1 ± 5	0 ± 3	1 ± 6	1 ± 8	1 ± 4	2 ± 12	2 ± 1	1 ± 6	0 ± 6	1 ± 6	2 ± 1	2 ± 7	1 ±2	3 ±1
Difference Between Means	0	-4	-5	0	-1	0	1	-2	-2	-5	-1	0	-2	-5
Post-thaw														
Pre- Treatment Mean	4 ± 1	4 ± 1	3 ± 9	4 ± 3	1 ± 8	2 ± 4	7 ± 2	4 ± 6	4 ± 3	2 ±3	2 ± 2	4 ± 7	3 ± 4	54 ± 6
Post-Treatment Mean	3 ± 1	4 ± 4	3 ± 1	2 ± 4	7 ± 15	3 ± 2	5 ± 2	3 ± 2	3 ± 2	2 ± 3	3 ± 3	11 ± 4	2 ± 2	9 ± 2
Difference Between Means	-1	0	0	-2	6	1	-2	-1	-1	0	1	7	-1	-45

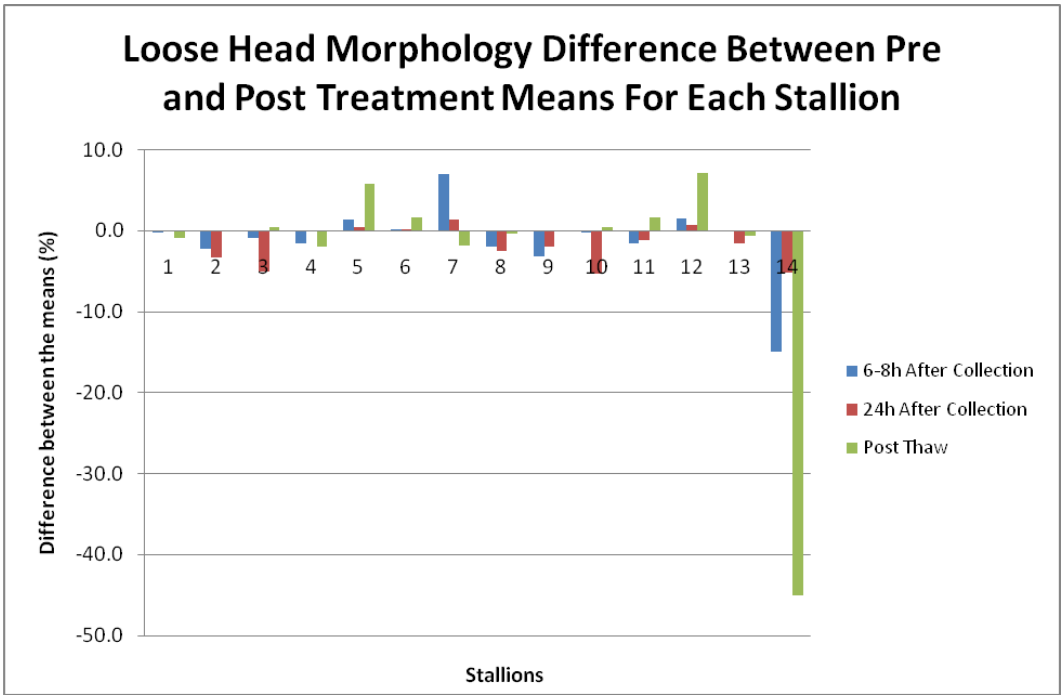


Figure 3. 6 The difference (post-treatment % - pre-treatment %) between the means of loose head defects in spermatozoa morphology at 6-8h, 24h, and post-thaw before and after supplementation of individual stallions (1-14). Stallions 1, 2, 3 (Control); 4, 5, 6 (Minerals and vitamin E); 7, 8 (Oil); 9, 10 (1/2 Control); 11, 12 (1/2 Minerals and vitamin E); 13, 14 (1/2 Oil).

The percentage of sperm bent midpiece defects was recorded and the averages of the semen samples collected before and after treatment for each stallion at each assessment time are shown in Table 3.7 The differences between the means (post-treatment- pre-treatment) are also shown in Table 3.7 and are graphed (Fig 3.7). A possible treatment effect was suggested by the small increase in the percentage if bent pieces at 6-8h after collection in stallion 4 (13%). There is also a small decrease in bent midpiece defects at 6-8h in stallion 8 (10%) and small decreases at 24h in stallions 8 (13%) and 11 (10%).

Table 3. 7 Percentage of bent midpiece defects in spermatozoa morphology at 6-8h, 24h, and post-thaw before and after supplementation of stallions (1-14) with minerals and vitamin E or oil compared with no supplementation (control). Stallions on the NRM diet received 1/2 the amount of supplement. The values are the means ± s.d. of the semen samples collected before and after supplementation. Differences between the means = post-treatment – pre-treatment (%).

Treatment Group	Control			Minerals and vitamin E			Oil		1/2 Control		1/2 Minerals and vitamin E		1/2 Oil	
Stallion	1	2	3	4	5	6	7	8	9	10	11	12	13	14
6-8h After Collection														
Pre- Treatment Mean	6 ± 5	11 ± 1	9 ± 8	5 ± 2	19 ± 4	6 ± 8	7 ± 5	23 ± 7	15 ± 4	12 ± 4	10 ± 4	15 ± 3	8 ± 4	11 ± 3
Post-Treatment Mean	9 ± 4	15 ± 6	11 ± 11	18 ± 1	14 ± 7	8 ± 6	9 ± 3	13 ± 7	16 ± 6	13 ± 8	13 ± 2	19 ± 9	14 ± 3	13 ±5
Difference Between Means	3	4	2	13	-5	2	2	-10	1	1	3	4	6	2
24h After Collection														
Pre- Treatment Mean	8 ± 3	13 ± 6	11 ± 4	10 ± 2	13 ± 12	7 ± 6	10 ± 2	24 ± 9	16 ± 7	14 ± 2	17 ± 9	17 ± 7	12 ± 4	11 ±5
Post-Treatment Mean	9 ± 5	10 ± 3	9 ± 6	11 ± 8	16 ± 4	13 ± 12	6 ± 1	12 ± 6	9 ± 6	8 ± 6	7 ± 1	11 ± 7	9 ± 2	5 ± 1
Difference Between Means	1	-3	-2	1	3	6	-4	-13	-7	-6	-10	-6	-3	-6
Post-thaw														
Pre- Treatment Mean	4 ±1	7 ± 1	7 ± 9	6 ± 3	14 ± 8	4 ± 4	7 ± 2	13 ± 6	11 ± 3	7 ± 3	4 ± 3	15 ± 2	8 ± 4	8 ± 6
Post-Treatment Mean	4 ± 1	5 ± 4	1 ± 1	6 ± 4	12 ± 15	3 ± 2	6 ± 2	7 ± 2	7 ± 2	8 ± 3	5 ± 3	10 ± 4	6 ± 2	5 ± 2
Difference Between Means	0	-2	-6	0	-2	-1	-1	-6	-4	1	1	-5	-2	-3

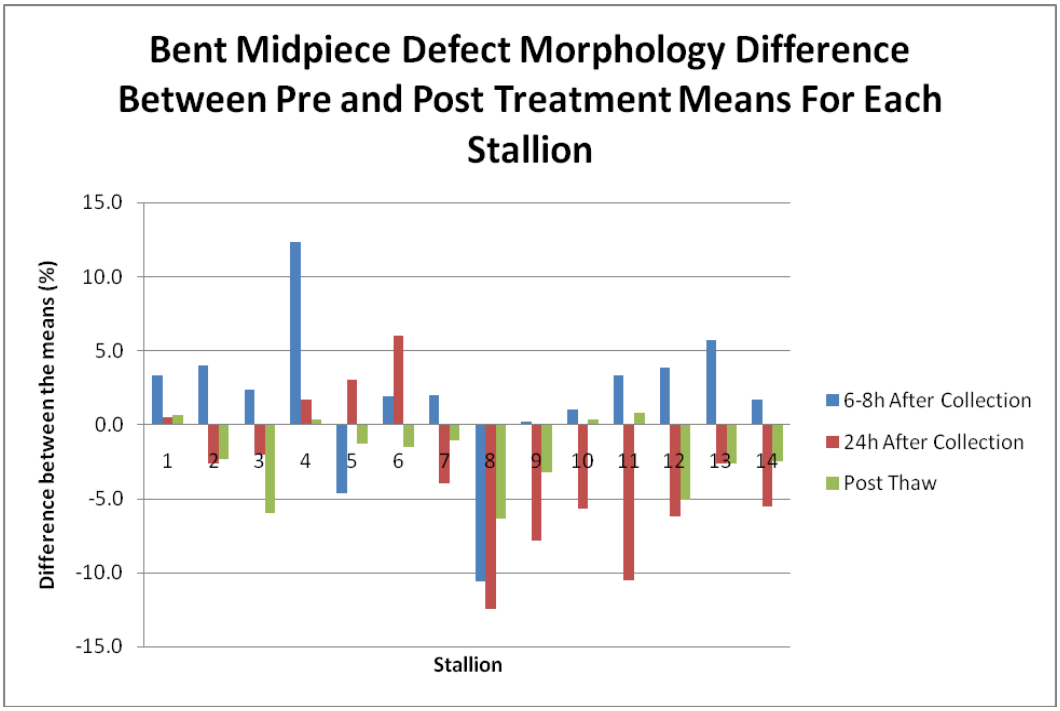


Figure 3. 7 The difference (post-treatment % - pre-treatment %) between the means of bent midpiece defects in spermatozoa morphology at 6-8h, 24h, 6-8h, 24h, and post-thaw before and after supplementation of individual stallions (1-14). Stallions 1, 2, 3 (Control); 4, 5, 6 (Minerals and vitamin E); 7, 8 (Oil); 9, 10 (1/2 Control); 11, 12 (1/2 Minerals and vitamin E); 13, 14 (1/2 Oil).

The percentage of sperm midpiece defects was recorded and the averages of the semen samples collected before and after treatment for each stallion at each assessment time are shown in Table 3.8. The differences between the pre and post-treatment means (post-treatment – pre-treatment) are also included in Table 3.8 and are graphed (Fig 3.8). A possible treatment effect was suggested by the small increase in the percentage of midpiece defects at 6-8h in stallion 4 (12%). However, midpiece defects also increased in control stallion 9 (14%) at 6-8h after collection and at 24h (11%). There is also a small decrease in midpiece defects at 6-8h in stallion 7 (10%).

Table 3. 8 Percentage of midpiece defect defects in spermatozoa morphology at 6-8h, 24h, and post-thaw before and after supplementation of stallions (1-14) with minerals and vitamin E or oil compared with no supplementation (control). Stallions on the NRM diet received 1/2 the amount of supplement. The values are the means ± s.d. of the semen samples collected before and after supplementation. Differences between the means = post-treatment – pre-treatment (%).

Treatment Group	Control			Minerals and vitamin E			Oil		1/2 Control		1/2 Minerals and vitamin E		1/2 Oil	
Stallion	1	2	3	4	5	6	7	8	9	10	11	12	13	14
6-8h After Collection														
Pre- Treatment Mean	6 ± 1	10 ± 3	11 ± 9	11 ± 1	16 ± 8	11 ± 2	23 ± 2	9 ± 2	10 ± 7	20 ± 2	16 ± 4	14 ± 4	16 ± 3	11 ± 2
Post-Treatment Mean	6 ± 1	13 ± 0	13 ± 1	23 ± 6	13 ± 2	9 ± 2	13 ± 1	13 ± 1	24 ± 1	13 ± 6	25 ± 3	12 ± 3	15 ± 2	11 ± 1
Difference Between Means	0	3	2	12	-3	-2	-10	4	14	-7	9	-2	-1	0
24h After Collection														
Pre- Treatment Mean	6 ± 3	13 ± 8	8 ± 2	11 ± 1	10 ± 14	13 ± 2	19 ± 14	8 ± 3	18 ± 5	15 ± 11	21 ± 6	21 ± 8	16 ± 5	12 ± 9
Post-Treatment Mean	4 ± 1	13 ± 4	8 ± 3	14 ± 3	7 ± 8	10 ± 1	13 ± 3	11 ± 1	29 ± 5	7 ± 12	15 ± 3	15 ± 9	9 ± 4	8 ± 3
Difference Between Means	-2	0	0	3	-3	-3	-6	3	11	-8	-6	-6	-7	-4
Post-thaw														
Pre- Treatment Mean	4 ± 4	8 ± 8	4 ± 5	5 ± 3	4 ± 6	4 ± 3	9 ± 2	4 ± 1	8 ± 6	6 ± 8	8 ± 6	6 ± 8	8 ± 2	2 ± 2
Post-Treatment Mean	6 ± 2	9 ± 5	6 ± 2	10 ± 2	7 ± 4	5 ± 1	16 ± 3	9 ± 1	6 ± 1	6 ± 1	12 ± 1	10 ± 5	6 ± 2	7 ± 1
Difference Between Means	2	1	2	5	3	1	7	5	-2	0	4	4	-2	5

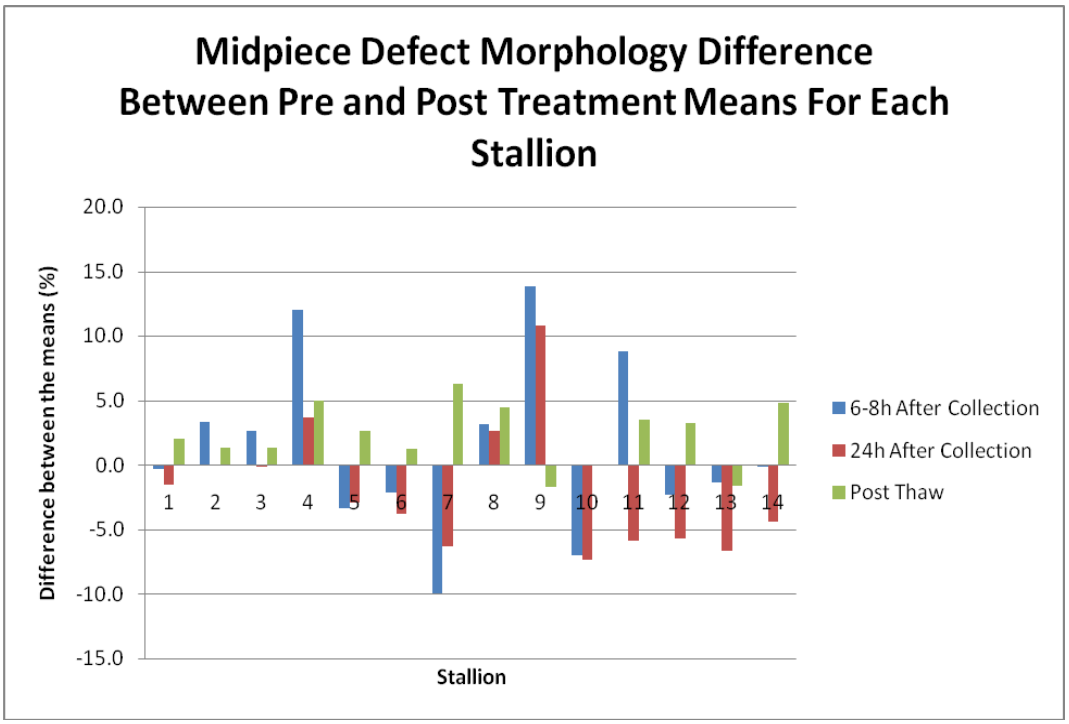


Figure 3. 8 The difference (post-treatment % - pre-treatment %) between the means of midpiece defects in spermatozoa morphology at 6-8h, 24h, and post-thaw before and after supplementation of individual stallions (1-14). Stallions 1, 2, 3 (Control); 4, 5, 6 (Minerals and vitamin E); 7, 8 (Oil); 9, 10 (1/2 Control); 11, 12 (1/2 Minerals and vitamin E); 13, 14 (1/2 Oil).

The percentage of sperm proximal droplet defects was recorded and the averages of the semen samples collected before and after treatment for each stallion at each assessment time are shown in Table 3.9. The differences between the pre and post-treatment means (post-treatment – pre-treatment) are also shown in Table 3.9 and are graphed (Fig 3.9). The results suggest a general small decrease over all stallions. Stallions 5 (15%) and 7 (11%) which belong to different treatment groups indicate larger decreases in the percentage of proximal droplets at 24h after collection and stallion 5 (11%) indicates a large decrease post-thaw.

Table 3. 9 Percentage of proximal droplet defects in spermatozoa morphology at 6-8h, 24h, and post-thaw before and after supplementation of stallions (1-14) with minerals and vitamin E or oil compared with no supplementation (control). Stallions on the NRM diet received 1/2 the amount of supplement. The values are the means ± s.d. of the semen samples collected before and after supplementation. Differences between the means = post-treatment – pre-treatment (%).

Treatment Group	Control			Minerals and vitamin E			Oil		1/2 Control		1/2 Minerals and vitamin E		1/2 Oil	
Stallion	1	2	3	4	5	6	7	8	9	10	11	12	13	14
6-8h After Collection														
Pre- Treatment Mean	5 ± 2	7 ± 3	2 ± 2	4 ± 4	2 ± 2	2 ± 2	5 ± 2	1 ± 2	6 ± 7	8 ± 2	13 ± 4	10 ± 4	11 ± 3	7 ± 2
Post-Treatment Mean	1 ± 1	1 ± 0	1 ± 1	5 ± 6	4 ± 2	3 ± 2	1 ± 1	1 ± 1	2 ± 1	5 ± 6	5 ± 3	3 ± 3	4 ± 2	2 ± 1
Difference Between Means	-4	-6	-1	1	2	1	-4	0	-4	-3	-8	-7	-7	-5
24h After Collection														
Pre- Treatment Mean	6 ± 3	12 ± 8	3 ± 2	8 ± 1	21 ± 14	2 ± 2	14 ± 14	3 ± 3	10 ± 5	14 ± 11	10 ± 6	9 ± 8	8 ± 5	10 ± 9
Post-Treatment Mean	2 ± 1	5 ± 4	4 ± 3	4 ± 3	6 ± 8	2 ± 1	3 ± 3	2 ± 1	13 ± 5	12 ± 12	10 ± 3	9 ± 9	9 ± 4	4 ± 3
Difference Between Means	-4	-7	1	-4	-15	0	-11	-1	3	-2	0	0	1	-6
Post-thaw														
Pre- Treatment Mean	4 ± 4	10 ± 8	8 ± 5	8 ± 3	15 ± 6	6 ± 3	10 ± 2	4 ± 1	10 ± 6	16 ± 8	12 ± 6	11 ± 8	10 ± 2	5 ± 2
Post-Treatment Mean	1 ± 2	8 ± 5	6 ± 2	4 ± 2	4 ± 4	5 ± 1	6 ± 3	2 ± 1	11 ± 1	8 ± 1	9 ± 1	7 ± 5	3 ± 2	6 ± 1
Difference Between Means	-3	-2	-2	-3	-11	-1	-4	-2	1	-8	-3	-4	-7	1

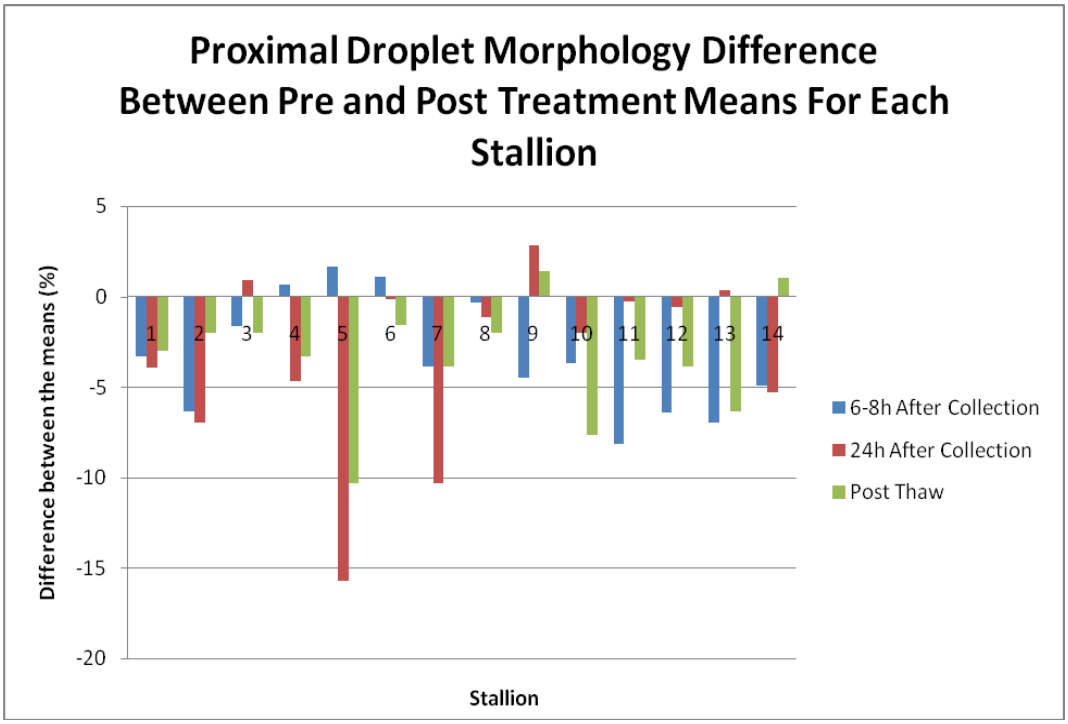


Figure 3. 9 The difference (post-treatment % - pre-treatment %) between the means of proximal droplet defects in spermatozoa morphology at 6-8h, 24h, and post-thaw before and after supplementation of individual stallions (1-14). Stallions 1, 2, 3 (Control); 4, 5, 6 (Minerals and vitamin E); 7, 8 (Oil); 9, 10 (1/2 Control); 11, 12 (1/2 Minerals and vitamin E); 13, 14 (1/2 Oil).

The percentage of sperm distal droplet defects was recorded and the averages of the semen samples collected before and after treatment for each stallion at each assessment time are shown in Table 3.10. The differences between the pre and post-treatment means (post-treatment – pre-treatment) are also included in Table 3.10 and are graphed (Fig 3.10). There are very small changes in the percentages of distal droplets defect spermatozoa. The largest change is the 5% decrease in stallions 5 and 13 post-thaw.

Table 3. 10 Percentage of distal droplet defects in spermatozoa morphology at 6-8h, 24h, and post-thaw before and after supplementation of stallions (1-14) with minerals and vitamin E or oil compared with no supplementation (control). Stallions on the NRM diet received 1/2 the amount of supplement. The values are the means ± s.d. of the semen samples collected before and after supplementation. Differences between the means = post-treatment – pre-treatment (%).

Treatment Group	Control			Minerals and vitamin E			Oil		1/2 Control		1/2 Minerals and vitamin E		1/2 Oil	
Stallion	1	2	3	4	5	6	7	8	9	10	11	12	13	14
6-8h After Collection														
Pre- Treatment Mean	2 ± 2	4 ± 2	4 ± 6	2 ± 11	1 ± 15	2 ± 4	2 ± 2	1 ± 5	1 ± 9	6 ± 9	2 ± 4	2 ± 7	2 ± 8	2 ± 9
Post-Treatment Mean	1 ± 9	2 ± 12	0 ± 12	2 ± 3	1 ± 21	1 ± 14	0 ± 5	1 ± 5	0 ± 1	7 ± 12	0 ± 11	1 ± 10	1 ± 3	1 ± 6
Difference Between Means	-1	-2	-4	0	0	-1	-2	0	-1	1	-2	-1	-1	-1
24h After Collection														
Pre- Treatment Mean	3 ± 8	4 ± 3	2 ± 6	3 ± 10	1 ± 7	4 ± 7	2 ± 8	1 ± 7	2 ± 9	5 ± 6	2 ± 11	1 ± 4	1 ± 6	2 ± 6
Post-Treatment Mean	3 ± 6	0 ± 18	1 ± 14	0 ± 6	1 ± 12	0 ± 11	0 ± 10	1 ± 10	3 ± 2	5 ± 7	2 ± 6	1 ± 13	4 ± 3	3 ± 4
Difference Between Means	0	-4	-1	-3	0	-4	-2	0	1	0	0	0	3	1
Post-thaw														
Pre- Treatment Mean	2 ± 7	3 ± 3	3 ± 7	2 ± 2	6 ± 3	7 ± 12	3 ± 6	4 ± 4	3 ± 7	3 ± 4	4 ± 5	2 ± 3	6 ± 5	1 ± 5
Post-Treatment Mean	3 ± 1	3 ± 8	4 ± 5	4 ± 9	1 ± 7	5 ± 14	1 ± 11	3 ± 11	2 ± 18	5 ± 3	2 ± 4	3 ± 3	1 ± 4	4 ± 3
Difference Between Means	1	0	1	2	-5	-2	-2	-1	-1	2	-2	1	-5	3

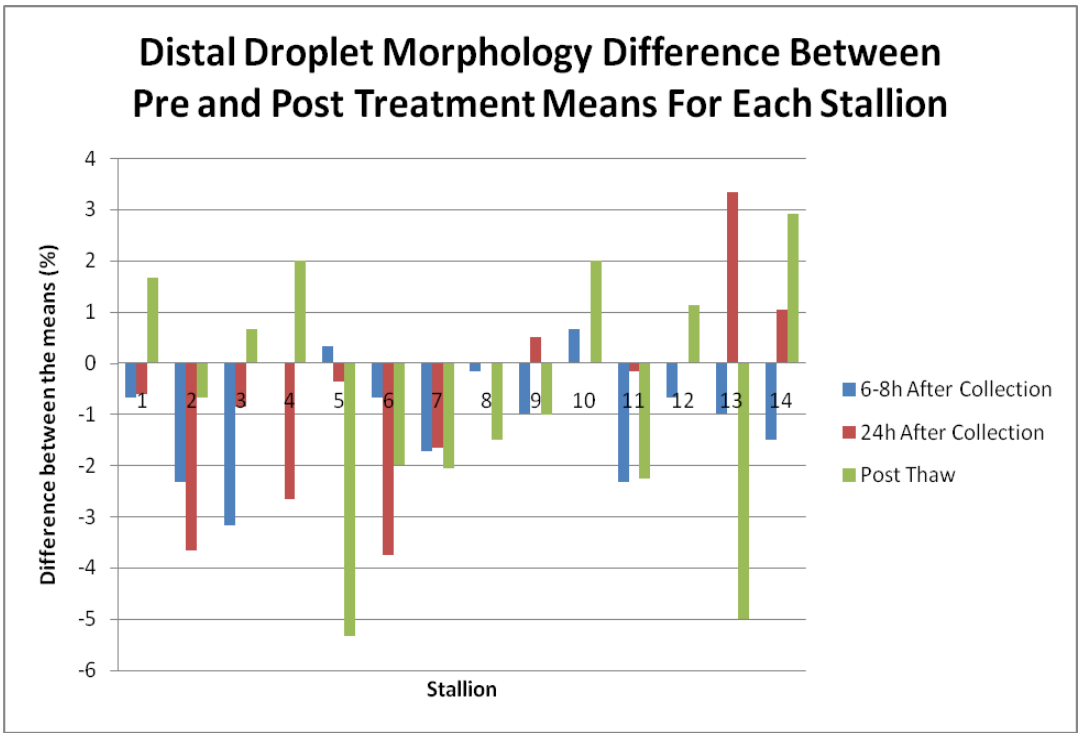


Figure 3. 10 The difference (post-treatment % - pre-treatment %) between the means of distal droplet defects in spermatozoa morphology at 6-8h, 24h, and post-thaw before and after supplementation of individual stallions (1-14). Stallions 1, 2, 3 (Control); 4, 5, 6 (Minerals and vitamin E); 7, 8 (Oil); 9, 10 (1/2 Control); 11, 12 (1/2 Minerals and vitamin E); 13, 14 (1/2 Oil).

The percentage of sperm bent tail defect were recorded and the averages of the semen samples collected before and after treatment for each stallion at each assessment time are shown in Table 3.11. The differences between the pre and post-treatment means (post – pre) are also included in Table 3.11 and are graphed (Fig 3.11). A possible treatment effect is indicated in the substantial increase in the percentage of bent tails post-thaw in stallion 13 (18%). There are also small increases in the percentage of bent tails at 24h in stallion 7 (11%), post-thaw in stallions 4 (9%), and 14 (11%). However there are also small increases at 6-8h in control groups stallions 2 (11%) and at 24h in stallion 10 (12%), and post-thaw in stallion 9 (10%).

There was also a range of decreases in the bent tails shown. Stallions 4 (19%) and 5 (14%) show large decreases in bent tails at 6-8h, and at 24h a large decrease is indicated in stallions 11 (15%) 14 (13%), and control groups stallion 9 (13%).

Table 3. 11 Percentage of bent tail defects in spermatozoa morphology at 6-8h, 24h, and post-thaw before and after supplementation of stallions (1-14) with minerals and vitamin E or oil compared with no supplementation (control). Stallions on the NRM diet received 1/2 the amount of supplement. The values are the means ± s.d. of the semen samples collected before and after supplementation. Differences between the means = post-treatment – pre-treatment (%).

Treatment Group	Control			Minerals and vitamin E			Oil		1/2 Control		1/2 Minerals and vitamin E		1/2 Oil	
Stallion	1	2	3	4	5	6	7	8	9	10	11	12	13	14
6-8h After Collection														
Pre- Treatment Mean	34 ± 8	28 ± 6	43 ± 6	42 ± 11	41 ± 15	46 ± 4	25 ± 2	30 ± 4	26 ± 9	23 ± 9	34 ± 4	28 ± 7	25 ± 8	20 ± 9
Post-Treatment Mean	40 ± 9	39 ± 12	38 ± 12	23 ± 3	27 ± 21	43 ± 14	29 ± 5	32 ± 5	20 ± 1	31 ± 12	30 ± 11	23 ± 10	26 ± 3	28 ± 6
Difference Between Means	6	11	-5	-19	-14	-3	4	2	-6	8	-4	-5	1	8
24h After Collection														
Pre- Treatment Mean	29 ± 8	24 ± 3	40 ± 6	32 ± 10	19 ± 7	29 ± 7	18 ± 8	27 ± 7	19 ± 9	16 ± 6	26 ± 11	20 ± 4	24 ± 6	24 ± 6
Post-Treatment Mean	23 ± 6	25 ± 18	32 ± 14	26 ± 6	20 ± 12	23 ± 11	29 ± 10	30 ± 10	6 ± 2	28 ± 7	11 ± 6	18 ± 13	22 ± 3	11 ± 4
Difference Between Means	-6	1	-8	-6	1	-6	11	3	-13	12	-15	-2	-2	-13
Post-thaw														
Pre- Treatment Mean	14 ± 7	17 ± 3	27 ± 7	13 ± 2	16 ± 3	25 ± 12	24 ±6	21 ± 4	12 ± 7	16 ± 4	16 ± 5	17 ± 3	15 ± 5	7 ± 5
Post-Treatment Mean	21 ± 6	21 ± 8	23 ± 5	22 ± 7	19 ± 7	24 ± 14	17 ± 11	29 ± 11	22 ± 18	21 ± 3	17 ± 4	14 ± 3	33 ± 4	18 ± 3
Difference Between Means	7	4	-4	9	3	-1	-7	8	10	5	1	-3	18	11

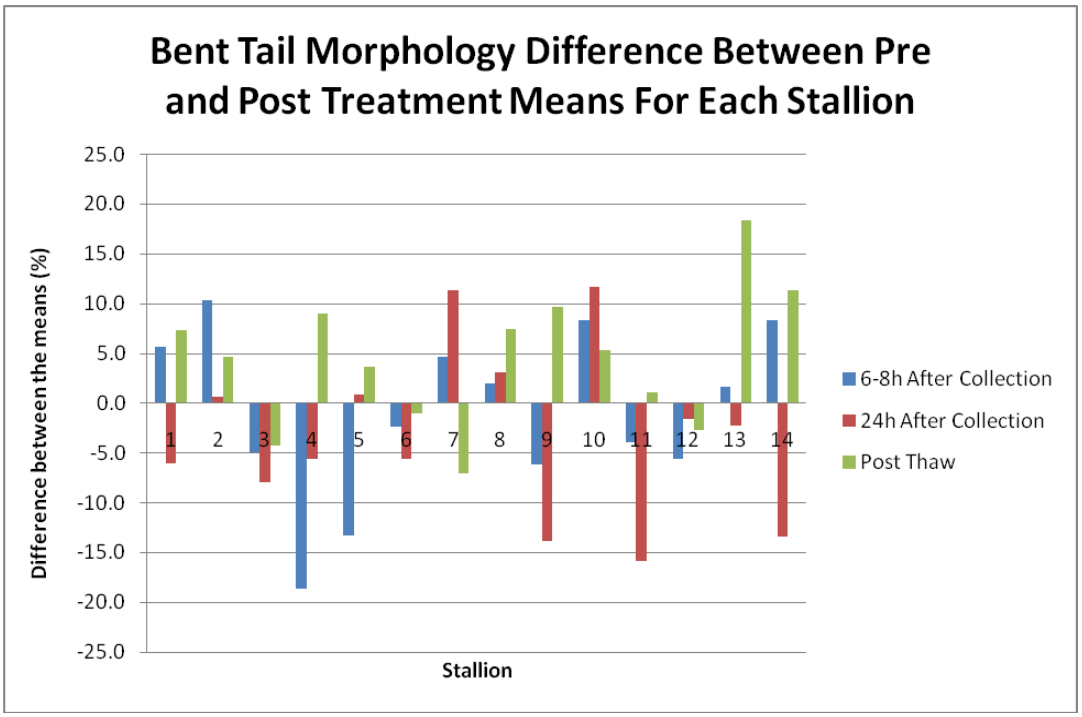


Figure 3. 11 The difference (post-treatment % - pre-treatment %) between the means of bent tail defects in spermatozoa morphology at 6-8h, 24h, and post-thaw before and after supplementation of individual stallions (1-14). Stallions 1, 2, 3 (Control); 4, 5, 6 (Minerals and vitamin E); 7, 8 (Oil); 9, 10 (1/2 Control); 11, 12 (1/2 Minerals and vitamin E); 13, 14 (1/2 Oil).

The percentage of sperm coiled tail defects was recorded and the averages of the semen samples collected before and after treatment for each stallion at each assessment time are shown in Table 3.12. The differences between the pre and post-treatment (post-pre) are also included in Table 3.12 and are graphed (Fig 3.12). The fewer coiled tail defects were recorded in comparison to other morphological defects and only very small changes are shown up to 2%.

Table 3. 12 Percentage of coiled tail defects in spermatozoa morphology at 6-8h, 24h, and post-thaw before and after supplementation of stallions (1-14) with minerals and vitamin E or oil compared with no supplementation (control). Stallions on the NRM diet received 1/2 the amount of supplement. The values are the means ± s.d. of the semen samples collected before and after supplementation. Differences between the means = post-treatment – pre-treatment (%).

Treatment Group	Control			Minerals and vitamin E			Oil		1/2 Control		1/2 Minerals and vitamin E		1/2 Oil	
Stallion	1	2	3	4	5	6	7	8	9	10	11	12	13	14
6-8h After Collection														
Pre- Treatment Mean	3 ± 1	0 ± 0	1 ± 1	1 ± 1	1 ± 1	1 ± 1	1 ± 1	1 ± 1	3 ± 1	2 ± 3	1 ± 1	4 ± 3	1 ± 1	3 ± 2
Post-Treatment Mean	1 ± 1	1 ± 1	1 ± 2	1 ± 1	1 ± 1	1 ± 2	0 ± 1	1 ± 1	1 ± 0	1 ± 2	1 ± 1	2 ± 1	2 ± 2	1 ± 0
Difference Between Means	-2	1	0	0	0	0	-1	0	-2	1	0	-2	1	-2
24h After Collection														
Pre- Treatment Mean	1 ± 2	0 ± 0	0 ± 0	1 ± 1	0 ± 0	1 ± 1	1 ± 2	1 ± 1	2 ± 1	1 ± 1	0 ± 1	2 ± 1	0 ± 1	0 ± 0
Post-Treatment Mean	1 ± 1	0 ± 0	1 ± 1	0 ± 1	0 ± 0	0 ± 0	0 ± 0	1 ± 1	2 ± 1	1 ± 1	2 ± 1	2 ± 1	0 ± 1	1 ± 1
Difference Between Means	0	0	1	-1	0	-1	-1	0	0	1	2	-1	0	1
Post-thaw														
Pre- Treatment Mean	0 ± 1	1 ± 1	1 ± 0	1 ± 1	0 ± 1	0 ± 0	2 ± 2	2 ± 1	2 ± 1	2 ± 1	2 ± 1	3 ± 1	1 ± 1	2 ± 0
Post-Treatment Mean	2 ± 2	0 ± 0	1 ± 1	1 ± 1	1 ± 1	0 ± 0	1 ± 1	2 ± 1	1 ± 0	1 ± 1	2 ± 2	3 ± 2	1 ± 1	2 ± 2
Difference Between Means	2	-1	0	0	1	0	-1	0	-1	-1	0	0	0	0

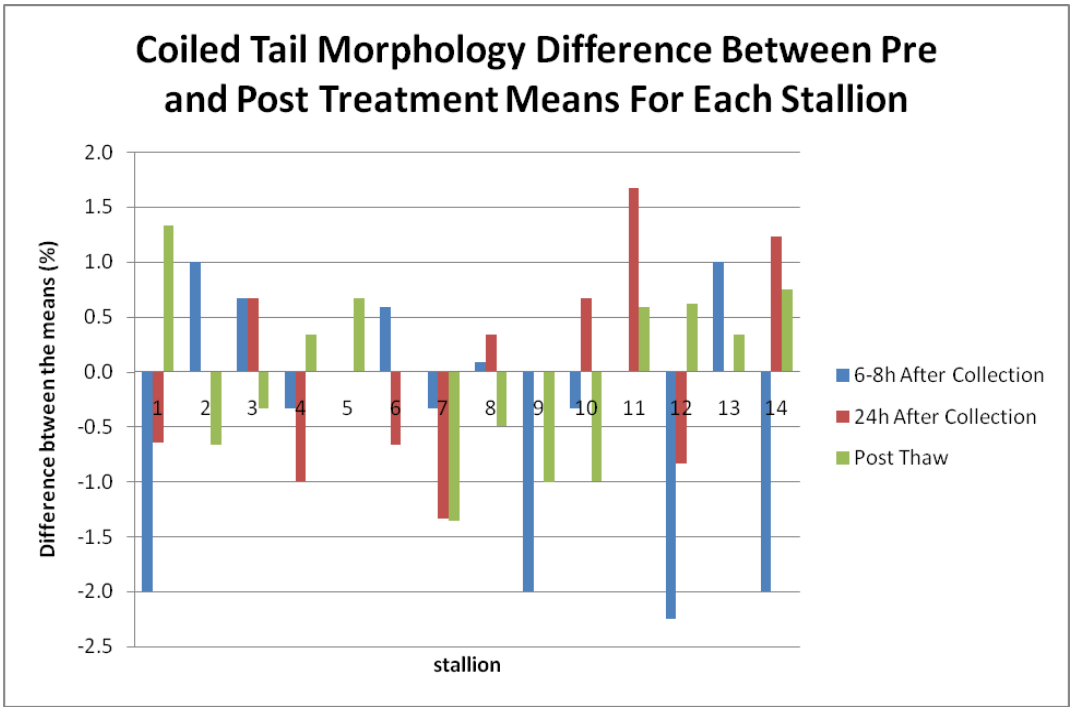


Figure 3. 12 The difference (post-treatment % - pre-treatment %) between the means of bent tail defects in spermatozoa morphology at 6-8h, 24h, and post-thaw before and after supplementation of individual stallions (1-14). Stallions 1, 2, 3 (Control); 4, 5, 6 (Minerals and vitamin E); 7, 8 (Oil); 9, 10 (1/2 Control); 11, 12 (1/2 Minerals and vitamin E); 13, 14 (1/2 Oil).

3.4 Acrosome Reaction

The acrosome status of the sperm was measured in the post-thaw spermatozoa and the means of the pre and post-treatment results are shown in Tables 3.13-3.17. The differences between the pre and post-treatment means (post-treatment – pre-treatment) are also shown in these Tables and are graphed (Fig 3.13). A possible treatment effect is indicated in the large decrease in the percentage of intact acrosome in stallion 6 (27%, Table 3.13), patchy acrosomes in stallion 13 (29%, 3.14) and reacted acrosome in stallion 14 (48%, Table 3.16). However large increases are also indicated in the percentage of 75% reacted acrosomes in stallion 13 (34%, Table 3.15) and stallions 14 show a large increase in intact acrosomes (23%, Table 3.13) and patchy acrosomes (31%, Table 3.14).

A treatment effect was also suggested by the large decrease in intact acrosomes in the minerals and vitamin E mix group (Fig 3.14). There also seem to be small increase in the percentage of intact acrosomes in the ½ minerals and vitamin E treatment groups, and a small increase in 75% reacted acrosomes in the minerals and vitamin E and the ½ oil treatment groups.

Overall, statistical analysis of the percentage intact acrosome showed no significant difference between pre and post-treatment in response to diet supplementation (P=0.219).

Table 3. 13 The percentage of intact acrosomes in post-thaw spermatozoa before and after (post-treatment – pre-treatment) supplementation of stallions (1-14) with minerals and vitamin E or oil compared with no supplementation (control). Stallions on the NRM diet received 1/2 the amount of supplement. The values are the means ± s.d. of the semen samples collected before and after supplementation.

Treatment Group	Control			Minerals and vitamin E			Oil		1/2 Control		1/2 Minerals and vitamin E		1/2 Oil	
Stallion	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Post-thaw														
Pre- Treatment Mean	23 ± 26	23 v 18	11 ± 16	33 ± 33	18 ± 14	46 ± 13	19 ± 9	22 ± 15	2 ± 4	19 ± 7	30 ± 11	4 ± 4	21 ± 8	0 ± 1
Post-Treatment Mean	27 ± 26	16 ± 5	20 ± 15	18 ± 25	9 ± 10	20 ± 21	36 ± 13	19 ± 17	18 ± 26	7 ± 5	44 ± 30	16 ± 15	13 ± 23	24 ± 13
Difference Between Means	4	-7	9	-16	-9	-27	17	-2	16	-12	14	12	-8	23

Table 3. 14 The percentage of patchy acrosomes in post-thaw spermatozoa before and after (post-treatment – pre-treatment) supplementation of stallions (1-14) with minerals and vitamin E or oil compared with no supplementation (control). Stallions on the NRM diet received 1/2 the amount of supplement. The values are the means ± s.d. of the semen samples collected before and after supplementation.

Treatment Group	Control			Minerals and vitamin E			Oil		1/2 Control		1/2 Minerals and vitamin E		1/2 Oil	
Stallion	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Post-thaw														
Pre- Treatment Mean	54 ± 15	50 ± 5	49 ± 21	51 ± 23	61 ± 1	44 ± 13	58 ± 10	53 ± 17	63 ± 3	56 ± 4	53 ± 16	72 ± 7	49 ± 4	30 ± 12
Post-Treatment Mean	51 ± 10	52 ± 20	54 ± 18	55 ± 15	54 ± 2	62 ± 15	42 ± 14	60 ± 13	60 ± 16	67 ± 8	47 ± 28	68 ± 17	20 ± 21	61 ± 5
Difference Between Means	-3	2	5	4	-7	18	-16	7	-3	11	-6	-4	-29	31

Table 3. 15 The percentage of 75% reacted acrosomes in post-thaw spermatozoa before and after (post-treatment – pre-treatment) supplementation of stallions (1-14) with minerals and vitamin E or oil compared with no supplementation (control). Stallions on the NRM diet received 1/2 the amount of supplement. The values are the means ± s.d. of the semen samples collected before and after supplementation.

Treatment Group	Control			Minerals and vitamin E			Oil		1/2 Control		1/2 Minerals and vitamin E		1/2 Oil	
Stallion	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Post-thaw														
Pre- Treatment Mean	15 ± 11	15 ± 5	29 ± 29	12 ± 13	12 ± 10	6 ± 4	15 ± 5	16 ± 2	24 ± 5	15 ± 1	14 ± 10	13 ± 5	21 ± 8	14 ± 4
Post-Treatment Mean	17 ± 12	25 ± 16	19 ± 18	25 ± 18	30 ± 13	17 ± 15	11 ± 4	16 ± 11	17 ± 3	20 ± 7	6 ± 5	9 ± 6	55 ± 39	9 ± 3
Difference Between Means	2	10	-10	13	18	11	-4	0	-7	5	-8	-4	34	-5

Table 3. 16 The percentage of reacted acrosomes in post-thaw spermatozoa before and after (post-treatment – pre-treatment) supplementation of stallions (1-14) with minerals and vitamin E or oil compared with no supplementation (control). Stallions on the NRM diet received 1/2 the amount of supplement. The values are the means ± s.d. of the semen samples collected before and after supplementation.

Treatment Group	Control			Minerals and vitamin E			Oil		1/2 Control		1/2 Minerals and vitamin E		1/2 Oil	
Stallion	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Post-thaw														
Pre- Treatment Mean	9 ± 13	7 ± 5	8 ± 6	3 ± 3	9 ± 9	2 ± 1	5 ± 3	9 ± 3	9 ± 2	8 ± 2	3 ± 2	10 ± 6	7 ± 4	56 ± 10
Post-Treatment Mean	4 ± 6	4 ± 7	6 ± 7	3 ± 4	6 ± 5	3 ± 3	10 ± 12	5 ± 5	5 ± 7	5 ± 4	2 ± 2	4 ± 3	12 ± 13	8 ± 8
Difference Between Means	-5	-3	-2	0	-3	1	5	-4	-4	-3	-1	-6	5	-48

Table 3. 17 The percentage of equatorial segment acrosomes in post-thaw spermatozoa before and after (post-treatment – pre-treatment) supplementation of stallions (1-14) with minerals and vitamin E or oil compared with no supplementation (control). Stallions on the NRM diet received 1/2 the amount of supplement. The values are the means ± s.d. of the semen samples collected before and after supplementation.

Treatment Group	Control			Minerals and vitamin E			Oil		1/2 Control		1/2 Minerals and vitamin E		1/2 Oil	
Stallion	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Post-thaw														
Pre- Treatment Mean	2 ± 3	6 ± 2	2 ± 2	0 ± 0	1 ± 1	2 ± 4	4 ± 6	0 ± 1	2 ± 3	1 ± 2	0 ± 1	1 ± 1	2 ± 3	0 ± 0
Post-Treatment Mean	1 ± 0	0 ± 0	1 ± 2	0 ± 0	1 ± 2	1 ± 1	1 ± 1	0 ± 0	0 ± 0	1 ± 1	2 ± 2	3 ± 4	1 ± 1	1 ± 1
Difference Between Means	-1	-6	-1	0	0	-1	-3	0	-2	0	2	2	-1	1

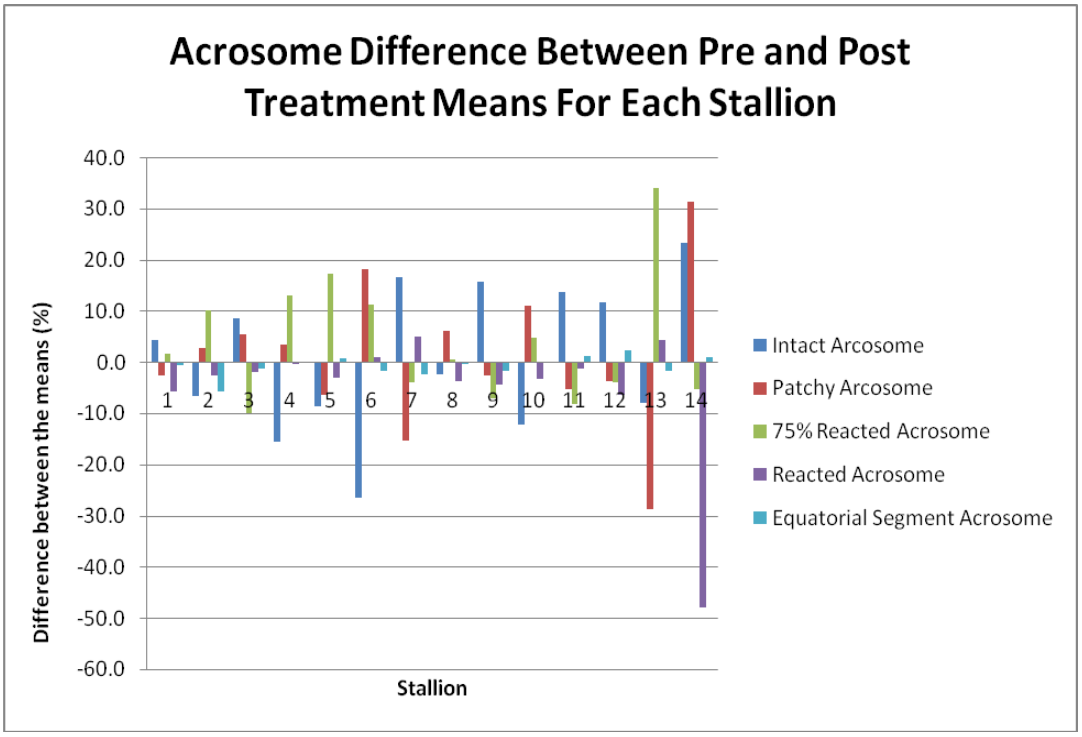


Figure 3. 13 The difference (post-treatment % - pre-treatment %) between the means of the degree of acrosome reaction (intact, patchy, 75% reacted, reacted and equatorial segment) in spermatozoa post-thaw before and after supplementation of individual stallions (1-14). Stallions 1, 2, 3 (Control); 4, 5, 6 (Minerals and vitamin E); 7, 8 (Oil); 9, 10 (1/2 Control); 11, 12 (1/2 Minerals and vitamin E); 13, 14 (1/2 Oil).

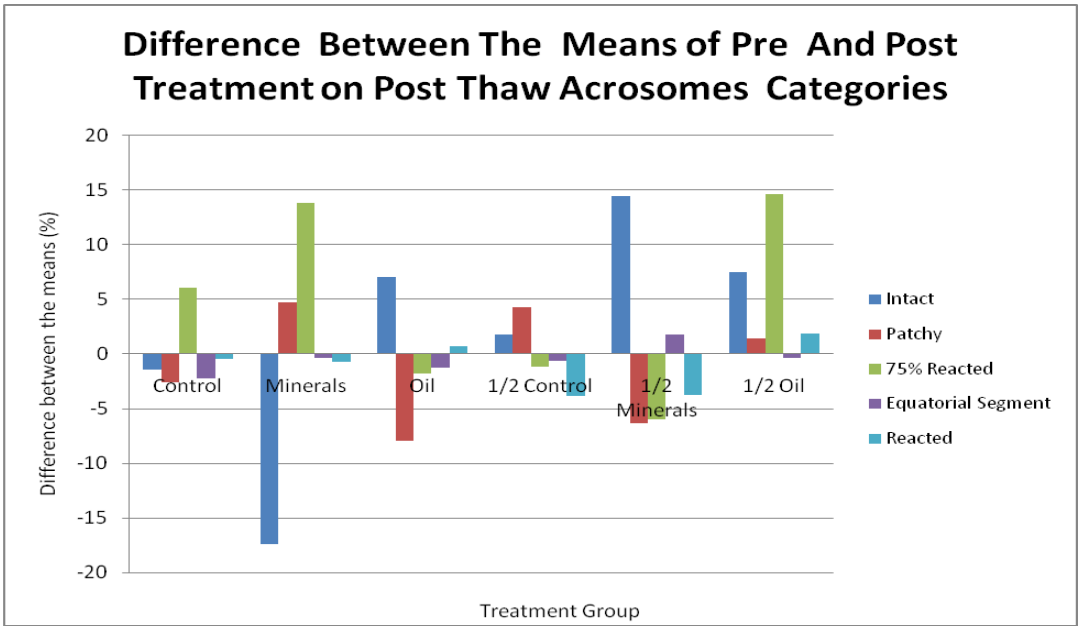


Figure 3. 14 The difference (post-treatment % - pre-treatment %) between the means of the degree of acrosome reaction (intact, patchy, 75% reacted, reacted and equatorial segment) in spermatozoa post-thaw before and after supplementation of the treatment groups (Control, Minerals and vitamin E, Oil, ½ Control, ½ Minerals and vitamin E and ½ Oil).

3.5 Hypo-osomotic Swelling Test

The hypo-osmotic swelling test (HOS) analysed the integrity of the spermatozoa membrane post-thaw. This was recorded in the percentage of coiled tails after incubation in the HOS media for 60 min. The averages of the semen samples collected before and after treatment for each stallion at each assessment time are shown in Table 1.18 along with the differences between the pre and post-treatment means (post-treatment – pre-treatment). The differences between the pre and post-treatment means are also graphed (Fig 3.13). A possible treatment effect is indicated in the large increase in stallion 6 (11%) and decrease in the number of coiled tails in the ½ minerals and vitamin E treatment group, stallions 11 (7%) & 13 (8%). However there was also a decrease in the control group stallion 9 (9%). Statistical analysis found no significant differences in all of the treatment groups pre and post-treatment means in response to diet (P=0.754).

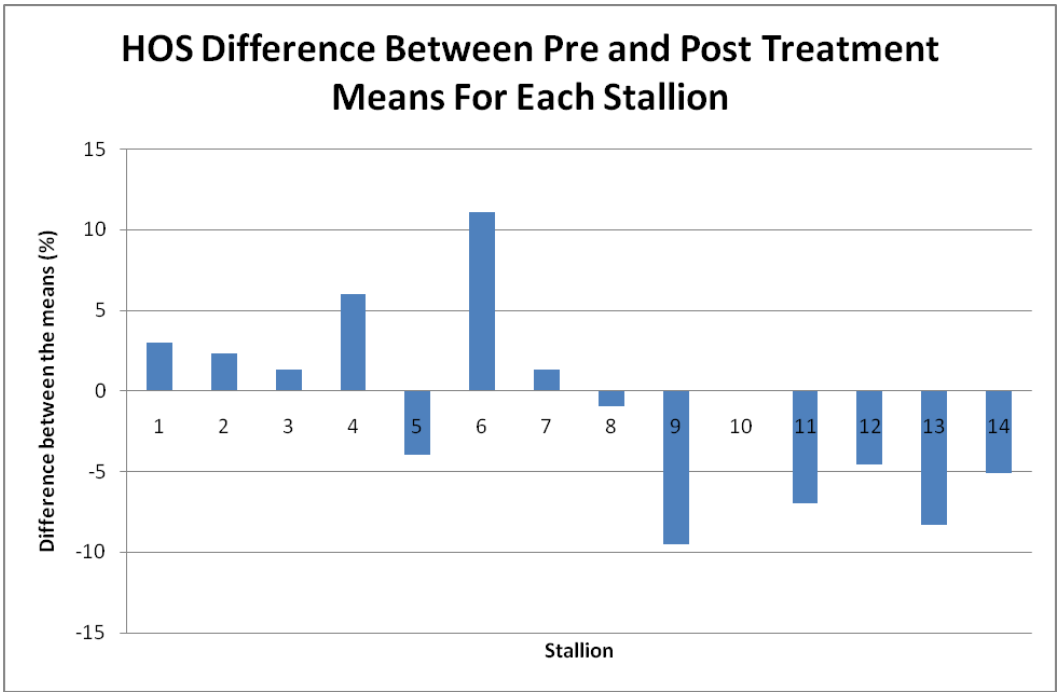


Figure 3. 15 The difference (post-treatment % - pre-treatment %) between the means of coiled tail spermatozoa post-thaw in the HOS analysis before and after supplementation of individual stallions (1-14). Stallions 1, 2, 3 (Control); 4, 5, 6 (Minerals and vitamin E); 7, 8 (Oil); 9, 10 (1/2 Control); 11, 12 (1/2 Minerals and vitamin E); 13, 14 (1/2 Oil).

Table 3. 18 Percentage of coiled tail spermatozoa in HOS analysis post-thaw before and after supplementation of stallions (1-14) with minerals and vitamin E or oil compared with no supplementation (control). Stallions on the NRM diet received 1/2 the amount of supplement. The values (%) are the means ± s.d. of the semen samples collected before and after supplementation. Differences between the means = post-treatment – pre-treatment (%).

Treatment Group	Control			Minerals and vitamin E			Oil		1/2 Control		1/2 Minerals and vitamin E		1/2 Oil	
Stallion	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Post-thaw														
Pre- Treatment Mean	30 ± 6	31 ± 13	32 ± 7	28 ± 3	41 ± 14	32 ± 4	47 ± 9	43± 9	53 ± 10	38 ± 9	40 ± 2	51 ± 14	36 ± 2	41 ± 5
Post-Treatment Mean	33 ± 4	33 ± 5	33 ± 5	34 ± 8	37 ± 9	43 ± 9	48 ± 12	42 ± 8	44 ± 6	38 ± 14	33 ± 8	47 ± 7	28 ± 5	36 ± 10
Difference Between Means	3	2	1	6	-4	11	1	-1	-9	0	-7	-4	-8	-5

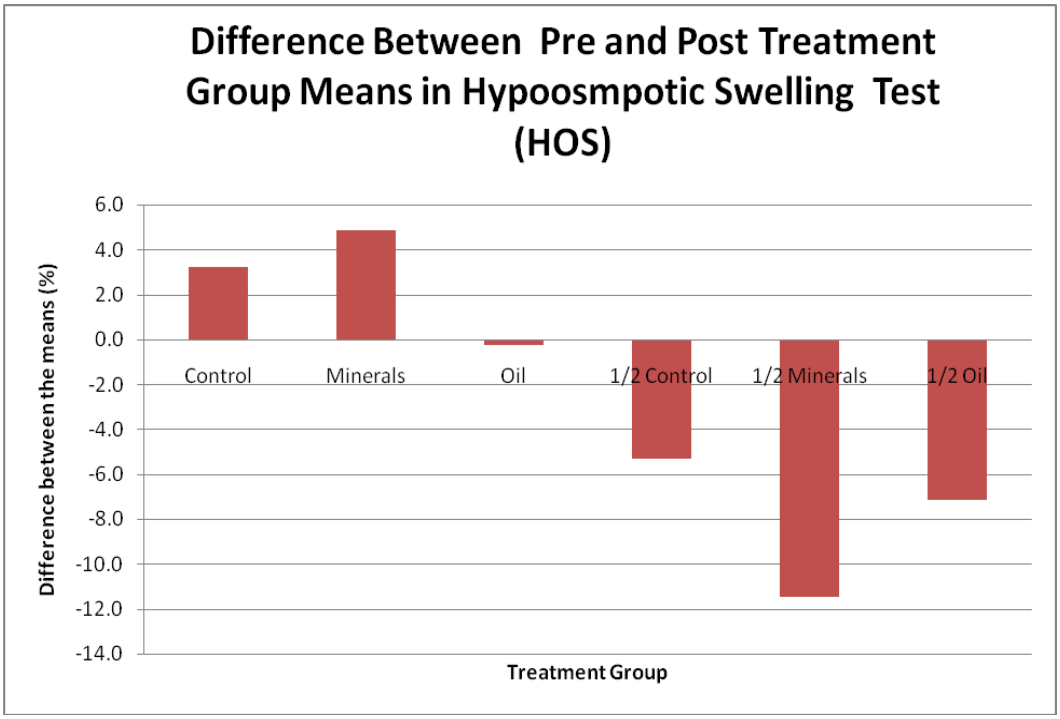


Figure 3. 16 The difference (post-treatment % - pre-treatment %) between the means of coiled tail spermatozoa post-thaw in the HOS analysis before and after supplementation of the treatment groups (Control, Minerals and vitamin E, Oil, ½ Control, ½ Minerals and vitamin E and ½ Oil).

3.6 Sperm Concentration

The sperm concentration (10^6.ml^{-1}) of the post-thaw samples was recorded and the averages of the semen samples collected before and after treatment for each stallion at each assessment time are shown in table 3.19. The difference between the pre and post-treatment means (post-treatment – pre-treatment) are also shown in table 3.19 and are graphed (Fig 3.17). A possible treatment effect was indicated in a very large decrease in the spermatozoa concentration in stallion 14 (-384) and the large decrease in stallions 4 (-104) and 6 (-125). However, large increases are also indicated in stallions 13 (113), and in control groups 3(103), and 1 (85).

Difference between the means of the treatment groups (fig 3.23) indicate a decrease in concentration of spermatozoa in minerals and vitamin E, and ½ oil treatment groups while both control group concentrations increased. However, statistical analysis of the treatment groups found no significant differences between pre and post-treatment in response to diet supplementation (P=0.494).

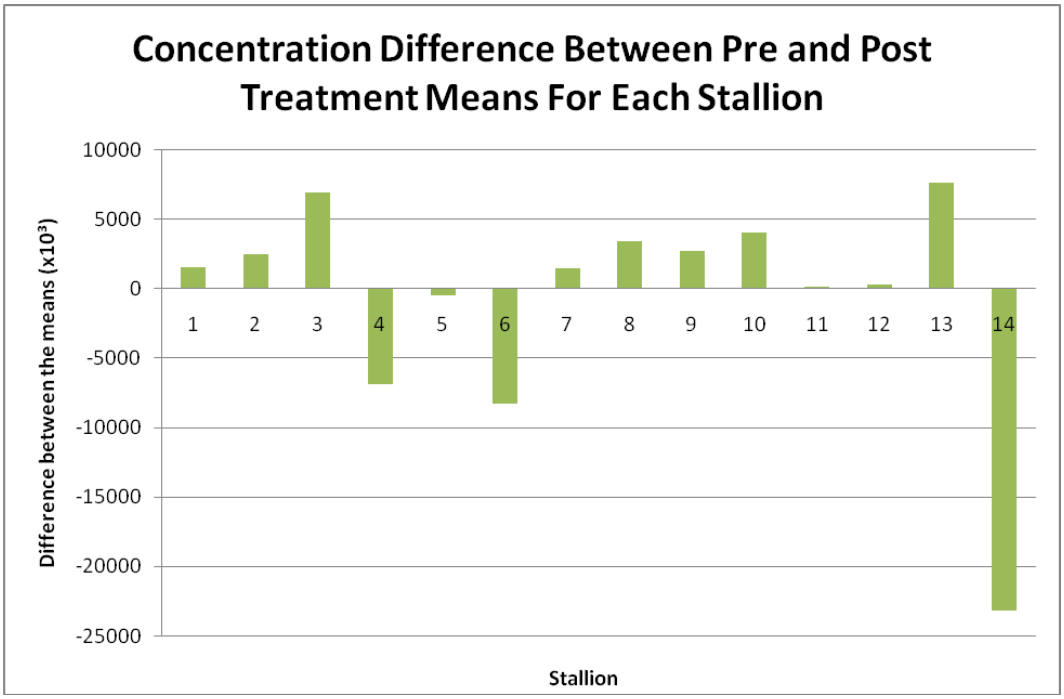


Figure 3. 17 The difference (post-treatment - pre-treatment 10^6.ml^{-1}) between the means of spermatozoa concentration post-thaw before and after supplementation of individual stallions (1-14). Stallions 1, 2, 3 (Control); 4, 5, 6 (Minerals and vitamin E); 7, 8 (Oil); 9, 10 (1/2 Control); 11, 12 (1/2 Minerals and vitamin E); 13, 14 (1/2 Oil).

Table 3. 19 The post-thaw spermatozoa concentration before and after supplementation of stallions (1-14) with minerals and vitamin E or oil compared with no supplementation (control). Stallions on the NRM diet received 1/2 the amount of supplement. The values are the means of the semen samples collected before and after supplementation. Differences between the means = post-treatment – pre-treatment (10^6.ml^{-1}).

Treatment Group	Control			Minerals and vitamin E			Oil		1/2 Control		1/2 Minerals and vitamin E		1/2 Oil	
Stallion	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Post-thaw														
Pre- Treatment Mean	14311	12378	10178	23000	8400	26133	11022	11711	12978	15133	20511	6222	16778	35256
Post-Treatment Mean	15778	14844	17022	16067	7889	17783	12467	15083	15633	19156	20600	6467	24333	12033
Difference Between Means	1467	2467	6844	-6933	-511	-8350	1444	3372	2656	4022	89	244	7556	-23222

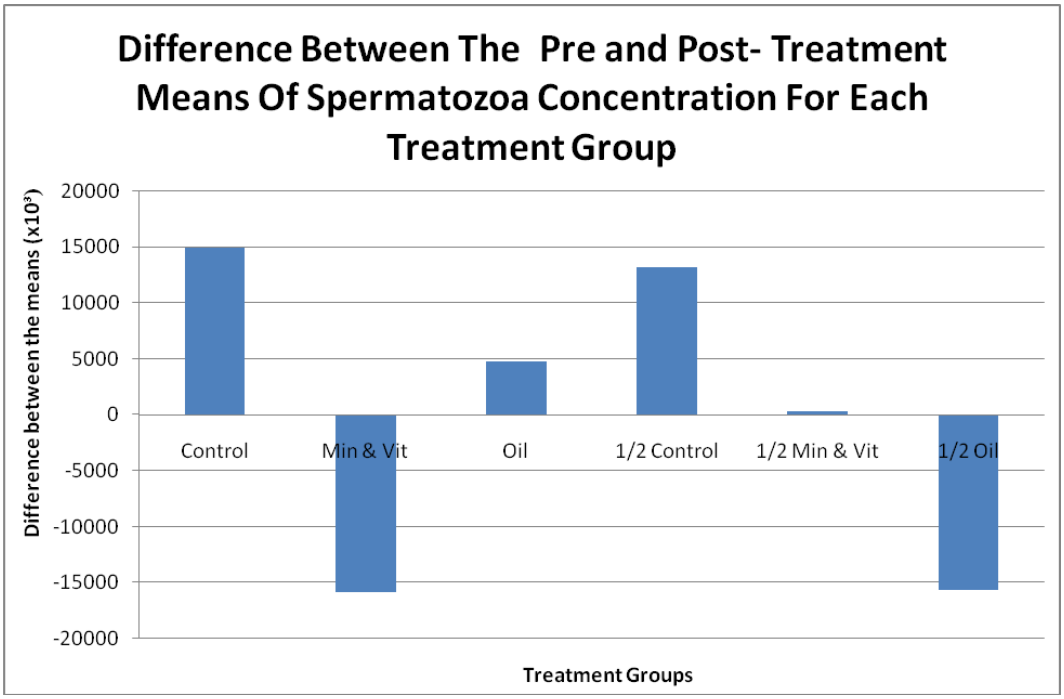


Figure 3. 18 The difference (post-treatment - pre-treatment $10^6.ml^{-1}$) between the means of spermatozoa concentration post-thaw before and after supplementation of the treatment groups (Control, Minerals and vitamin E, Oil, ½ Control, ½ Minerals and vitamin E and ½ Oil).

3.7 Blood Mineral Levels

The concentration of selenium, copper and zinc in blood were measured before and after supplementation for each stallion (Table 3.20). Unfortunately a post-treatment blood sample was unable to be taken from two of the stallions in oil treatment groups due to uncontrollable circumstances. The differences between pre and post-treatment blood mineral levels for the individual stallions are included in Table 3.20 and are graphed (Fig 3.19 & Fig 3.20). These results indicate a large increase in selenium blood levels in the stallions in the control, minerals and vitamin E, and oil group occurred, whereas the other stallions in the ½ treatment groups showed a substantial decrease (except stallion 13). All stallions were getting approximately 2mg/kg of selenium in the minerals and vitamin E supplement daily (Appendix 3 Fig A3.2).

An increase in copper blood levels in the control and oil group and a decrease in copper in the ½ control and ½ oil group. Zinc blood levels decreased in the ½ control and oil group. There was little change in copper and zinc blood levels in the minerals and vitamin E treatment group stallions (Fig.3.20).

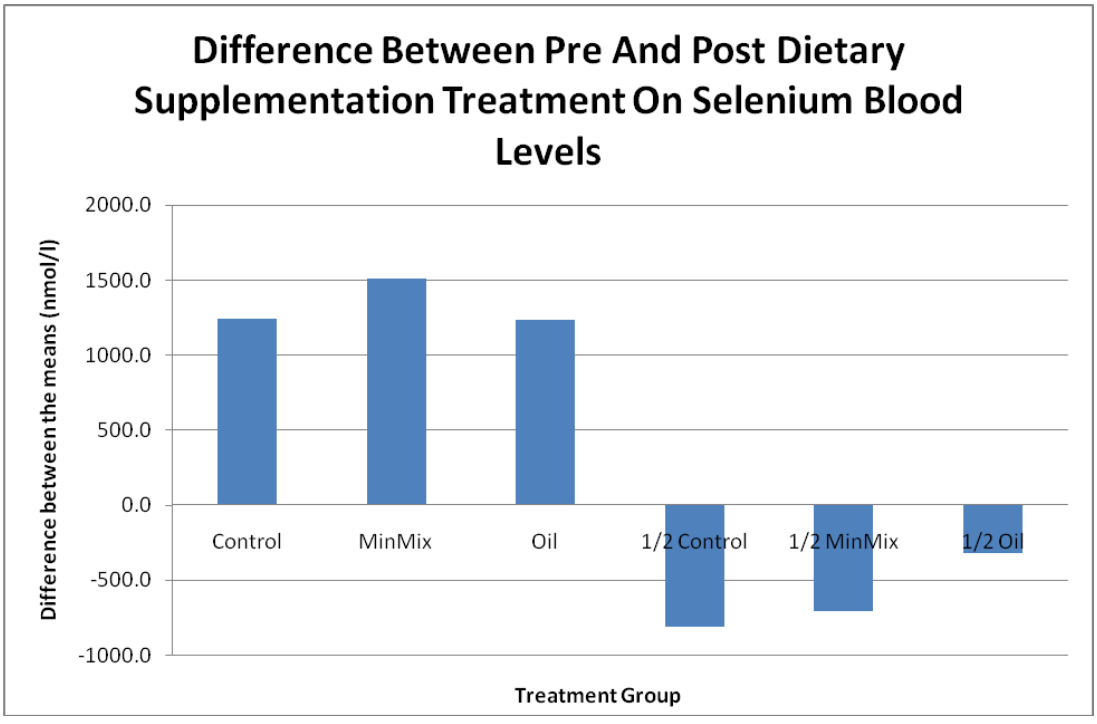


Figure 3. 19 The difference (nmol/l) between the means of selenium blood levels before and after (post-treatment-pre-treatment) supplementation of the treatment groups (Control, Minerals and vitamin E, Oil, ½ Control, ½ Minerals and vitamin E and ½ Oil).

Table 3. 20 Blood mineral (selenium, copper, zinc) levels before and after dietary supplementation of stallions (1-14) with minerals and vitamin E or oil compared with no supplementation (control). Stallions on the NRM (Stud C) diet received 1/2 the amount of supplement. Differences between the pre and post-treatment = post-treatment – pre-treatment.

Treatment Group	Control			Minerals and vitamin E			Oil		1/2 Control		1/2 Minerals and vitamin E		1/2 Oil	
Studs	A	B	B	A	B	B	B	B	C	C	C	C	C	C
Stallion	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Seleinum (nmol/l)														
Pre- Treatment	2160	260	790	2280	360	380	430	1050	3200	2790	3050	3070	2590	3360
Post-Treatment	3780	1570	1590	3580	2110	1860		1970	2350	2010	2220		2540	2760
Difference Between Pre & Post Treatment	1620	1310	800	1300	1750	1480		920	-850	-780	-830		-50	-600
Copper (umol/l)														
Pre- Treatment Mean	20	18	20	20	17	27	19	28	21	26	24	22	20	24
Post-Treatment Mean	25	21	25	16	21	31		26	15	18	22		20	19
Difference Between Means	5	3	5	-4	4	4		-2	-6	-8	-2		1	-5
Zinc (umol/l)														
Pre- Treatment Mean	8	18	18	8	15	24	18	21	13	18	15	12	14	13
Post-Treatment Mean	23	16	16	23	17	17		17	12	10	14		13	17
Difference Between Means	15	-2	-2	15	2	-7		-4	-1	-8	-1		-1	4

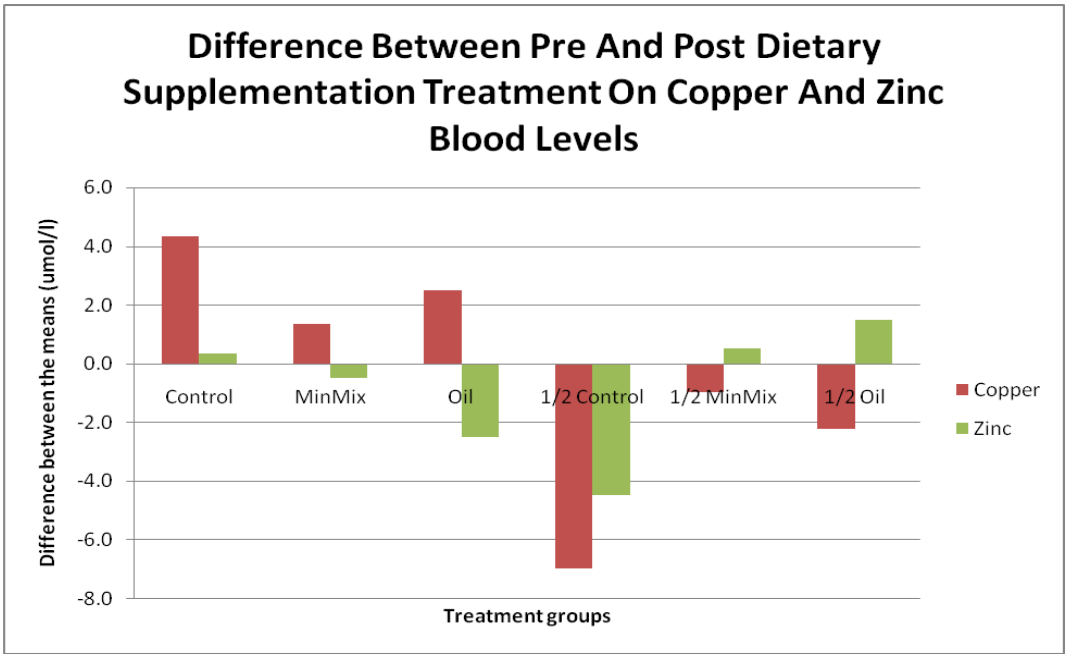


Figure 3. 20 The difference (umol/l) between the means of zinc and copper blood levels before and after (post-treatment-pre-treatment) supplementation of the treatment groups (Control, Minerals and vitamin E, Oil, ½ Control, ½ Minerals and vitamin E and ½ Oil).

3.8 Combined Diet Analysis

After performing this first set of analysis, a second analysis was performed after combining the Dunstans dietary treatment groups with their corresponding NRM dietary treatment groups. Therefore the control group's results were combined with the ½ control group, the minerals and vitamin E supplement group's results were combined with the ½ minerals and vitamin E supplement group and the Oil supplement group's results were combine with the ½ oil supplement group. Statistical analysis was performed (Table 3.21) and no statistical significance of dietary supplementation on fertility parameters were was found except for an increase in total motility 24h after collection in the Oil supplement group (P=0.019).

Table 3. 21 P values for effect of supplement additions (post-treatment – pre-treatment) in combined treatment groups (Control + ½ control; minerals and vitamin E + ½ mineral and vitamin E; Oil + ½ Oil) at 6-8h, 24h, post-thaw and 30mins after thawing (Post-thaw + 30min) for various semen parameters.

	6-8h	24h	Post-Thaw	Post-Thaw + 30 min
Total Motility	0.126	0.019*	0.785	0.491
Progressive Motility	0.636	0.409	0.875	0.895
Total Progressive Motility	0.339	0.554	0.869	0.812
Normal Morphology	0.073	0.961	0.972	
Acrosome			0.503	
HOS				0.488
Concentration				0.324

*Statistical significance <0.05

3.9 Per Cycle Pregnancy Rate

The per cycle pregnancy rate data was analysed from two of the three studs, both before and after treatment. Two studs (A & C) provided accurate information on the per cycle pregnancy rate for each one of their stallions from the breeding season both before and after treatment with dietary supplements. The per cycle pregnancy data provided from the third stud (B) was incomplete and unsuitable for analysis.

The effect of treatment in the combined control, and minerals and vitamin E groups was not statistically significant after Chi-square analysis. However there was a statistically significant effect of dietary supplementation with oil on the per cycle pregnancy rates in the stallions housed at these two studs (A&C).

Table 3. 22 Per-cycle pregnancy rates before and after supplement for each treatment group for the stallions (1,4 and 9-14) housed at stud A and C. N = the number of cycles, and the percentage cycles in brackets (%).

CONTROL GROUP	Pre-Treatment N (%)	Post-Treatment N (%)	
Number of Pregnant cycles	61 (70.1)	18 (72.0)	
Number of Not pregnant cycles	26 (29.9)	7 (28.0)	p = 0.855

VITAMIN & MINERAL GROUP	Pre-Treatment N (%)	Post-Treatment N (%)	
Number of Pregnant cycles	106 (61.3)	21 (56.8)	
Number of Not pregnant cycles	67 (38.7)	16 (43.2)	p = 0.260

OIL GROUP	Pre-Treatment N (%)	Post-Treatment N (%)	
Number of Pregnant cycles	14 (48.3)	9 (100.0)	
Number of Not pregnant cycles	15 (51.7)	0 (0.0)	p = 0.006*

*Statistically significant p<0.05

CHAPTER FOUR: DISCUSSION

4.1 Discussion

The aim of this study was to investigate the effects of antioxidant dietary supplementation on semen parameters in New Zealand Standardbred stallions. This was the first study to examine the potential for a different effect of mineral and vitamin El antioxidant supplementation in comparison with oil antioxidant supplementation. In this study we found that there was no effect of supplementation with minerals and vitamin E on the fertility parameters assessed. However, there was an effect of oil supplementation on both the longevity of the chilled semen at 24h after collection and also on the per cycle pregnancy rates in the data provided from studs A and C.

Initially statistical analyses were done by using a one-way ANOVA analysing differences between pre- and post-treatment means for each stallion on the two different basal diets (Dunstans and NRM). As there was no statistical difference between the stallions on the Dunstans basal diet and the NRM basal diet, their results were pooled for each treatment group. Subsequent analysis revealed a treatment effect of oil supplementation on total motility 24h after semen collection and also on the per cycle pregnancy rates. The per cycle pregnancy rates were significantly increased in the mares which had been inseminated with semen from the stallions supplemented with oil but not in the mares inseminated with semen from the stallions not treated or supplemented with mineral and vitamin E supplement. These results suggest there is a beneficial effect of oil supplementation on stallion fertility that warrants further study.

There was a high degree of individual variation amongst stallions in their response to the diets. For future prospective studies a large number of stallions and mares should be included and factors analysed by way of a logistic regression to account for the variables encountered in this study.

4.2 Motility

Initial motility results indicated no significant effect of dietary supplementation on total motility, progressive motility, or total progressive motility at 6-8h and 24h after semen was collected or after freezing and thawing the semen when stallions were grouped according to their basal diet (first analysis). However, a subsequent analysis performed when both basal diet groups were combined showed a significant effect of oil on increased total motility at 24h after collection. As spermatozoa are very complex cells that require a number of different attributes for successful fertilisation more than one semen parameter would need to improve to suggest antioxidant supplementation increased fertility in stallions.

Similar results are found in the study by Deishsel, et al. (2008) which found no significant effect of feeding antioxidants for four weeks to Shetland pony stallions. Grady, et al. (2009) found Omega-3 dietary supplementation did not improve motility parameters in fresh ejaculates, chilled or frozen semen of miniature horse stallions after 84 days of feeding the flaxseed/algae based or fish-based omega-3 supplements. Harris, et al. (2005) also found no difference in motility in fresh, chilled or frozen semen after feeding omega-3 supplements for 90 days.

However, Brinko, et al., (2005) found that feeding docosahexaenoic acid omega-3 supplementations for 126 days significantly increased total motility, progressive motility and spermatozoa velocity after 48h of storage in an equitainer, and after freezing and thawing the semen. Elhordoy, et al., (2008) supported these finding and showed stallion spermatozoa motility significantly increased after 80 days supplementary feeding docosahexaenoic acid. A Colorado State University study also found that sperm motility increased in fresh semen and after 24h of chilled storage when feeding omega-3 to stallions (Squires, 2008).

Studies in other species have shown a significant increase in the proportion of progressively motile spermatozoa after feeding boars fish oil supplements for 8 weeks (Rooke, et al., 2001). However, Estienne, et al. (2008) found no differences in the motility of boar spermatozoa after omega-3 dietary supplementation. The difference between the equine and boar studies may be a result of different types of omega-3 supplement used in each study and the

variation in the duration of treatment relative to the spermatogenic cycle. A study in rabbits (Gliozzi, et al., 2009) showed that feeding fish oil, vitamin E or both together had no effect on semen parameters. High antioxidant intake in human males has been shown to be associated with higher motility in a nonclinical study (Eskenazi, et al., 2005).

4.3 Morphology

No significant effects of feeding the antioxidant supplements were found on spermatozoa morphology at 6-8h and 24h after collection or after stressing the cells by freezing and thawing the semen. Grady, et al. (2009) also found the percentage of morphologically normal stallion spermatozoa was not affected by feeding two different forms of omega-3 supplements. On the other hand Deishsel, et al. (2008) found a reduction in morphologically abnormal spermatozoa in pony stallions after feeding the antioxidant supplemented diet for 28 days. Elhordoy, et al. (2008) also found a decrease in the percentage of abnormal stallion spermatozoa especially in acrosome and midpiece defects. Harris, et al. (2005) found a higher percentage of morphologically normal stallion spermatozoa. Rooke, et al. (2001) found a reduction in the percentage of morphologically abnormal boar spermatozoa and a reduction in abnormal boar spermatozoa tails was observed by Estienne, et al. (2008).

4.4 Acrosome Reaction

Acrosome status of post-thawed spermatozoa was also not significantly affected by antioxidant supplementation in this study. Grady, et al. (2009) supports these findings as they found no effect on the percentage of intact acrosomes when feeding different omega-3 supplements to miniature stallions.

4.5 HOS – Membrane Integrity

Analysis of the spermatozoa membrane functional integrity (HOS test) after freezing and thawing indicated no significant effect of feeding antioxidants

to Standardbred stallions. Deichsel, et al. (2008) supports these results as they also found no effect of feeding pony stallion antioxidants on membrane integrity up to 24h after collection.

4.6 Concentration of spermatozoa

The concentration of spermatozoa has been shown to increase when feeding fatty-acids to horses (Harris, et al., 2005), however this was not the case in this study on Standardbred stallions' spermatozoa post-thaw. This may be the result of not feeding the supplement for long enough or feeding a different type of supplement at a lower concentration as Elhordoy, et al. (2008) also found an increase in the concentration of spermatozoa in stallion ejaculates.

Eskenazi, et al. (2005) showed that higher sperm concentrations have been associated with high antioxidant intakes in human males in a non-clinical trial. Feeding omega-3 oil to boars (Estienne, et al., 2008) and rabbits (Gliozzi, et al., 2009) has also been shown to result in an increase concentration of spermatozoa in ejaculates. However the differences in physiology of different species may be the reason for the different results observed.

4.7 Blood Mineral Levels

This study showed no correlation between blood mineral levels and semen parameters investigated and was not analysed for significant differences before and after treatment. The interesting results from this study are the decreases in selenium blood levels in the NRM ½ supplemented group of six stallions. Difference between the control, minerals and vitamin E, and oil groups when compared to the half treatment groups were to be expected as they were put onto the Dunstans diet at the same time as the supplement whereas the ½ treatment groups stayed on the same basal diet. The decrease in selenium levels in the ½ treatment groups would suggest that the stallions were feed an antioxidant supplement before the fertility trial, however communications with the stud have assured this was not the case. Another possibility is something in the base diet may have affected the absorption of these minerals and vitamin E as the

supplements were designed to be added to the modified Dunstans feed and not the NRM feed. Also it has been reported that selenium does not always correlate with dietary selenium concentrations and hence the analysis may not be valid (NRC, 2007). This has lead to the conclusion that interactions with other minerals, vitamins, or even enzymes may influence the absorption of dietary selenium and therefore affect the blood results.

There was also an unexpected increase in the Dunstans control group's blood selenium levels, although this may have been caused by changing the base diets. The pre-treatment analyses were performed when the stallions were being feed their original stud diets. When supplementary treatments were started in Studs A and B their basal diet was also changed to the Dunstans modified feed. This change in feed may have affected mineral levels and changes in the control group's selenium blood levels may be been a result of this.

However aside from stud A, studs B and C post-treatment selenium levels were similar. This would lead one to believe that the effect of the breeding season caused large amounts of stress on the stallions and therefore a drop in selenium levels in the blood is observed because selenium is being metabolised at higher rates. The supplements may have stopped selenium levels from dropping beyond the blood selenium levels observed in stud C's stallions. An increase is observed in stud B because the change of diet caused the base selenium level to increase more than the pre-treatment diet and therefore we see a selenium saturation blood level that is similar to stud C post-treatment. However this does not account for the large increase in selenium levels observed in stallions housed at stud A. Therefore differences in selenium may be a result of the change in selenium available in pastures, baylage or hay in different areas of New Zealand.

4. 8 Per Cycle Pregnancy Rates

This study revealed that there was a beneficial effect of dietary supplementation with oil in the per cycle pregnancy rates from the two studs (A & C) that were analysed. Despite the small sample size analysed, these pregnancy results are also consistent with the improvement in sperm longevity observed at 24h after semen collection in the stallions fed the oil supplement when compared

with control and mineral and vitamin E treatment groups. These results are of great interest and warrant further investigation with larger sample sizes.

4.9 Why Didn't Minerals and Vitamin E Antioxidants Improve Semen Parameters?

Our results indicate that there may be a physiological difference in the way oil antioxidants affect fertility parameters when compared with mineral and vitamin E antioxidants. It is of great interest that the dietary supplementation with oil improves both the longevity of the chilled spermatozoa and also the pregnancy rates in the data provided. However, there was no such effect of supplementation with minerals and vitamin E when compared with the control, untreated stallions. There are various possibilities as to why this might be the case. There are a number of different antioxidants that control excessive production of ROS during spermatogenesis and perhaps oil antioxidants is better than minerals and vitamins in horses already on a balance diet.

The differences between this study's results compared to other studies may have been as a result of differences in the supplement concentrations, amounts of supplements given and length of time the supplement was given. Like this study, Deichsel, et al. (2008) also found that feeding mineral and vitamin E antioxidant supplement had no effect of semen fertility parameters. However, they did find a significant decrease in morphological defects. During their trial period they also gave mineral supplements as part of their basal diet which may very well have affected their results. They also had three pony stallions in each treatment group and fed the supplement for 28 days. Perhaps, in horses that are fed a complete diet the levels of antioxidant minerals and vitamins needs to be increased beyond those levels investigated to obtain a beneficial effect. Indeed, any such changes must be made with care to avoid toxicity.

In contrast Grady, et al. (2009) found no changes in motility as a result of feeding two different types of omega-3 fatty acid supplement for 84 days. However, the experimental design of this study differs from many of the other as they did not have a control group and the nine stallions used in this study were split into two different treatment groups, one group was fed 60g/d of a flaxseed

and algae based omega-3 supplement to five stallions and the other group was fed150g/d of a fish-based omega-3 fatty acid to four other stallions.. Therefore it is difficult to conclude that their results were not affected by seasonal or environmental changes.

Elhordoy, et al. (2008) feed 30g/d DHA supplement for 80 days and found an increase in spermatozoa concentration in ejaculates, increase in motility, and a reduction morphological defects and dead spermatozoa. The difference between their study results and the current study may be as a result of the type of supplement given for a longer length of time or because their experimental design allowed them to have larger groups sizes. In this study they only had six stallions. However, because they split the stallions into two groups (treatment and control groups) and then after 80 days of feeding the supplement the groups were reversed (treatment group became the control group and visa versa) this allowed them to have a larger sample group sizes.

Elhordoy, et al. (2008) found increased progressive motility after cryopreservation in only some of the stallions fed the antioxidant supplement, while Brinsko, et al. (2005) feed 250g of DHA to stallions for 98 days and found a significant increase in total motility, progressive motility, and what they call rapid motility after cryopreservation. We found no effect of feeding antioxidant supplements on frozen-thawed spermatozoa. The differences between the results may be due to the centrifugation step in the cryopreservation process. During centrifugation much of the seminal plasma is removed and with it many of the antioxidants. It is unclear whether the cryopreservation process differed in Elhordoy, et al. (2008) and Brinkso, et al., (2005) studies or whether the conflicting motility results after cryopreservation is due to a variation in the amount of seminal plasma antioxidants removed during centrifugation and removal of the supernatant. Although, differences in the results of cryopreservation is more likely to have been a result of the semen being frozen 6-8h after collection and chilled storage as opposed to being frozen straight away. This was used in this study as an extreme spermatozoa stress test. However, Backman, et al. (2004) study contradicts this theory as they found that chilling stallion semen for 18h before cryopreservation may not decrease the fertility of the semen.

Harris, et al (2005) feed 550g/d of a polyunsaturated fatty acid supplement for 90 days and found no changes in motility in fresh, chilled stored (24h and 48h) or after cryopreservation. They did however find a significant increase in spermatozoa concentrations in ejaculates and the percentage of morphologically normal spermatozoa. Again these conflicting results may be a result of small sample group sizes and Harris, et al. (2005) agree that further antioxidant dietary supplementation studies should be performed with a larger number of stallions.

Inevitably it is hard to compare results between different studies due to the wide variation in husbandry and management practices, which would lead to different stress levels, environmental and seasonal factors.

There are several other possibilities as to why mineral and vitamin antioxidants did not improve semen parameters as expected in this study that may be related to the effect of an already balanced diet, supplementation composition and experimental design.

4.9.1 Effects of Balanced Diet

4.9.1.1 Saturation

The levels of minerald and vitamin E antioxidants needed by horses may be sufficiently provided by their basic forage. The phytonutrients in the grass may provide the majority of the antioxidants needed for physiological reactions. Therefore the small proportions of essential nutrients that are necessary and not manufactured within the body could easily be provided in a balanced diet. Hence it is possible that all the mineral and vitamin E stallions in this trial already had significant levels of these antioxidants and that further supplementation had no further effect. Inevitably the most obvious way of testing this theory would be to experimentally deprive the horses of their endogenous antioxidants, and mirroring studies that have been performed in mice (Behne, et al., 1982). However this would be unfeasible, impractical, and difficult to do.

The effect of forage in the diet might be better controlled in stallions that were kept in a stable for 24h a day and therefore not allowed access to grass enabling a more controlled assessment of the total dietary intake, but again it would be difficult and unhealthy to limit access to forage.

4.9.1.2 Absorption and Composition

The absorption of the oral minerals and vitamin E supplements in the gut may also have prevented the expected effects on semen parameters. It is possible that the oral uptake of the minerals and vitamin E supplement was poor in terms of stallions feeding behavioural habits which may include spilling the feed on the ground or the supplements may not have been palatable despite best efforts to ensure that the stallions were getting all of the supplements. As the minerals and vitamin E supplement was in a powder form it is possible that the stallions may have been able to avoid eating the supplement by spilling the feed on the ground and then only eating some of the feed. Oil on the other hand is very sticky and stallions would not have been able to avoid eating the supplement as easily. Also as mentioned earlier the basal diet may have affected the absorption of the minerals and vitamin E in the gut and therefore these types antioxidants may need to be given intravenously (IV).

The concentration and amount of supplements may have also been effected by the gut's ability to absorb certain compounds and how different compounds interact with one another during metabolism. If so, the absorption and effective use of these antioxidants to improve semen fertility parameter would have been affected by the composition of the supplements and the different base diets. It is possible that extreme quantities of minerals and vitamin E antioxidant are required before improvement in semen parameters would be observed. Some of the studies that have found significant improvements in semen parameters as a result of feeding antioxidants have often feed supplements for 80-100 days (almost two spermatogenesis cycles). A study in rabbits showed that it took longer for significant vitamins E levels to be reached in the spermatozoa membrane than in the blood or seminal plasma (Castellini, et al., 2007). Therefore antioxidant supplement in the diet may require a longer time to build up to higher concentrations and therefore warrants further investigations into more effective ways of administering antioxidants in the diet. However there was a concern that feeding these supplements in excessive amounts could have lead to other problems such as too much zinc, manganese and copper which can cause reductions in the body ability to utilise very important iron in the diet, selenium can be extremely toxic at high levels and high levels of omega-3 have also been suggested to reduce vitamin E absorption (NRC, 2007; NRC, 1989).

Both low and high levels of selenium have been suggested to be detrimental to spermatogenesis (Shanlini & Bansal, 2006). Another study has found low to moderate levels of vitamin E is absorbed in the blood, liver and testis but high level of vitamin E supplementation had similar results to control non-supplemented diets in goats (Hong, et al., 2010). Therefore it is possible that the body regulates the absorption of minerals and vitamins to ensure damage to spermatogenesis does not occur as a result of high concentrations of antioxidants as ROS still play an important role in capacitation and acrosome reactions.

Beneficial effect of feeding oil the other sperm parameters may have been observed if a fish-based omega-3 oil would have been used in this study as fish oils and linseed oils are richer in Omega-3 than canola oil (Pagan, 2008). However, finding a palatable fish oil in New Zealand proved to be very difficult due MAF regulations limiting the possibility of getting fish-oil supplements from overseas, and fish-oil supplements proved to be expensive, thus the canola oil was chosen for its availability and low cost.

Antioxidants are found in many of the body's cells in the control of excessive ROS production and are essential not only for fertility but for many other biological functions as well. Mineral levels were investigated in the blood before and after treatment, though there was no analysis to investigate whether the minerals and vitamin E were being absorbed and utilised in the male reproductive tract instead of being metabolised elsewhere in the body. It may have been worthwhile analysing the levels of minerals and vitamin E in the seminal plasma for the raw ejaculates and DHA concentrations in the plasma membranes. This may have also been useful to ensure that high levels of antioxidants were being reached in the blood and semen before semen parameters were investigated.

4.9.2 Possible Study Limitations

The experimental design of this study may have contributed to the results. The experimental design of this study was to originally include twenty-four Standardbred stallions. However fourteen stallions participate in this study for the duration of the trial. Results from small group sample sizes may have compromised the statistical analysis as individual stallion variation would have

influenced the statistical results more than if there had been larger sample group sizes.

Other studies have used the same stallions in a cross over experimental design where the stallions in the study were split into a control group and a treatment group, supplement was feed to the treatment group for 80 days and then after a 'wash out' period of no supplementation the groups reverse so the control group became the treatment group, and visa versa. This is a very effective way of increasing the experimental group sizes and further studies may consider this experimental design if time permitted it.

There were also a lot of variables in this study resulting from the stallions being housed at different studs around New Zealand, therefore it was hard to control for all possible environmental and treatment effects; such as husbandry practices, semen handling procedures, sterility of the AV's used, cooling rates of semen before it was packaged for chilled semen shipping, per cycle pregnancy data that was collected, and many other factors. This could have been controlled better if the stallion were all housed at the same place for the duration of the study.

4.10 Logistical Problems Encountered During This Study

There were a several logistical problems encountered during this study. The original experiment design of this study was to include 24 stallions of varying fertility from around New Zealand. Eighteen stallions were volunteered at the beginning of the study and a further 4 stallions were withdrawn during the trial period for reasons unrelated to this study.

The three studs involved in this study were located some distance from Equibreed NZ Ltd (one in the North Island and two in the South Island). Therefore I was not able to personally supervise all the aspects the trial procedures and was dependent upon the cooperation of others on site.

There were also problems associated with starting the supplemented diet at one stud resulting in a delayed start, and complete pregnancy data required to calculate the per cycle pregnancy rates was not able to be supplied from that same stud.

All of the information and cooperation given during this study by the staff at the different studs was much appreciated and although there were logistical problems encountered the experience that has been obtained from this study will be invaluable for further research.

These logistical problems were managed by the investigator making regular contact by phone or questionnaire with each of the studs. Furthermore the supplements were prelabelled and pre-measured to ensure ease of application and compliance with the study design.

CHAPTER FIVE: CONCLUSION AND FURTHER STUDIES

5.1 Conclusion

This study demonstrates that there was a statistically significant effect of feeding oil as a dietary supplement on sperm motility at 24h after collection and also on the per cycle pregnancy rates. Consequently, we were able to elucidate a difference in the actions of mineral and vitamin E supplementation when compared with oil supplementation on fertility parameters measured in this trial. Other beneficial effects of antioxidant supplementation on sperm parameters were suggested but were not found to be statistically significant.

5.2 Further Study

The effect of antioxidants in the control of ROS production is important to investigate as excessive ROS production is believed to be one of the leading causes of reduced fertility in many species. Although this study has shown that mineral and vitamin E antioxidant supplementation had no effect on semen parameters in Standardbred stallions, the effect of oil supplementation on semen fertility parameters warrants further investigation in more controlled environments with larger numbers of stallions. The different mechanisms of the two types of antioxidants and their effects on fertility is of considerable interest as we do not know enough about the role of antioxidants and spermatogenesis and fertilisation to clearly understand their physiological role and how ROS's affect fertility in horses. This is extremely difficult to study as there are many enzymes, molecules, and hormones that contribute to the process of spermatogenesis and fertilisation.

Human studies have shown improvements in sperm-oocyte fusion despite observing no improvements in semen parameters (Aitkens & Clarkson, 1988). Therefore further studies should include analyses of the effect of antioxidant dietary supplementation on sperm-oocyte fusion. As shown in this study per cycle

pregnancy rates significantly increase when supplementing the diet with oil even though supplement did not significantly improve other sperm fertility parameters.

Per cycle pregnancy rates should not only be performed in further antioxidant supplementations studies with larger sample sizes, but should also be investigated during the middle of the breeding season to reduce the effect of problem mares that are usually being bred towards the end of the breeding season however, in our study the control group accounted for these factors over time.

Other aspects of the stallion diet such as different minerals, vitamins or oils, or feeding higher concentrations of these supplements may be worthwhile investigating in further studies. However the most effective way to investigate the effects of various minerals, vitamins or oils may have on semen parameters is by making the stallions deficient in them before beginning the experimental treatment. Though it is unlikely this type of experiment would receive ethical approval due to the detrimental effects deficiency in these mineral, vitamins and oils may cause.

Also dietary action of antioxidants has still not been established (Zini, et al., 2009). Further study is required to determine how dietary antioxidants may be utilised in the male reproductive tract and if so where and when. For example it may be that antioxidants are more important during the embryonic development of the male reproductive tract, or even during puberty. However this would be very difficult to determine as spermatogenesis is very complicated with many spermatozoa at different stages of development and the development of an embryo is still being studied.

Further studies should investigate when the addition of antioxidants would be the most effect. Previous studies have been performed to investigate the effects of adding antioxidants to semen extender to improve semen quality have had varying success (Ball, et al., 2001b; Bruemmer, et al., 2008; Dennistion, et al., 2000; Maldjianm, et al., 2005). Further study in this area may be needed to find reliable combinations of antioxidants to be added to semen extenders.

Future studies should include DNA fragmentation analysis if the funding is available as there are no studies to our knowledge that have looked at the effects of feeding antioxidants on DNA fragmentation in horses. Other studies have also included membrane compositions in their analyses and this may also be worthwhile to perform in further studies.

A previous study has looked at the genetics of Hanoverian warmblood stallions in relation to per cycle pregnancy rates and has found a CRISP3 gene that is associated with fertility (Hamann, et al., 2007). Further studies should look at genetics in stallion for common pattern in genome deletions that correlation to ROS production and similar fertility associated genes in different horse breeds. This information may be useful to develop a way to monitor young colts for their fertility success rather than having to wait until they mature. Associations may even be able to incorporate a genetic requirement of no more than x amount of Y chromosome deletions in a stallion is allowed for it to be registered. This may help to improve the fertility genetics of stallions worldwide by adding genetic fertility characteristic into selective breeding options along with performance and conformation.

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APPENDIX 1: MEDIA AND STAINS

Table A1. 1 Media and Stain Components

Stallion Freezing Extender (Cochran et al., 1984)

Ingredients	Amount
Component 1	
Lactose	44g
Penicillin G	0.32g
Streptomycin Sulphate	0.32g
Sterile Water	up to 400ml
Component 2	
Glucose	12g
Tri Sodium Citrate	0.74g
EDTA	0.74g
Sodium Bicarbonate	0.24g
Penicillin G	0.16g
Streptomycin Sulphate	0.16g
Sterile Water	up to 200ml
Final	
Component 1	400ml
Component 2	200ml
Egg Yolk	160ml
Equex paste	4ml
Centrifuge prior to storage	
Add 5% glycerol prior to use	

Eosin-Nigrosin Stain (Barth & Oko, 1989)

Ingredients	Amount
Eosin (water soluble)	1.67g
Nigrosin (water soluble)	10g
Sodium Citrate	2.9g
Pure water	up to 100mls
filter prior to use	

FITC-PNA Stain (Cheng et al., 1996)

Ingredients	Amount
Peanut aggluthinin FITC conjugated	1000ug
PBS	10ml
Final concentration = 100ug/ml	

UCD Mounting Media (Weston, 2005)

Ingredients	Amount
Sodium Azide	0.005g
p-phenylenediamine	0.005g
PBS	0.5 ml
Glycerol	4.5 ml

APPENDIX 2: INTRUMENTS, MATERIALS AND CHEMICALS

Table A2. 1 Instruments and materials used in this study.

Item	Company	Specifications
Semen Straws	MINITUBE	0.25cc
Breakers	BIOLAB NZ	2 250ml, 70mm glass
Centrifuge	MINITUBE	Centra CL2
Slides	BIOLAB NZ	Plain 25 x 75 x 1mm
Slides	BIOLAB NZ	Frosted 25 x 75 x 1mm
Cover Slides	BIOLAB NZ	22 x 22mm
Centrifuge Tube	BIOLAB NZ	15ml
Haemocytometer	MINITUBE	
Immersion Oil	BIOLAB NZ	Type A 4oz
Liquid Nitrogen Tank	Gases	
Olympus Microscope	Olympus NZ	BHB
WILD Microscope	Heerbrugg, Switzerland	M20
Fluorescent Microscope	Leica DMRE	50 watt mercury vapour lamp, Olympus D70, Ex filter BP 450-490
Pipette	BIOLAB NZ	3ml, Disposable
Pipette	BIOLAB NZ	Eppendorf 2-20µl
Pipette	BIOLAB NZ	Eppendorf 200-1000µl
Pipette Tips	BIOLAB NZ	2-200µl
Pipette Tips	BIOLAB NZ	50-1000µl

Table A2. 2 Chemicals used in this study.

Item	Company	Specifications
Ethanol	BIOLAB NZ	70% Absolute
Sterile Water	Rurakura NZ	
Penicillin G	Sigma	P3032-100 million units
EDTA	Sigma	E1644-100G
Streptomycin Sulfate	Sigma	S-9137
Equex Paste	MINITUBE	8oz (STM)
Glycerol	BUOLAB	Molecular Biology Grade
Glucose	BIOLAB	
Lactose	Sigma	L3750-500G
Tri Sodium Citrate	Sigma	AH86048 335
Sodium Bicarbonate	Sigma	55761-500G

APPENDIX 3: SUPPLEMENTS AND DIET

Nutritech NZ 'Stallion Feed Supplement' used as the minerals and vitamin E supplement in this study. The supplement contained Oilseed meals, Vitamin E, Zinc Protienate, Yeast Extracts, Molasses, yeast by products (yeast cell wall) and Antioxidants. Any further details of the supplement are confidential and therefore cannot be included in this study.

Table A3. 1 Canola Oil used as the omega-3 oil supplement in this study. Budget brand. Ingredients include Canola Oil (9-11% omega-3), Antioxidants (319, 320, 321). Serving per pack: 150. Serving Size: 20ml

	Average Quantity	
Nutrient	Per Serving	Per 100ml
Energy	681kJ	3400kJ
Protein	Nil	Nil
Fat - Total	18g	92g
- Saturated	1g	7g
Carbohydrate	Nil	Nil
- Sugar	Nil	Nil
Sodium	Nil	Nil

Table A3. 2 Amount of minerals in total diet. NRC (mg) = recommended level of each mineral in complete diet, trial supplement = Amount of minerals in 30gm of the mineral and vitamin E trial supplement. Amounts of minerals in Dunstan and NRM diets feed daily and the total amounts of mineral included in the supplemented diets. Information provided by Dr. Lucy Tucker from Waiti Hill Ltd, Palmerston North, NZ (2009).

Minerals	NRC (gm)	Trial Supplement (mg/30g)	Dunstan Diet (mg)	Dunstan Total + Trial Supplement (mg)	NRM Diet (mg)	NRM Total +1/2 Trial Supplement (mg)
Se (Sel-Plex)	0.1	0.3	0.9	1.2	3	3.2
Zn (Bioplex)	40	80	359	439	728	768
Cu (Bioplex)	10	10	126	136	271	276
Mn (Bioplex)	40	40	558	598	742	762

Table A3. 3 Modified Dunstans diet. Dunstans Cool Feed Extra (mg/Kg) = amount of each nutrient in one kg of feed. NRC (100%) recommended levels of nutrients. NRM (50%) half the amount of supplement recommended compared to half the amount of nutrient provided in the feed and half in hay. NRC good grass hay is an approximation of the amount of nutrient provided in hay fed to stallions. Nutrients in the complete diet of hay and Dunstans Cool Feed Extra in one kilo of complete diet fed to stallions during the treatment period of the study and the difference between the NRC recommended levels and the Dunstans Cool Feed Xtra + Hay diet. The Dunstans feed was modified to include only half of their usual additives. The modified Dunstans feed + hay are also shown in this table. Information provided by Dr. Lucy Tucker (pers. Comm. 2009)

Nutrient	**Dunstans Cool Feed Extra spec (% or mg/kg)**	Daily intake NRC (50%) (gm)	Daily intake NRC (100%) (gm)	Amount of nutrient from feed (gm_	NRC good grass hay (% or mg)	Nutrients from hay supplies (gm)	Nutrients in Complete Diet (gm)	Difference between NRC & Dunstans Diet (gm)	Modified Dunstans feed + Hay (gm)
Dig energy Mcal/kg	3.178	10.9	21.8	3.430	2.18	5.00	21.8	0	
Crude protein %	11.4	394.5	789	391	13.3	665	1056	267	
Lysine %	0.493	16.95	33.9	17	0.46	23	40	6	
Ca %	0.899	15	30	31	0.66	33	64	34	48.4
P %	0.499	9	18	17	0.29	15	32	14	23.1
Na %	0.537	6.95	13.9	18	0.08	4	22	9	13.2
Cl %	0.994	23.3	46.6	34	0.92	46	80	33	63.0
K %	1.154	14.25	28.5	40	2.13	107	146	118	126.3
Mg %	0.241	4.75	9.5	8	0.23	12	20	10	15.6
Fe % (0.016%)	160	200	400	549	194	970	1519	1119	1244.4
Zn mg/kg	136.6	200	400	469	25	125	594	194	359.3
Mn mg/kg	115.6	200	400	396	72	360	756	356	558.2
Cu mg/kg	46.92	50	100	161	9	45	206	106	125.5
I mg/kg	1.238	1.75	3.5	4	0	0	4	1	2.1
Co mg/kg	1.193	0.25	0.5	4	0	0	4	4	2.0
Se mg/kg	0.37	0.5	1	1	0.06	0	2	1	0.9
S %	0.177	7.5	15	6	0.24	12	18	3	15.0

Table A3. 4 NRM Diet. NRM (mg/Kg) = amount of each nutrient in one kg of feed. NRC (100%) recommended levels of nutrients. NRM (50%) half the amount of supplement recommended compared to half the amount of nutrient provided in the feed and hay. NRC good grass hay is an approximation of the amount of nutrient provided in hay fed to stallions. Nutrients in the complete diet of hay and NRM feed in one kilo of complete diet fed to stallions during the study and the difference between the NRC recommended levels and the NRM + Hay diet. Information provided by Dr. Lucy Tucker (pers. Comm. 2009).

Nutrient	**NRM (mg/kg)**	Daily intake NRC (50%) (gm)	Daily intake NRC (100%) (gm)	Amount of nutrients from feed (gm)	NRC good grass hay (% or mg)	Nutrient from hay supplies (gm)	Nutrients in Complete Diet (gm)	Difference between NRC & NRM Diets (gm)
Dig energy Mcal/kg	2.8	10.9	21.8	3.893	2.18	5.00	21.8	0
Crude protein %	13.5	394.5	789	526	13.3	665	1191	402
Lysine %	0.55	16.95	33.9	21	0.46	23	44	11
Ca %	0.8	15	30	31	0.66	33	64	34
P %	0.6	9	18	23	0.29	15	38	20
Na %	0.43	6.95	13.9	17	0.08	4	21	7
Cl %	0.6	23.3	46.6	23	0.92	46	69	23
K %	0.95	14.25	28.5	37	2.13	107	143	115
Mg %	0.2	4.75	9.5	8	0.23	12	19	10
Fe % (0.016%)		200	400	0	194	970	970	570
Zn mg/kg	155	200	400	603	25	125	728	328
Mn mg/kg	98	200	400	382	72	360	742	342
Cu mg/kg	58	50	100	226	9	45	271	171
I mg/kg	0.55	1.75	3.5	2	0	0	2	-1
Co mg/kg		0.25	0.5	0	0	0	0	-1
Se mg/kg	0.67	0.5	1	3	0.06	0	3	2
S %		7.5	15	0	0.24	12	12	-3

APPENDIX 4: CORRESPONDENCE

Address
Address
Address

22-05-08

Dear Stud Owners

DO YOU KNOW IF YOUR STALLION'S DIETARY SUPPLEMENTS WORK?

EquiBreed NZ Ltd is collaborating with Dr Lucy Tucker and The University of Waikato in a study to evaluate the efficacy of dietary supplementation with antioxidants on fertility parameters in stallions.

As you may be aware, stallions have poor semen quality in comparison to cattle, sheep, dogs and pigs. Stallions that are under high stress levels, often due to exercise or a busy breeding season, produce metabolic breakdown products called reactive oxygen species, which damage sperm cells and reduce their longevity and viability. This results in the break down of the sperm plasma membranes and reduces fertility. Previous studies have suggested that supplementation with dietary antioxidants helps protect the sperm cells by preventing damage to the sperm plasma membrane and improving sperm motility and morphology. To date, however, no studies have actually evaluated the effect of these dietary supplements on the longevity of chilled semen, frozen semen or indeed the gold standard of fertility – actual pregnancy rates.

The purpose of this study is to investigate if antioxidant supplementation does actually increase the fertility in your stallions. If your stallions are in good health and on a balanced diet, then perhaps supplementation with dietary antioxidants is not justified, and simply expensive. Alternatively, if it does work then this research will pave the way for further study that will narrow down the key components that are required to increase fertility in your stallions.

This is an independent scientific study that will be performed under the supervision of Dr Lee Morris from Equibreed NZ Ltd, Dr Lucy Tucker and the University of Waikato. The stallions will be coded and not identified by name or stud, so you can be assured that all results will be kept confidential. This will be the first large scale fertility trial in horses on nutrition and reproduction and the results from this study will hopefully optimize the semen quality and longevity in your stallions, and ultimately your stallion's pregnancy rates.

We would like to invite you to provide semen from your stallions for this study. We hope to recruit a minimum of 24 stallions of varying fertilities to participate in this trial. All of the stallions will be fed a balanced diet providing the recommended NRC levels for good nutrition. Stallions will be allocated to different supplements (including a control group), and a single dose of semen will be required once a fortnight to be shipped to Equibreed NZ Ltd (Hamilton airport) for analysis. During the trial we will evaluate the semen upon arrival at our laboratory and again 24h later and also freeze 2 straws of semen to test the "freezability" of the semen. All semen will be discarded after evaluation and will not be used to inseminate any mares. At the end of the trial we will be able to provide you with an objective report on the semen quality of your stallions throughout the season. We would then like to evaluate the per cycle pregnancy rates of these stallions and correlate those results with the semen evaluation and dietary supplementation.

We would be delighted to include your stallions in our trial and we hope that we can provide you with useful information about your stallions. If you are interested in being a part of this study please fill out the enclosed questionnaire and return to EquiBreed NZ Ltd with your details. Once you have expressed your interest in this trial then we would like to arrange to meet you to discuss the details of the trial and ensure that it will be rewarding for us all.

If you have any further questions please contact Dr Lee Morris at Equibreed NZ Ltd.

Yours Sincerely

Lee Morris BVSc DVSc DipACT
Registered Specialist – Veterinary Reproduction

Figure A4. 1 Letter sent to Standardbred studs all over New Zealand to inform they about this study and to ask them to volunteer their stallions to participate in this study.

Research Consent Form

I have read the attached letter of information and agree to my stallion's participation in this research project - "Evaluation of dietary supplementation with antioxidants on fertility parameters in stallions".

I understand that:

1. My participation in this study is voluntary.
2. I have the right to withdraw my stallion(s) from this research at any time.
3. Data may be collected through questionnaires and semen samples. This data will be kept confidential and securely stored.
4. Data obtained during the research project will be used for the purpose of writing a thesis in partial fulfilment of a Master of Science degree. This data will be reported anonymously.
5. I can direct any questions to Jody Blomfield, University of Waikato (email: jab55@waikato.ac.nz, Ph: 021 293 4022).
6. For any unresolved issues I can seek advice from the research's mentor Dr. Lee Morris (email: lee@equibreed.co.nz, Ph: 07 827 9312).

I give consent for my stallion to be involved in this project under the conditions set out above.

Name: _____
Signed: _____
Date: _____

Please return to Equibreed Ltd via the self addresses envelope included.

Figure A4. 2 Research consent form sent out to all of the studs along with the letter (Fig A4.2). The studs that volunteer stallions to participate in this study filled out this form and return along with questionnaire (Fig A4.4).

Name of Stallion Owner: _____ _____

Name of Stud: _____

Address:_____

_____

Ph No.: _____ Fax:_____

Email: _____

Confidential Questionnaire

Name of Stallion:_____

Age of Stallion:_____ _____

Number of breeding seasons:_____

Exercised during breeding season: Yes / No

End of season pregnancy rates: (Please answer the following as 'No. of mare pregnant / No. of mare breed)

Pregnant rates from the pervious breeding season: _____

Pregnancy rates from chilled semen last season: _____

Pregnancy rates from frozen semen last season: _____

How often is this stallion collected from each week on average during the breeding season? _____

Basic Diet from last breeding season: _____

_____

What sort of fertility supplements have you used previously? _____

Please return Questionnaire by 30th June, 2008 to:

EquiBreed NZ Ltd, 673 State Hwy 1, RD 4 ,Cambridge 3496

Figure A4. 3 Questionnaire sent out to all the studs before the study started. Information from this questionnaire was used to separate the stallions into treatment groups and gain background information about each stallion.

Table A4. 1 Information provided by the studs in reply to the first questionnaire.

Standardbred Stud	Stallion ID	Age	# Breeding Seasons	Exercised	Preg Rates (# Preg/# Mares Breed)	Chilled Preg Rate (#Preg/# Mares AI)	Frozen Preg Rate (#Preg/# Mares AI)	Semen Collected	Diet	Supplements Given Previously
A	1	6	1	No	N/A	N/A	N/A	Every 2nd Day	-	-
B	2	11	6	No	N/A	95%/ 45	N/A	Every 2nd Day	Oats, Small amount of hay	
B	3	22	16	No	N/A	80% / 250	N/A	Every 2nd Day	Protein mix, Oats, Chaff, Lucerne Hay	Hyronate in the vein for arthritic Joints every month
A	4	8	3	No	94% /65	80% /96	N/A	Every 2nd Day	Pasture leucerne balage, Sweetfeed	Selenium Gold, Vit E
B	5	6	1	No		80% / 85	N/A	Every 2nd Day	Oats, Leucerne balage	
B	6	14	8	No	N/A	80% / 75	N/A	Every 2nd Day	Oats, Lucerne balage	
B	7	13	6	Yes-Walks pad	N/A	N/A	N/A	Every 2nd Day	Oats, Leucerne balage	
B	8	6	0	-	N/A	N/A	N/A		-	
C	9	19	14	No	81% / 100	83%/ 66	N/A	Every 2nd Day	Pasture + 3KG Asset Daily	Omega 3 (Fish Oil)
C	10	-	0	No	N/A	N/A	N/A	Every 2nd Day	-	-
C	11	9	3	No	85% /189	83% / 141	N/A	Every 2nd Day	Pasture + 3KG Asset Daily	-
C	12	5	1	No	N/A	N/A	N/A	Every 2nd Day	-	-
C	13	12	7	No	78% /69	86% /35	-	Every 2nd Day	Pasture + 2KG Asset Daily	-
C	14	16	12	No	77% /84	73% /71	N/A	Every 2nd Day	Pasture + 2KG Asset Daily	Omega 3 (Fish Oil)
A	15	15	10	No	88%/113	92%/130	N/A	Every 2nd Day	Pasture, leucerne balage, Sweetfeed	Selenium Gold, Vit E
B	16	16	11	No	N/A	30-40%	N/A	Every 2nd Day	Protein mix, Oats, Chaff, Lucerne Hay	
B	17	14	8	Yes-Walks pad	80% / 60	N/A	N/A	Every 2nd Day	Oats, Lucerne balage	
B	18	11	5	No	100% / 3	N/A	N/A	?	Oats, Hay	

29 October, 2008

Address
Address
Address

Dear Stud Owner

Thank you very much for your participation in our stallion semen trial to date. For your interest, I have enclosed a copy of the average pretreatment semen motility assessments on the samples that your stallions have provided thus far.

We are now about to start the exciting part of the trial and to include the antioxidant supplements in their basal diets as described below:

Stallion name	**Treatment group**	**Amount daily**
11	Powder	1 pottel (30g)
12	Powder	
13	Oil	1 pottel (30mls)
14	Oil	
9	No treatment (control)	Zero
10	No treatment (control)	Zero

Start date: 15th November, 2008.

The resealable bags are also labelled to ensure that each stallion is provided with the correct treatment.

Please note that the group of stallions not fed supplement are a control group to allow us to make comparisons between groups and evaluate the supplement.

The next series of semen collections required for completion of the trial will be January. We will require 3 more ejaculates from each stallion. I will contact you shortly to arrange the best dates that will suit you at that time of the year.

Please do not hesitate to contact me if you have any questions about these treatments. Thank-you very much again for you participation in this fertility trial and we look to your continued support.

Yours Sincerely

Jody Blomfield

Figure A4. 4 Letter Sent out with pre-measured pottels of supplements to give stud owners and stud staff detailed information about which stallion is to be give what supplement and when to begin feeding the supplements.

9 January, 2009

Address
Address
Address

Dear Stud Owner

Thank you very much for your participation in our stallion semen trial to date. For you interest, I have enclosed a copy of the results from the pre-treatment blood samples. Please note that these blood samples are taken to compare the effect the supplementary diet has on the mineral levels in the stallion's blood.

I have also enclosed a questionnaire about the stallions' behavioural responses to the supplementary diet. I would be grateful if you would mind asking the member of your team that feeds your stallions to fill out this questionnaire and send it back to me at Equibreed NZ Ltd.

We are now coming towards the end of this trial and I look forward to receiving the semen samples from your stallions. Also we will require another blood sample from each of your stallions to compare to the pre-treatment blood samples. I will send you the needles and tubes required for collecting these samples shortly.

Please do not hesitate to contact me if you have any questions. Thank-you very much for your participation in this fertility trial and we look forward to your continued support and hope that you find the results interesting.

Yours Sincerely

Jody Blomfield

Figure A4. 5 Cover letter sent out with questionnaire about that stallions' behavioural responses to the supplemented diets (Fig A4.6) to ensure that they were being feed the supplements and if the stallions' eating habits meant they were eating all of the supplement.

Confidential Questionnaire

Name of Person Feeding Stallion: _____
Date that the stallions were started on the supplement: _____/_____/_____
Date that the stallions were started on the Dunstans Feed: _____/_____/_____
Which stallions are being feed a supplement and what type of supplement have they been receiving?

_____

How much supplement are the stallions being feed each day?

_____

Are the supplements being divided up over a couple of feeds or are they being feed in one feed?

_____

Are the stallions eating all of their supplemented feeds? Yes / No
How have the stallions responded to the supplementary diet? Is the diet very palatable?

_____

Have there been any problems associated with the supplementary diet? If so Please explain:

_____

Please comment on each stallion's behavioural responses to the supplementary diet: (e.g. 'Doesn't eat it straight away but does eat it all' or 'drops a lot of his feed as he eats').
13: _____
9:_____
11: _____
12:_____
14:_____
10:_____
Please return Questionnaire by 2nd February, 2009 to:
EquiBreed NZ Ltd, 673 State Hwy 1, RD 4 ,Cambridge 3496

Figure A4. 6 Questionnaire sent out to all of the studs asking about the stallions' behavioural responses to the supplemented diets to ensure that they were being feed the supplements and if the stallions' eating habits meant they were eating all of the supplement. Unfortunately the time that this questionnaire was sent out was around the peak of the breeding season when the studs were their busiest and this questionnaire was not returned. However stud were contacted and interviewed regarding how the stallions were going on the supplemented diets and ensured that the stallions were getting the all of the supplements.

24 July, 2009

Address
Address
Address

Dear Stud Owner

Thank you very much for your participation in our stallion semen trial. For your interest, I have enclosed a summary of the motility and blood results from this fertility trial. We hope that you find the results interesting and informative.

As you can see from the motility results, although the semen was frozen 6-8 hours after it had been collected the overall post thaw motility rates looked good. Please don't hesitate to contact us at Equibreed NZ Ltd if you wish to send us semen to be frozen in the future.

I have also enclosed a questionnaire about the stallions' per cycle pregnancy rates for the 2008-2009 breeding season. I would be very grateful if you could please fill out these questionnaires and send them back to me at Equibreed NZ Ltd by 7th of August, 2009.

We have now come to the end of this fertility trial and I have enjoyed working with you. Without your help this fertility trial would not have been possible. Upon completion of the statistical analysis, I will forward you a summary of all the results.

Please do not hesitate to contact me if you have any questions. Thank-you very much for your participation in this fertility trial and we look forward to your questionnaire.

We hope that you had a very successful breeding season and wish you well for the upcoming season.

Yours Sincerely

Jody Blomfield

Figure A4. 7 Cover Letter sent out with the per cycle pregnancy rate questionnaire (Fig A4.8) after the supplement trial period.

Confidential Questionnaire

Name of Stallion:_____ **13**_____

Exercised during breeding season: Yes / No

Please fill in the table below:

	No. Of Mares	No. Of Cycles	No. Of Inseminations	No. Of pregnancies at 15 days
Last breeding season				
From the 15th of September till 15th of November				
From the 16th of November till 14th of January				
From the 15th of January till 15th March				

Name of Stallion:_____ **9**_____

Exercised during breeding season: Yes / No

Please fill in the table below:

	No. Of Mares	No. Of Cycles	No. Of Inseminations	No. Of pregnancies at 15 days
Last breeding season				
From the 15th of September till 15th of November				
From the 16th of November till 14th of January				
From the 15th of January till 15th March				

Please return Questionnaire by 7th August, 2009 to:

EquiBreed NZ Ltd, 673 State Hwy 1, RD 4 ,Cambridge 3496

Figure A4. 8 Questionnaire sent out after the supplementation trial period to obtain information required to work out per cycle pregnancy rates.

APPENDIX 5: STATISTICAL GRAPHS

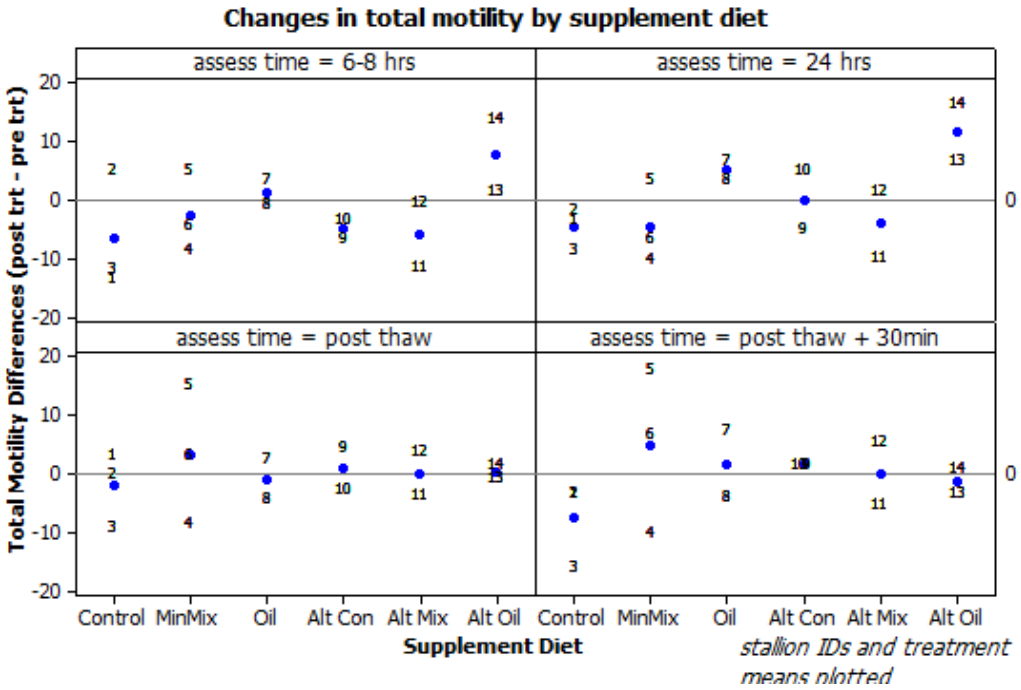

Figure A5. 1 This graph shows the differences between the means of pre and post treatment for total motility. The circle plots represent the mean differences for the treatment groups. The numbers plotted represent the differences between the means of before and after treatment (post-pre) for each stallion in each treatment group. Figure provided by Ray Littler.

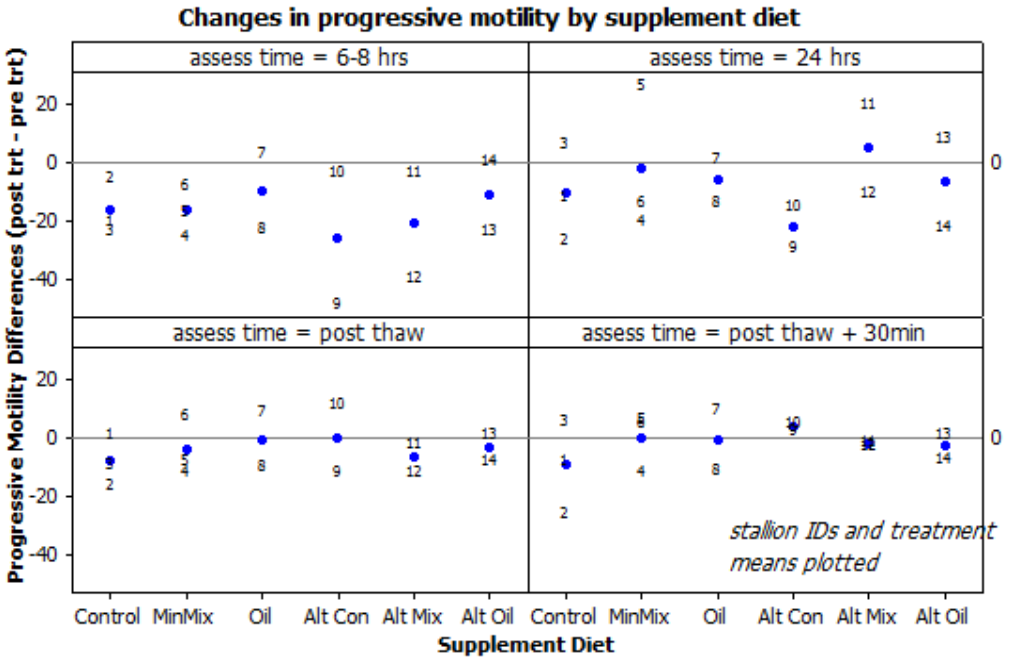

Figure A5. 2 This graph shows the differences between the means of pre and post treatment for progressive motility. The circle plots represent the mean differences for the treatment groups. The numbers plotted represent the differences between the means of before and after treatment (post-pre) for each stallion in each treatment group. Figure provided by Ray Littler.

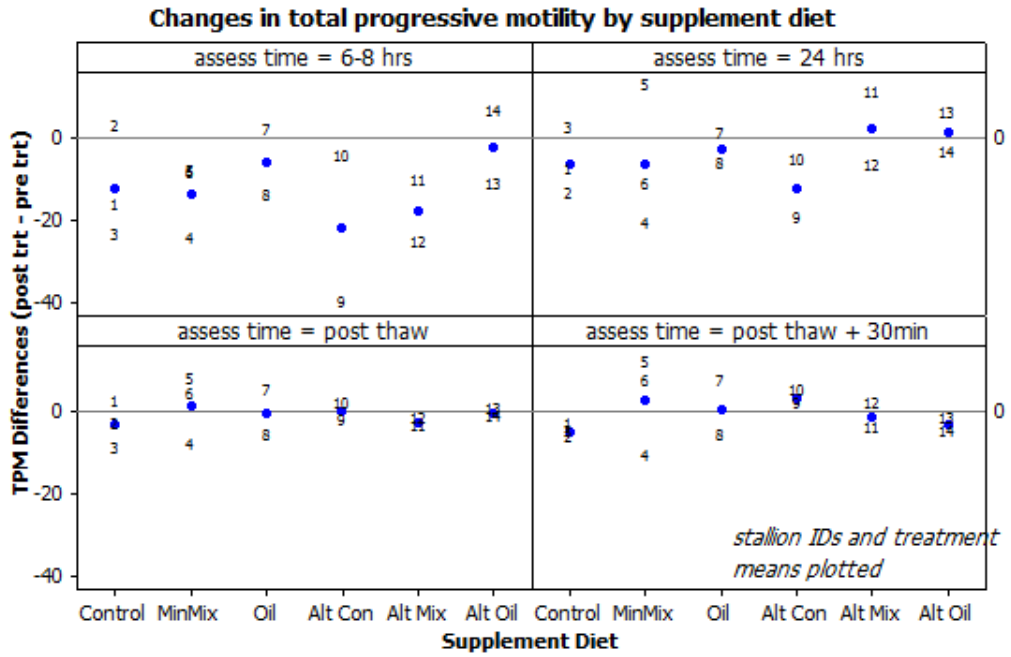

Figure A5. 3 This graph shows the differences between the means of pre and post treatment for total progressive motility. The circle plots represent the mean differences for the treatment groups. The numbers plotted represent the differences between the means of before and after treatment (post-pre) for each stallion in each treatment group. Figure provided by Ray Littler.

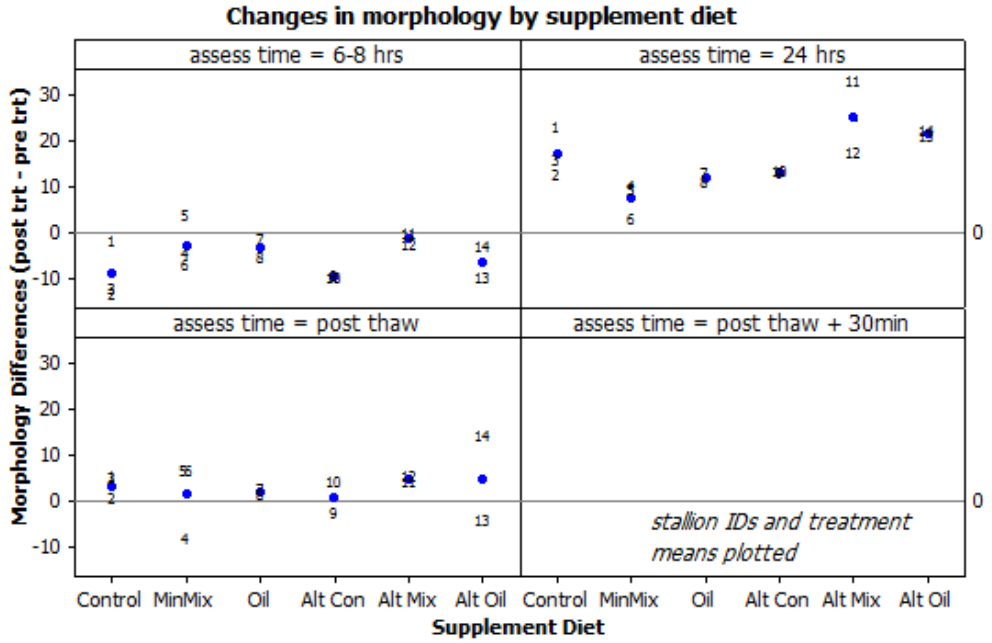

Figure A5. 4 This graph shows the differences between the means of pre and post treatment for the percentage of morphologically normal spermatozoa. The circle plots represent the mean differences for the treatment groups. The numbers plotted represent the differences between the means of before and after treatment (post-pre) for each stallion in each treatment group. Figure provided by Ray Littler.

APPENDIX 6: RAW DATA

Table A6. 1 Raw data of all semen parameter analysed. Tx= Treatment, Assesst= Assessment, PM= Progressive Motility, TPM, Total Progressive Motility, Acrosome = Intact Acrosome, HOS = Hypo-osmotic Swelling, Conc= Concentration of Spermatozoa.

Stallion ID	Treatment Diet	Sample	Date	Tx Period	Assesst time	Total motility (%)	PM (%)	TPM (%)	Morphology (%)	Acrosome (%)	HOS (%)	Conc ($x10^6.ml^{-1}$)
1	Control	1	15/09/2008	Pre Tx	6-8h	60	70	42	20			
1	Control	1	15/09/2008	Pre Tx	24h	60	50	30	26			
1	Control	1	15/09/2008	Pre Tx	Post thaw 0	30	67.5	20.3	48	13		
1	Control	1	15/09/2008	Pre Tx	Post thaw 30	27.5	62.5	17.2			37	18867
1	Control	2	22/09/2008	Pre Tx	6-8h	80	50	40	17			
1	Control	2	22/09/2008	Pre Tx	24h	75	50	37.5	17			
1	Control	2	22/09/2008	Pre Tx	Post thaw 0	22.5	57.5	12.9	48	4		
1	Control	2	22/09/2008	Pre Tx	Post thaw 30	22.5	52.5	11.8			28	9867
1	Control	3	30/09/2008	Pre Tx	6-8h	70	30	21	18			
1	Control	3	30/09/2008	Pre Tx	24h	40	10	4	15			
1	Control	3	30/09/2008	Pre Tx	Post thaw 0	32.5	62.5	20.3	40	52		
1	Control	3	30/09/2008	Pre Tx	Post thaw 30	30	70	21.0			25	14200
2	Control	1	26/09/2008	Pre Tx	6-8h	60	80	48	18			
2	Control	1	26/09/2008	Pre Tx	24h	50	60	30	19			
2	Control	1	26/09/2008	Pre Tx	Post thaw 0	25	80	20.0	32	19		
2	Control	1	26/09/2008	Pre Tx	Post thaw 30	22.5	70	15.8			44	6000
2	Control	2	6/10/2008	Pre Tx	6-8h	60	70	42	24			
2	Control	2	6/10/2008	Pre Tx	24h	50	60	30	30			
2	Control	2	6/10/2008	Pre Tx	Post thaw 0	30	75	22.5	43	7		
2	Control	2	6/10/2008	Pre Tx	Post thaw 30	30	80	24.0			19	22067
2	Control	3	6/01/2009	Pre Tx	6-8h	80	40	32	33			
2	Control	3	6/01/2009	Pre Tx	24h	60	20	12	24			
2	Control	3	6/01/2009	Pre Tx	Post thaw 0	30	60	18.0	46	42		
2	Control	3	6/01/2009	Pre Tx	Post thaw 30	27.5	60	16.5			30	9067

3	Control	1	26/09/2008	Pre Tx	6-8h	85	80	68	25			
3	Control	1	26/09/2008	Pre Tx	24h	60	40	24	22			
3	Control	1	26/09/2008	Pre Tx	Post thaw 0	42.5	77.5	32.9	34	5		
3	Control	1	26/09/2008	Pre Tx	Post thaw 30	57.5	60	34.5			34	17333
3	Control	2	6/10/2008	Pre Tx	6-8h	80	30	24	19			
3	Control	2	6/10/2008	Pre Tx	24h	70	10	7	27			
3	Control	2	6/10/2008	Pre Tx	Post thaw 0	45	62.5	28.1	47	0		
3	Control	2	6/10/2008	Pre Tx	Post thaw 30	45	55	24.8			24	7200
3	Control	3	6/01/2009	Pre Tx	6-8h	80	40	32	28			
3	Control	3	6/01/2009	Pre Tx	24h	60	5	3	20			
3	Control	3	6/01/2009	Pre Tx	Post thaw 0	40	72.5	29.0	44	29		
3	Control	3	6/01/2009	Pre Tx	Post thaw 30	42.5	60	25.5			38	6000
4	Min & Vit	1	15/09/2008	Pre Tx	6-8h	80	80	64	26			
4	Min & Vit	1	15/09/2008	Pre Tx	24h	80	50	40	19			
4	Min & Vit	1	15/09/2008	Pre Tx	Post thaw 0	35	72.5	25.4	45	5		
4	Min & Vit	1	15/09/2008	Pre Tx	Post thaw 30	40	70	28.0			24	27200
4	Min & Vit	2	22/09/2008	Pre Tx	6-8h	90	80	72	17			
4	Min & Vit	2	22/09/2008	Pre Tx	24h	80	90	72	30			
4	Min & Vit	2	22/09/2008	Pre Tx	Post thaw 0	45	77.5	34.9	61	26		
4	Min & Vit	2	22/09/2008	Pre Tx	Post thaw 30	42.5	82.5	35.1			29	21400
4	Min & Vit	3	30/09/2008	Pre Tx	6-8h	70	80	56	17			
4	Min & Vit	3	30/09/2008	Pre Tx	24h	60	20	12	14			
4	Min & Vit	3	30/09/2008	Pre Tx	Post thaw 0	35	65	22.8	41	69		
4	Min & Vit	3	30/09/2008	Pre Tx	Post thaw 30	40	65	26.0			30	20400
5	Min & Vit	1	26/09/2008	Pre Tx	6-8h	40	80	32	11			
5	Min & Vit	1	26/09/2008	Pre Tx	24h	30	20	6	26			
5	Min & Vit	1	26/09/2008	Pre Tx	Post thaw 0	17.5	70	12.3	20	31		
5	Min & Vit	1	26/09/2008	Pre Tx	Post thaw 30	17.5	42.5	7.4			56	7667
5	Min & Vit	2	6/10/2008	Pre Tx	6-8h	75	90	67.5	18			
5	Min & Vit	2	6/10/2008	Pre Tx	24h	55	10	5.5	28			
5	Min & Vit	2	6/10/2008	Pre Tx	Post thaw 0	30	80	24.0	31	4		
5	Min & Vit	2	6/10/2008	Pre Tx	Post thaw 30	35	82.5	28.9			38	13867
5	Min & Vit	3	7/11/2008	Pre Tx	6-8h	80	90	72	9			
5	Min & Vit	3	7/11/2008	Pre Tx	24h	70	70	49	28			

5	Min & Vit	3	7/11/2008	Pre Tx	Post thaw 0	27.5	72.5	19.9	45	18		
5	Min & Vit	3	7/11/2008	Pre Tx	Post thaw 30	17.5	57.5	10.1			29	3667
6	Min & Vit	1	26/09/2008	Pre Tx	6-8h	90	70	63	37			
6	Min & Vit	1	26/09/2008	Pre Tx	24h	70	50	35	36			
6	Min & Vit	1	26/09/2008	Pre Tx	Post thaw 0	50	80	40.0	30	31		
6	Min & Vit	1	26/09/2008	Pre Tx	Post thaw 30	42.5	50	21.3			34	7467
6	Min & Vit	2	6/10/2008	Pre Tx	6-8h	70	70	49	15			
6	Min & Vit	2	6/10/2008	Pre Tx	24h	70	40	28	38			
6	Min & Vit	2	6/10/2008	Pre Tx	Post thaw 0	40	75	30.0	63	53		
6	Min & Vit	2	6/10/2008	Pre Tx	Post thaw 30	40	75	30.0			34	30200
6	Min & Vit	3	7/11/2008	Pre Tx	6-8h	70	30	21	25			
6	Min & Vit	3	7/11/2008	Pre Tx	24h	60	5	3	41			
6	Min & Vit	3	7/11/2008	Pre Tx	Post thaw 0	27.5	50	13.8	32	53		
6	Min & Vit	3	7/11/2008	Pre Tx	Post thaw 30	30	77.5	23.3			27	40733
7	Oil	1	6/10/2008	Pre Tx	6-8h	70	50	35	8			
7	Oil	1	6/10/2008	Pre Tx	24h	60	20	12	18			
7	Oil	1	6/10/2008	Pre Tx	Post thaw 0	37.5	60	22.5	28	22		
7	Oil	1	6/10/2008	Pre Tx	Post thaw 30	35	60	21.0			55	13067
7	Oil	2	7/11/2008	Pre Tx	6-8h	50	10	5	9			
7	Oil	2	7/11/2008	Pre Tx	24h	30	0	0	7			
7	Oil	2	7/11/2008	Pre Tx	Post thaw 0	35	32.5	11.4	26	26		
7	Oil	2	7/11/2008	Pre Tx	Post thaw 30	20	40	8.0			37	14400
7	Oil	3	6/01/2009	Pre Tx	6-8h	60	10	6	22			
7	Oil	3	6/01/2009	Pre Tx	24h	60	10	6	16			
7	Oil	3	6/01/2009	Pre Tx	Post thaw 0	32.5	65	21.1	22	9		
7	Oil	3	6/01/2009	Pre Tx	Post thaw 30	37.5	60	22.5			48	5600
8	Oil	1	26/09/2008	Pre Tx	6-8h	70	70	49	18			
8	Oil	1	26/09/2008	Pre Tx	24h	60	70	42	20			
8	Oil	1	26/09/2008	Pre Tx	Post thaw 0	37.5	77.5	29.1	24	25		
8	Oil	1	26/09/2008	Pre Tx	Post thaw 30	27.5	72.5	19.9			47	10733
8	Oil	2	6/10/2008	Pre Tx	6-8h	60	80	48	23			
8	Oil	2	6/10/2008	Pre Tx	24h	50	30	15	20			
8	Oil	2	6/10/2008	Pre Tx	Post thaw 0	37.5	70	26.3	46	35		
8	Oil	2	6/10/2008	Pre Tx	Post thaw 30	40	70	28.0			32	9600

8	Oil	3	7/11/2008	Pre Tx	6-8h	60	30	18	14			
8	Oil	3	7/11/2008	Pre Tx	24h	60	5	3	10			
8	Oil	3	7/11/2008	Pre Tx	Post thaw 0	42.5	70	29.8	38	5		
8	Oil	3	7/11/2008	Pre Tx	Post thaw 30	40	70	28.0			50	14800
9	1/2 Control	1	15/09/2008	Pre Tx	6-8h	85	90	76.5	19			
9	1/2 Control	1	15/09/2008	Pre Tx	24h	70	70	49	10			
9	1/2 Control	1	15/09/2008	Pre Tx	Post thaw 0	40	55	22.0	42	0		
9	1/2 Control	1	15/09/2008	Pre Tx	Post thaw 30	40	50	20.0			45	13133
9	1/2 Control	2	22/09/2008	Pre Tx	6-8h	75	95	71.3	25			
9	1/2 Control	2	22/09/2008	Pre Tx	24h	80	20	16	11			
9	1/2 Control	2	22/09/2008	Pre Tx	Post thaw 0	37.5	62.5	23.4	36	7		
9	1/2 Control	2	22/09/2008	Pre Tx	Post thaw 30	32.5	45	14.6			58	11733
9	1/2 Control	3	30/09/2008	Pre Tx	6-8h	70	80	56	18			
9	1/2 Control	3	30/09/2008	Pre Tx	24h	60	50	30	23			
9	1/2 Control	3	30/09/2008	Pre Tx	Post thaw 0	40	60	24.0	31	0		
9	1/2 Control	3	30/09/2008	Pre Tx	Post thaw 30	42.5	50	21.3			56	14067
10	1/2 Control	1	15/09/2008	Pre Tx	6-8h	70	80	56	18			
10	1/2 Control	1	15/09/2008	Pre Tx	24h	50	40	20	15			
10	1/2 Control	1	15/09/2008	Pre Tx	Post thaw 0	22.5	70	15.8	38	15		
10	1/2 Control	1	15/09/2008	Pre Tx	Post thaw 30	27.5	75	20.6			30	29400
10	1/2 Control	2	22/09/2008	Pre Tx	6-8h	75	40	30	16			
10	1/2 Control	2	22/09/2008	Pre Tx	24h	40	50	20	10			
10	1/2 Control	2	22/09/2008	Pre Tx	Post thaw 0	37.5	65	24.4	31	16		
10	1/2 Control	2	22/09/2008	Pre Tx	Post thaw 30	40	57.5	23.0			48	7267
10	1/2 Control	3	30/09/2008	Pre Tx	6-8h	70	80	56	19			
10	1/2 Control	3	30/09/2008	Pre Tx	24h	50	40	20	24			
10	1/2 Control	3	30/09/2008	Pre Tx	Post thaw 0	40	72.5	29.0	37	27		
10	1/2 Control	3	30/09/2008	Pre Tx	Post thaw 30	32.5	72.5	23.6			36	8733
11	1/2 Min & Vit	1	15/09/2008	Pre Tx	6-8h	80	70	56	17			
11	1/2 Min & Vit	1	15/09/2008	Pre Tx	24h	80	50	40	6			
11	1/2 Min & Vit	1	15/09/2008	Pre Tx	Post thaw 0	42.5	75	31.9	44	42		
11	1/2 Min & Vit	1	15/09/2008	Pre Tx	Post thaw 30	45	70	31.5			40	14933
11	1/2 Min & Vit	2	22/09/2008	Pre Tx	6-8h	85	90	76.5	15			
11	1/2 Min & Vit	2	22/09/2008	Pre Tx	24h	75	40	30	15			

11	1/2 Min & Vit	2	22/09/2008	Pre Tx	Post thaw 0	37.5	75	28.1	37	26		
11	1/2 Min & Vit	2	22/09/2008	Pre Tx	Post thaw 30	42.5	75	31.9			38	20267
11	1/2 Min & Vit	3	30/09/2008	Pre Tx	6-8h	90	70	63	15			
11	1/2 Min & Vit	3	30/09/2008	Pre Tx	24h	80	30	24	14			
11	1/2 Min & Vit	3	30/09/2008	Pre Tx	Post thaw 0	52.5	80	42.0	41	22		
11	1/2 Min & Vit	3	30/09/2008	Pre Tx	Post thaw 30	57.5	77.5	44.6			42	26333
12	1/2 Min & Vit	1	15/09/2008	Pre Tx	6-8h	60	80	48	8			
12	1/2 Min & Vit	1	15/09/2008	Pre Tx	24h	75	20	15	8			
12	1/2 Min & Vit	1	15/09/2008	Pre Tx	Post thaw 0	42.5	67.5	28.7	32	2		
12	1/2 Min & Vit	1	15/09/2008	Pre Tx	Post thaw 30	42.5	65	27.6			66	7533
12	1/2 Min & Vit	2	24/09/2008	Pre Tx	6-8h	70	60	42	8			
12	1/2 Min & Vit	2	24/09/2008	Pre Tx	24h	40	5	2	10			
12	1/2 Min & Vit	2	24/09/2008	Pre Tx	Post thaw 0	32.5	65	21.1	27	8		
12	1/2 Min & Vit	2	24/09/2008	Pre Tx	Post thaw 30	20	55	11.0			39	3400
12	1/2 Min & Vit	3	30/09/2008	Pre Tx	6-8h	70	80	56	17			
12	1/2 Min & Vit	3	30/09/2008	Pre Tx	24h	60	50	30	9			
12	1/2 Min & Vit	3	30/09/2008	Pre Tx	Post thaw 0	27.5	62.5	17.2	9	2		
12	1/2 Min & Vit	3	30/09/2008	Pre Tx	Post thaw 30	30	60	18.0			49	7733
13	1/2 Oil	1	15/09/2008	Pre Tx	6-8h	60	80	48	22			
13	1/2 Oil	1	15/09/2008	Pre Tx	24h	60	30	18	5			
13	1/2 Oil	1	15/09/2008	Pre Tx	Post thaw 0	42.5	52.5	22.3	38	12		
13	1/2 Oil	1	15/09/2008	Pre Tx	Post thaw 30	45	65	29.3			38	21400
13	1/2 Oil	2	22/09/2008	Pre Tx	6-8h	70	80	56	10			
13	1/2 Oil	2	22/09/2008	Pre Tx	24h	50	65	32.5	10			
13	1/2 Oil	2	22/09/2008	Pre Tx	Post thaw 0	30	65	19.5	41	22		
13	1/2 Oil	2	22/09/2008	Pre Tx	Post thaw 30	30	70	21.0			36	11200
13	1/2 Oil	3	2/10/2008	Pre Tx	6-8h	50	60	30	22			
13	1/2 Oil	3	2/10/2008	Pre Tx	24h	30	10	3	9			
13	1/2 Oil	3	2/10/2008	Pre Tx	Post thaw 0	15	70	10.5	29	29		
13	1/2 Oil	3	2/10/2008	Pre Tx	Post thaw 30	22.5	70	15.8			34	17733
14	1/2 Oil	1	15/09/2008	Pre Tx	6-8h	30	50	15	7			
14	1/2 Oil	1	15/09/2008	Pre Tx	24h	35	70	24.5	11			
14	1/2 Oil	1	15/09/2008	Pre Tx	Post thaw 0	22.5	45	10.1	19	0		
14	1/2 Oil	1	15/09/2008	Pre Tx	Post thaw 30	22.5	50	11.3			36	44867

14	1/2 Oil	2	22/09/2008	Pre Tx	6-8h	50	85	42.5	15			
14	1/2 Oil	2	22/09/2008	Pre Tx	24h	40	70	28	23			
14	1/2 Oil	2	22/09/2008	Pre Tx	Post thaw 0	27.5	60	16.4	6	1		
14	1/2 Oil	2	22/09/2008	Pre Tx	Post thaw 30	26.25	43.75	23.4			46	40500
14	1/2 Oil	3	30/09/2008	Pre Tx	6-8h	40	20	8	30			
14	1/2 Oil	3	30/09/2008	Pre Tx	24h	30	20	6	18			
14	1/2 Oil	3	30/09/2008	Pre Tx	Post thaw 0	37.5	60	22.5	23	0		
14	1/2 Oil	3	30/09/2008	Pre Tx	Post thaw 30	32.5	62.5	20.3			42	20400
1	Control	4	12/01/2009	Post Tx	6-8h	70	40	28	24			
1	Control	4	12/01/2009	Post Tx	24h	60	20	12.0	28			
1	Control	4	12/01/2009	Post Tx	Post thaw 0	30	67.5	20.3	47	26		
1	Control	4	12/01/2009	Post Tx	Post thaw 30	27.5	62.5	17.2			37	18867
1	Control	5	20/01/2009	Post Tx	6-8h	60	30	18	5			
1	Control	5	20/01/2009	Post Tx	24h	70	50	35.0	43			
1	Control	5	20/01/2009	Post Tx	Post thaw 0	35	65	22.8	47	54		
1	Control	5	20/01/2009	Post Tx	Post thaw 30	10	40	4.0			29	11467
1	Control	6	28/01/2009	Post Tx	6-8h	40	20	8	20			
1	Control	6	28/01/2009	Post Tx	24h	35	5	1.8	55			
1	Control	6	28/01/2009	Post Tx	Post thaw 0	30	60	18.0	57	2		
1	Control	6	28/01/2009	Post Tx	Post thaw 30	32.5	60	19.5			33	17000
2	Control	4	14/01/2009	Post Tx	6-8h	80	90	72	14			
2	Control	4	14/01/2009	Post Tx	24h	50	20	10.0	27			
2	Control	4	14/01/2009	Post Tx	Post thaw 0	22.5	32.5	7.3	49	10		
2	Control	4	14/01/2009	Post Tx	Post thaw 30	10	20	2.0			33	21333
2	Control	5	28/01/2009	Post Tx	6-8h	70	65	45.5	9			
2	Control	5	28/01/2009	Post Tx	24h	55	30	16.5	59			
2	Control	5	28/01/2009	Post Tx	Post thaw 0	25	60	15.0	39	20		
2	Control	5	28/01/2009	Post Tx	Post thaw 30	20	50	10.0			38	10000
2	Control	6	30/01/2009	Post Tx	6-8h	65	20	13	11			
2	Control	6	30/01/2009	Post Tx	24h	50	10	5.0	24			
2	Control	6	30/01/2009	Post Tx	Post thaw 0	37.5	75	28.1	34	18		
2	Control	6	30/01/2009	Post Tx	Post thaw 30	40	63	25.2			29	13200
3	Control	4	14/01/2009	Post Tx	6-8h	60	40	24	12			
3	Control	4	14/01/2009	Post Tx	24h	55	25	13.8	20			

3	Control	4	14/01/2009	Post Tx	Post thaw 0	22.5	50	11.3	44	0		
3	Control	4	14/01/2009	Post Tx	Post thaw 30	15	37.5	5.6			29	12200
3	Control	5	28/01/2009	Post Tx	6-8h	70	30	21	13			
3	Control	5	28/01/2009	Post Tx	24h	50	30	15.0	52			
3	Control	5	28/01/2009	Post Tx	Post thaw 0	42.5	67.5	28.7	42	1		
3	Control	5	28/01/2009	Post Tx	Post thaw 30	42.5	87.5	37.2			33	23467
3	Control	6	30/01/2009	Post Tx	6-8h	80	10	8	10			
3	Control	6	30/01/2009	Post Tx	24h	60	20	12.0	44			
3	Control	6	30/01/2009	Post Tx	Post thaw 0	35	67.5	23.6	53	27		
3	Control	6	30/01/2009	Post Tx	Post thaw 30	40	67.5	27.0			38	15400
4	Min & Vit	4	12/01/2009	Post Tx	6-8h	75	50	37.5	16			
4	Min & Vit	4	12/01/2009	Post Tx	24h	70	10	7.0	29			
4	Min & Vit	4	12/01/2009	Post Tx	Post thaw 0	35	65	22.8	39	46		
4	Min & Vit	4	12/01/2009	Post Tx	Post thaw 30	30	65	19.5			27	19000
4	Min & Vit	5	20/01/2009	Post Tx	6-8h	70	45	31.5	14			
4	Min & Vit	5	20/01/2009	Post Tx	24h	60	40	24.0	39			
4	Min & Vit	5	20/01/2009	Post Tx	Post thaw 0	37.5	77.5	29.1	45	6		
4	Min & Vit	5	20/01/2009	Post Tx	Post thaw 30	30	60	18.0			32	16867
4	Min & Vit	6	26/01/2009	Post Tx	6-8h	70	70	49	15			
4	Min & Vit	6	26/01/2009	Post Tx	24h	60	50	30.0	25			
4	Min & Vit	6	26/01/2009	Post Tx	Post thaw 0	17.5	37.5	6.6	38	1		
4	Min & Vit	6	26/01/2009	Post Tx	Post thaw 30	32.5	57.5	18.7			42	12333
5	Min & Vit	4	14/01/2009	Post Tx	6-8h	80	70	56	15			
5	Min & Vit	4	14/01/2009	Post Tx	24h	55	60	33.0	20			
5	Min & Vit	4	14/01/2009	Post Tx	Post thaw 0	35	67.5	23.6	48	6		
5	Min & Vit	4	14/01/2009	Post Tx	Post thaw 30	32.5	72.5	23.6			27	10333
5	Min & Vit	4	28/01/2009	Post Tx	6-8h	60	70	42	19			
5	Min & Vit	5	28/01/2009	Post Tx	24h	50	60	30.0	45			
5	Min & Vit	5	28/01/2009	Post Tx	Post thaw 0	40	67.5	27.0	45	20		
5	Min & Vit	5	28/01/2009	Post Tx	Post thaw 30	50	60	30.0			42	5733
5	Min & Vit	6	30/01/2009	Post Tx	6-8h	70	70	49	14			
5	Min & Vit	6	30/01/2009	Post Tx	24h	60	60	36.0	45			
5	Min & Vit	6	30/01/2009	Post Tx	Post thaw 0	45	65	29.3	22	1		
5	Min & Vit	6	30/01/2009	Post Tx	Post thaw 30	40	70	28.0			42	7600

6	Min & Vit	4	6/01/2009	Post Tx	6-8h	90	40	36	22			
6	Min & Vit	4	6/01/2009	Post Tx	24h	80	20	16.0	24			
6	Min & Vit	4	6/01/2009	Post Tx	Post thaw 0	40	80	32.0	51	2		
6	Min & Vit	4	6/01/2009	Post Tx	Post thaw 30	40	67.5	27.0			46	8200
6	Min & Vit	5	14/01/2009	Post Tx	6-8h	85	70	59.5	18			
6	Min & Vit	5	14/01/2009	Post Tx	24h	55	20	11.0	27			
6	Min & Vit	5	14/01/2009	Post Tx	Post thaw 0	37.5	80	30.0	61	32		
6	Min & Vit	5	14/01/2009	Post Tx	Post thaw 30	42.5	72.5	30.8			35	14400
6	Min & Vit	6	28/01/2009	Post Tx	6-8h	65	35	22.8	9			
6	Min & Vit	6	28/01/2009	Post Tx	24h	55	2	1.1	58			
6	Min & Vit	6	28/01/2009	Post Tx	Post thaw 0	42.5	80	34.0	47	40		
6	Min & Vit	6	28/01/2009	Post Tx	Post thaw 30	45	80	36.0			54	23067
6	Min & Vit	7	30/01/2009	Post Tx	6-8h	50	50	25	25			
6	Min & Vit	7	30/01/2009	Post Tx	24h	50	30	15.0	56			
6	Min & Vit	7	30/01/2009	Post Tx	Post thaw 0	50	65	32.5	33	0		
6	Min & Vit	7	30/01/2009	Post Tx	Post thaw 30	50	70	35.0			36	25467
7	Oil	4	14/01/2009	Post Tx	6-8h	70	40	28	18			
7	Oil	4	14/01/2009	Post Tx	24h	55	10	5.5	20			
7	Oil	4	14/01/2009	Post Tx	Post thaw 0	32.5	60	19.5	30	27		
7	Oil	4	14/01/2009	Post Tx	Post thaw 30	32.5	62.5	20.3			55	7467
7	Oil	5	28/01/2009	Post Tx	6-8h	60	30	18	7			
7	Oil	5	28/01/2009	Post Tx	24h	55	5	2.8	24			
7	Oil	5	28/01/2009	Post Tx	Post thaw 0	45	65	29.3	35	51		
7	Oil	5	28/01/2009	Post Tx	Post thaw 30	42.5	62.5	26.6			55	13533
7	Oil	6	30/01/2009	Post Tx	6-8h	60	10	6	9			
7	Oil	6	30/01/2009	Post Tx	24h	60	20	12.0	35			
7	Oil	6	30/01/2009	Post Tx	Post thaw 0	35	60	21.0	18	29		
7	Oil	6	30/01/2009	Post Tx	Post thaw 30	40	65	26.0			34	16400
8	Oil	4	6/01/2009	Post Tx	6-8h	50	30	15	20			
8	Oil	4	6/01/2009	Post Tx	24h	70	40	28.0	20			
8	Oil	4	6/01/2009	Post Tx	Post thaw 0	37.5	60	22.5	26	44		
8	Oil	4	6/01/2009	Post Tx	Post thaw 30	30	65	19.5			45	12533
8	Oil	5	14/01/2009	Post Tx	6-8h	80	50	40	6			
8	Oil	5	14/01/2009	Post Tx	24h	60	15	9.0	26			

8	Oil	5	14/01/2009	Post Tx	Post thaw 0	45	79	35.6	48	16		
8	Oil	5	14/01/2009	Post Tx	Post thaw 30	37.5	67.5	25.3			51	22133
8	Oil	6	28/01/2009	Post Tx	6-8h	60	40	24	18			
8	Oil	6	28/01/2009	Post Tx	24h	50	10	5.0	41			
8	Oil	6	28/01/2009	Post Tx	Post thaw 0	25	57.5	14.4	45	5		
8	Oil	6	28/01/2009	Post Tx	Post thaw 30	30	57.5	17.3			39	23867
8	Oil	7	30/01/2009	Post Tx	6-8h	60	30	18	7			
8	Oil	7	30/01/2009	Post Tx	24h	60	20	12.0	23			
8	Oil	7	30/01/2009	Post Tx	Post thaw 0	32.5	55	17.9	30	12		
8	Oil	7	30/01/2009	Post Tx	Post thaw 30	30	50	15.0			33	1800
9	1/2 Control	4	22/01/2009	Post Tx	6-8h	70	50	35	8			
9	1/2 Control	4	22/01/2009	Post Tx	24h	70	30	21.0	30			
9	1/2 Control	4	22/01/2009	Post Tx	Post thaw 0	37.5	45	16.9	41	0		
9	1/2 Control	4	22/01/2009	Post Tx	Post thaw 30	30	52.5	15.8			39	6333
9	1/2 Control	5	30/01/2009	Post Tx	6-8h	70	30	21	14			
9	1/2 Control	5	30/01/2009	Post Tx	24h	60	5	3.0	25			
9	1/2 Control	5	30/01/2009	Post Tx	Post thaw 0	50	50	25.0	26	36		
9	1/2 Control	5	30/01/2009	Post Tx	Post thaw 30	50	50	25.0			48	24933
10	1/2 Control	4	16/01/2009	Post Tx	6-8h	70	65	45.5	5			
10	1/2 Control	4	16/01/2009	Post Tx	24h	50	5	2.5	22			
10	1/2 Control	4	16/01/2009	Post Tx	Post thaw 0	30	70	21.0	37	12		
10	1/2 Control	4	16/01/2009	Post Tx	Post thaw 30	22.5	45	10.1			39	9200
10	1/2 Control	5	20/01/2009	Post Tx	6-8h	65	80	52	7			
10	1/2 Control	5	20/01/2009	Post Tx	24h	50	60	30.0	29			
10	1/2 Control	5	20/01/2009	Post Tx	Post thaw 0	22.5	85	19.1	45	3		
10	1/2 Control	5	20/01/2009	Post Tx	Post thaw 30	32.5	85	27.6			24	30600
10	1/2 Control	6	28/01/2009	Post Tx	6-8h	70	45	31.5	11			
10	1/2 Control	6	28/01/2009	Post Tx	24h	55	20	11.0	37			
10	1/2 Control	6	28/01/2009	Post Tx	Post thaw 0	40	87.5	35.0	36	6		
10	1/2 Control	6	28/01/2009	Post Tx	Post thaw 30	50	90	45.0			51	17667
11	1/2 Min & Vit	4	16/01/2009	Post Tx	6-8h	85	90	76.5	16			
11	1/2 Min & Vit	4	16/01/2009	Post Tx	24h	80	70	56.0	51			
11	1/2 Min & Vit	4	16/01/2009	Post Tx	Post thaw 0	40	75	30.0	43	10		
11	1/2 Min & Vit	4	16/01/2009	Post Tx	Post thaw 30	50	80	40.0			29	16600

11	1/2 Min & Vit	5	20/01/2009	Post Tx	6-8h	80	60	48	9			
11	1/2 Min & Vit	5	20/01/2009	Post Tx	24h	75	60	45.0	34			
11	1/2 Min & Vit	5	20/01/2009	Post Tx	Post thaw 0	40	82.5	33.0	52	69		
11	1/2 Min & Vit	5	20/01/2009	Post Tx	Post thaw 30	42.5	80	34.0			24	23333
11	1/2 Min & Vit	6	26/01/2009	Post Tx	6-8h	70	75	52.5	10			
11	1/2 Min & Vit	6	26/01/2009	Post Tx	24h	70	70	49.0	49			
11	1/2 Min & Vit	6	26/01/2009	Post Tx	Post thaw 0	45	75	33.8	51	70		
11	1/2 Min & Vit	6	26/01/2009	Post Tx	Post thaw 30	42.5	67.5	28.7			43	21733
11	1/2 Min & Vit	7	28/01/2009	Post Tx	6-8h	60	70	42	26			
11	1/2 Min & Vit	7	28/01/2009	Post Tx	24h	50	40	20.0	44			
11	1/2 Min & Vit	7	28/01/2009	Post Tx	Post thaw 0	37.5	67.5	25.3	32	26		
11	1/2 Min & Vit	7	28/01/2009	Post Tx	Post thaw 30	37.5	65	24.4			36	20733
12	1/2 Min & Vit	4	24/01/2009	Post Tx	6-8h	75	40	30	2			
12	1/2 Min & Vit	4	24/01/2009	Post Tx	24h	60	10	6.0	16			
12	1/2 Min & Vit	4	24/01/2009	Post Tx	Post thaw 0	37.5	67.5	25.3	21	11		
12	1/2 Min & Vit	4	24/01/2009	Post Tx	Post thaw 30	30	70	21.0			54	10133
12	1/2 Min & Vit	5	26/01/2009	Post Tx	6-8h	70	55	38.5	5			
12	1/2 Min & Vit	5	26/01/2009	Post Tx	24h	60	40	24.0	26			
12	1/2 Min & Vit	5	26/01/2009	Post Tx	Post thaw 0	35	40	14.0	32	37		
12	1/2 Min & Vit	5	26/01/2009	Post Tx	Post thaw 30	35	30	10.5			45	2933
12	1/2 Min & Vit	6	28/01/2009	Post Tx	6-8h	60	20	12	7			
12	1/2 Min & Vit	6	28/01/2009	Post Tx	24h	60	5	3.0	40			
12	1/2 Min & Vit	6	28/01/2009	Post Tx	Post thaw 0	40	62.5	25.0	37	14		
12	1/2 Min & Vit	6	28/01/2009	Post Tx	Post thaw 30	40	65	26.0			38	9200
12	1/2 Min & Vit	7	1/02/2009	Post Tx	6-8h	60	20	12	18			
12	1/2 Min & Vit	7	1/02/2009	Post Tx	24h	60	5	3.0	23			
12	1/2 Min & Vit	7	1/02/2009	Post Tx	Post thaw 0	40	45	18.0	22	1		
12	1/2 Min & Vit	7	1/02/2009	Post Tx	Post thaw 30	40	65	26.0			50	3600
13	1/2 Oil	4	24/01/2009	Post Tx	6-8h	60	70	42	14			
13	1/2 Oil	4	24/01/2009	Post Tx	24h	50	60	30.0	31			
13	1/2 Oil	4	24/01/2009	Post Tx	Post thaw 0	22.5	75	16.9	36	0		
13	1/2 Oil	4	24/01/2009	Post Tx	Post thaw 30	22.5	80	18.0			24	12000
13	1/2 Oil	5	28/01/2009	Post Tx	6-8h	75	70	52.5	4			
13	1/2 Oil	5	28/01/2009	Post Tx	24h	60	60	36.0	22			

13	1/2 Oil	5	28/01/2009	Post Tx	Post thaw 0	35	57.5	20.1	34	0		
13	1/2 Oil	5	28/01/2009	Post Tx	Post thaw 30	40	70	28.0			33	28267
13	1/2 Oil	6	30/01/2009	Post Tx	6-8h	50	10	5	6			
13	1/2 Oil	6	30/01/2009	Post Tx	24h	50	10	5.0	33			
13	1/2 Oil	6	30/01/2009	Post Tx	Post thaw 0	27.5	60	16.5	25	39		
13	1/2 Oil	6	30/01/2009	Post Tx	Post thaw 30	25	60	15.0			26	32733
14	1/2 Oil	4	16/01/2009	Post Tx	6-8h	50	70	35	11			
14	1/2 Oil	4	16/01/2009	Post Tx	24h	50	60	30.0	32			
14	1/2 Oil	4	16/01/2009	Post Tx	Post thaw 0	27.5	42.5	11.7	33	17		
14	1/2 Oil	4	16/01/2009	Post Tx	Post thaw 30	17.5	15	2.6			49	6867
14	1/2 Oil	5	22/01/2009	Post Tx	6-8h	60	50	30	14			
14	1/2 Oil	5	22/01/2009	Post Tx	24h	50	30	15.0	48			
14	1/2 Oil	5	22/01/2009	Post Tx	Post thaw 0	25	32.5	8.1	18	35		
14	1/2 Oil	5	22/01/2009	Post Tx	Post thaw 30	22.5	60	13.5			40	16467
14	1/2 Oil	6	26/01/2009	Post Tx	6-8h	55	60	33	3			
14	1/2 Oil	6	26/01/2009	Post Tx	24h	55	30	16.5	40			
14	1/2 Oil	6	26/01/2009	Post Tx	Post thaw 0	32.5	60	19.5	36	8		
14	1/2 Oil	6	26/01/2009	Post Tx	Post thaw 30	35	55	19.3			29	11333
14	1/2 Oil	7	28/01/2009	Post Tx	6-8h	50	30	15	28			
14	1/2 Oil	7	28/01/2009	Post Tx	24h	50	5	2.5	37			
14	1/2 Oil	7	28/01/2009	Post Tx	Post thaw 0	37.5	55	20.6	33	31		
14	1/2 Oil	7	28/01/2009	Post Tx	Post thaw 30	37.5	50	18.8			27	13467

Table A6. 2 Morphology raw data. TX= treatment, Assesst+ Assessment, HD= Head Defect, LH= Loose Head, BM= Bent Midpiece, Pro-D= Proximal Droplet, Dis-D= Distal Droplet, BT= Bent Tail, CT= Coiled Tail, CRT= Curved Tail. Values are in percentages (%).

Stallion ID	Treatment Diet	Sample	Date	Tx Period	Assesst time	Straight Tail	HD	LH	BM	MP	Prox-D	Dis-D	BT	CT	CRT	Other	Normal
1	Control	1	15/09/2008	Pre Tx	6-8h	5	28	1	11	6	3	1	26	4	15	0	20
1	Control	1	15/09/2008	Pre Tx	24h	1	33	0	5	10	4	2	20	0	25	0	26
1	Control	1	15/09/2008	Pre Tx	Post Thaw	2	17	4	4	8	7	0	11	0	46	1	48
1	Control	2	22/09/2008	Pre Tx	6-8h	2	22	1	4	6	5	1	41	3	15	0	17
1	Control	2	22/09/2008	Pre Tx	24h	2	22	2	10	1	9	4	32	3	15	0	17
1	Control	2	22/09/2008	Pre Tx	Post Thaw	4	28	2	4	3	6	0	9	0	44	0	48
1	Control	3	30/09/2008	Pre Tx	6-8h	5	23	3	2	7	6	4	35	2	13	0	18
1	Control	3	30/09/2008	Pre Tx	24h	0	23	1	10	6	6	4	34	1	15	1	15
1	Control	3	30/09/2008	Pre Tx	Post Thaw	2	21	5	3	2	0	5	22	1	38	1	40
2	Control	1	26/09/2008	Pre Tx	6-8h	3	12	4	11	7	11	5	32	0	15	0	18
2	Control	1	26/09/2008	Pre Tx	24h	0	8	5	15	20	8	3	22	0	19	0	19
2	Control	1	26/09/2008	Pre Tx	Post Thaw	3	18	0	8	2	19	4	14	1	29	2	32
2	Control	2	6/10/2008	Pre Tx	6-8h	1	9	4	10	10	6	5	32	0	23	0	24
2	Control	2	6/10/2008	Pre Tx	24h	1	5	5	6	8	21	2	23	0	29	0	30
2	Control	2	6/10/2008	Pre Tx	Post Thaw	2	3	2	7	13	6	5	20	1	41	0	43
2	Control	3	6/01/2009	Pre Tx	6-8h	4	12	3	11	13	5	2	21	0	29	0	33
2	Control	3	6/01/2009	Pre Tx	24h	1	4	1	18	11	8	6	28	0	23	0	24
2	Control	3	6/01/2009	Pre Tx	Post Thaw	7	8	10	6	8	4	1	16	0	39	1	46
3	Control	1	26/09/2008	Pre Tx	6-8h	2	10	0	9	9	1	2	44	0	23	0	25
3	Control	1	26/09/2008	Pre Tx	24h	1	5	14	8	5	5	4	34	0	21	2	22
3	Control	1	26/09/2008	Pre Tx	Post Thaw	5	5	2	4	6	3	2	33	1	39	0	44
3	Control	2	6/10/2008	Pre Tx	6-8h	6	8	5	16	3	1		48	0	13	0	19
3	Control	2	6/10/2008	Pre Tx	24h	1	6	3	15	5	1	0	42	0	26	1	27
3	Control	2	6/10/2008	Pre Tx	Post Thaw	1	3	3	17	4	10	7	20	1	33	1	34
3	Control	3	6/01/2009	Pre Tx	6-8h	0	2	1	1	20	5	5	36	2	28	0	28
3	Control	3	6/01/2009	Pre Tx	24h	2	7	0	11	13	2	1	45	0	18	1	20
3	Control	3	6/01/2009	Pre Tx	Post Thaw	2	4	3	0	3	12	1	29	1	45	0	47
4	Min & Vit	1	15/09/2008	Pre Tx	6-8h	5	16	2	4	11	8	1	30	2	21	0	26
4	Min & Vit	1	15/09/2008	Pre Tx	24h	3	20	1	8	10	9	1	31	1	16	0	19

4	Min & Vit	1	15/09/2008	Pre Tx	Post Thaw	2	21	2	6	4	5	0	15	2	43	0	45
4	Min & Vit	2	22/09/2008	Pre Tx	6-8h	1	10	4	4	11	0	3	50	1	16	0	17
4	Min & Vit	2	22/09/2008	Pre Tx	24h	3	14	0	9	12	9	2	22	2	27	0	30
4	Min & Vit	2	22/09/2008	Pre Tx	Post Thaw	5	8	5	3	3	7	1	11	0	56	1	61
4	Min & Vit	3	30/09/2008	Pre Tx	6-8h	3	9	1	8	10	4	3	46	1	14	1	17
4	Min & Vit	3	30/09/2008	Pre Tx	24h	0	6	3	12	10	7	5	42	1	14	0	14
4	Min & Vit	3	30/09/2008	Pre Tx	Post Thaw	5	7	4	8	9	11	6	13	0	36	1	41
5	Min & Vit	1	26/09/2008	Pre Tx	6-8h	1	3	4	22	26	4	2	27	1	10	0	11
5	Min & Vit	1	26/09/2008	Pre Tx	24h	1	7	1	20	7	27	0	11	0	25	1	26
5	Min & Vit	1	26/09/2008	Pre Tx	Post Thaw	2	7	2	22	5	20	5	19	0	18	0	20
5	Min & Vit	2	6/10/2008	Pre Tx	6-8h	8	5	5	20	10	1	0	39	0	10	2	18
5	Min & Vit	2	6/10/2008	Pre Tx	24h	0	9	0	0	7	31	1	24	0	28	0	28
5	Min & Vit	2	6/10/2008	Pre Tx	Post Thaw	2	12	0	12	5	16	10	13	1	29	0	31
5	Min & Vit	3	7/11/2008	Pre Tx	6-8h	0	3	1	15	12	2	1	56	1	9	0	9
5	Min & Vit	3	7/11/2008	Pre Tx	24h	1	4	0	20	17	6	2	23	0	27	0	28
5	Min & Vit	3	7/11/2008	Pre Tx	Post Thaw	7	16	1	7	3	8	4	15	0	38	1	45
6	Min & Vit	1	26/09/2008	Pre Tx	6-8h	6	10	0	1	4	2	3	42	1	31	0	37
6	Min & Vit	1	26/09/2008	Pre Tx	24h	2	6	3	7	11	2	1	33	0	34	1	36
6	Min & Vit	1	26/09/2008	Pre Tx	Post Thaw	1	15	3	3	4	10	11	24	0	29	0	30
6	Min & Vit	2	6/10/2008	Pre Tx	6-8h	4	5	2	16	7	3	2	49	1	11	0	15
6	Min & Vit	2	6/10/2008	Pre Tx	24h	3	5	1	1	15	3	4	32	1	35	0	38
6	Min & Vit	2	6/10/2008	Pre Tx	Post Thaw	1	4	2	1	2	5	8	14	0	62	1	63
6	Min & Vit	3	7/11/2008	Pre Tx	6-8h	3	6	0	2	21	0	0	46	0	22	0	25
6	Min & Vit	3	7/11/2008	Pre Tx	24h	5	2	1	13	14	0	7	21	1	36	0	41
6	Min & Vit	3	7/11/2008	Pre Tx	Post Thaw	5	10	0	8	6	4	2	38	0	27	0	32
7	Oil	1	6/10/2008	Pre Tx	6-8h	2	17	2	7	29	7	3	26	0	6	1	8
7	Oil	1	6/10/2008	Pre Tx	24h	4	15	2	11	14	29	1	8	1	14	1	18
7	Oil	1	6/10/2008	Pre Tx	Post Thaw	0	17	3	7	5	11	2	23	4	28	0	28
7	Oil	2	7/11/2008	Pre Tx	6-8h	1	25	5	3	29	4	0	22	2	8	1	9
7	Oil	2	7/11/2008	Pre Tx	24h	2	26	0	8	30	3	0	23	3	5	0	7
7	Oil	2	7/11/2008	Pre Tx	Post Thaw	2	16	6	5	12	11	4	19	1	24	0	26
7	Oil	3	6/01/2009	Pre Tx	6-8h	4	21	2	12	11	3	3	26	0	18	0	22
7	Oil	3	6/01/2009	Pre Tx	24h	0	24	1	10	14	9	4	22	0	16	0	16
7	Oil	3	6/01/2009	Pre Tx	Post Thaw	1	4	12	8	11	8	4	30	1	21	0	22

8	Oil	1	26/09/2008	Pre Tx	6-8h	1	13	0	16	13	4	2	33	1	17	0	18
8	Oil	1	26/09/2008	Pre Tx	24h	0	23	0	18	1	6	3	27	2	20	0	20
8	Oil	1	26/09/2008	Pre Tx	Post Thaw	4	12	4	20	8	5	0	24	3	20	0	24
8	Oil	2	6/10/2008	Pre Tx	6-8h	14	16	5	24	7	0	0	25	0	9	0	23
8	Oil	2	6/10/2008	Pre Tx	24h	0	12	0	20	14	1	0	33	0	20	0	20
8	Oil	2	6/10/2008	Pre Tx	Post Thaw	3	11	4	11	3	3	4	17	1	43	0	46
8	Oil	3	7/11/2008	Pre Tx	6-8h	5	10	5	30	8	0	0	32	1	9	0	14
8	Oil	3	7/11/2008	Pre Tx	24h	2	16	9	34	10	1	0	20	0	8	0	10
8	Oil	3	7/11/2008	Pre Tx	Post Thaw	1	10	3	9	2	4	8	23	2	37	1	38
9	1/2 Control	1	15/09/2008	Pre Tx	6-8h	2	15	2	11	16	13	3	18	3	17	0	19
9	1/2 Control	1	15/09/2008	Pre Tx	24h	2	19	2	9	15	8	2	19	3	21	0	23
9	1/2 Control	1	15/09/2008	Pre Tx	Post Thaw	1	13	7	8	7	7	1	12	3	41	0	42
9	1/2 Control	2	22/09/2008	Pre Tx	6-8h	6	9	8	17	8	5	0	24	2	19	2	25
9	1/2 Control	2	22/09/2008	Pre Tx	24h	1	12	1	18	13	15	1	28	2	9	0	10
9	1/2 Control	2	22/09/2008	Pre Tx	Post Thaw	0	16	1	10	10	17	2	5	2	36	1	36
9	1/2 Control	3	30/09/2008	Pre Tx	6-8h	7	4	16	18	5	0	0	35	4	11	0	18
9	1/2 Control	3	30/09/2008	Pre Tx	24h	0	18	3	22	25	6	3	11	1	11	0	11
9	1/2 Control	3	30/09/2008	Pre Tx	Post Thaw	0	14	3	14	6	6	6	19	1	31	0	31
10	1/2 Control	1	15/09/2008	Pre Tx	6-8h	3	7	2	8	26	9	9	21	0	15	0	18
10	1/2 Control	1	15/09/2008	Pre Tx	24h	1	9	15	12	17	13	4	12	0	14	3	15
10	1/2 Control	1	15/09/2008	Pre Tx	Post Thaw	0	8	2	5	8	24	2	13	0	38	0	38
10	1/2 Control	2	22/09/2008	Pre Tx	6-8h	2	17	1	15	23	10	3	15	0	14	0	16
10	1/2 Control	2	22/09/2008	Pre Tx	24h	0	15	1	13	18	26	2	13	2	10	0	10
10	1/2 Control	2	22/09/2008	Pre Tx	Post Thaw	0	10	3	7	9	15	1	21	3	31	0	31
10	1/2 Control	3	30/09/2008	Pre Tx	6-8h	1	7	2	12	10	6	6	33	5	18	0	19
10	1/2 Control	3	30/09/2008	Pre Tx	24h	1	13	3	16	9	4	8	23	0	23	0	24
10	1/2 Control	3	30/09/2008	Pre Tx	Post Thaw	0	20	1	10	1	9	6	14	2	37	0	37
11	1/2 Min & Vit	1	15/09/2008	Pre Tx	6-8h	5	2	2	8	14	17	4	34	2	12	0	17
11	1/2 Min & Vit	1	15/09/2008	Pre Tx	24h	0	5	0	14	28	14	0	33	0	6	0	6
11	1/2 Min & Vit	1	15/09/2008	Pre Tx	Post Thaw	0	10	5	5	7	11	1	13	3	44	1	44
11	1/2 Min & Vit	2	22/09/2008	Pre Tx	6-8h	1	5	1	14	18	10	0	37	0	14	0	15
11	1/2 Min & Vit	2	22/09/2008	Pre Tx	24h	2	12	1	10	14	13	3	32	0	13	0	15
11	1/2 Min & Vit	2	22/09/2008	Pre Tx	Post Thaw	5	13	0	5	8	18	4	13	1	32	1	37
11	1/2 Min & Vit	3	30/09/2008	Pre Tx	6-8h	1	8	5	7	15	11	3	30	1	14	5	15

11	1/2 Min & Vit	3	30/09/2008	Pre Tx	24h	5	11	8	27	20	3	2	14	1	9	0	14
11	1/2 Min & Vit	3	30/09/2008	Pre Tx	Post Thaw	4	12	0	2	9	7	7	21	1	37	0	41
12	1/2 Min & Vit	1	15/09/2008	Pre Tx	6-8h	0	9	5	16	14	13	3	25	7	8	0	8
12	1/2 Min & Vit	1	15/09/2008	Pre Tx	24h	0	15	3	23	20	1	1	22	3	9	3	9
12	1/2 Min & Vit	1	15/09/2008	Pre Tx	Post Thaw	3	16	6	7	4	17	1	14	2	29	1	32
12	1/2 Min & Vit	2	24/09/2008	Pre Tx	6-8h	2	14	1	17	8	11	2	36	3	6	0	8
12	1/2 Min & Vit	2	24/09/2008	Pre Tx	24h	0	19	0	10	27	16	2	15	3	8	0	8
12	1/2 Min & Vit	2	24/09/2008	Pre Tx	Post Thaw	0	15	4	18	10	2	1	18	4	27	1	27
12	1/2 Min & Vit	3	30/09/2008	Pre Tx	6-8h	0	17	3	11	21	5	0	24	2	17	0	17
12	1/2 Min & Vit	3	30/09/2008	Pre Tx	24h	2	20	2	18	16	11	0	22	1	8	0	10
12	1/2 Min & Vit	3	30/09/2008	Pre Tx	Post Thaw	0	26	2	21	5	14	3	19	2	9	0	9
13	1/2 Oil	1	15/09/2008	Pre Tx	6-8h	7	18	4	5	5	9	3	33	1	15	0	22
13	1/2 Oil	1	15/09/2008	Pre Tx	24h	2	23	5	15	11	10	0	31	0	3	0	5
13	1/2 Oil	1	15/09/2008	Pre Tx	Post Thaw	7	14	6	12	7	8	2	13	0	31	0	38
13	1/2 Oil	2	22/09/2008	Pre Tx	6-8h	4	12	3	12	25	14	1	23	0	6	0	10
13	1/2 Oil	2	22/09/2008	Pre Tx	24h	1	40	1	8	7	12	0	21	1	9	0	10
13	1/2 Oil	2	22/09/2008	Pre Tx	Post Thaw	0	14	1	4	4	9	6	20	0	41	1	41
13	1/2 Oil	3	2/10/2008	Pre Tx	6-8h	3	13	6	8	19	10	2	18	1	19	1	22
13	1/2 Oil	3	2/10/2008	Pre Tx	24h	2	22	2	12	29	3	2	20	0	7	1	9
13	1/2 Oil	3	2/10/2008	Pre Tx	Post Thaw	0	14	1	9	12	12	9	11	3	29	0	29
14	1/2 Oil	1	15/09/2008	Pre Tx	6-8h	0	14	30	13	14	6	3	11	2	7	0	7
14	1/2 Oil	1	15/09/2008	Pre Tx	24h	1	17	0	9	11	19	4	29	0	10	0	11
14	1/2 Oil	1	15/09/2008	Pre Tx	Post Thaw	0	9	46	6	3	3	0	12	2	19	0	19
14	1/2 Oil	2	22/09/2008	Pre Tx	6-8h	2	13	20	8	8	5	0	28	2	13	1	15
14	1/2 Oil	2	22/09/2008	Pre Tx	24h	0	19	18	7	7	8	1	17	0	23	0	23
14	1/2 Oil	2	22/09/2008	Pre Tx	Post Thaw	0	9	72	3	3	5	1	6	2	10	1	10
14	1/2 Oil	3	30/09/2008	Pre Tx	6-8h	2	7	10	13	10	9	3	21	5	20	0	22
14	1/2 Oil	3	30/09/2008	Pre Tx	24h	1	13	7	16	18	2	0	26	0	17	0	18
14	1/2 Oil	3	30/09/2008	Pre Tx	Post Thaw	1	6	45	14	1	6	2	2	1	22	0	23
1	Control	4	12/01/2009	Post Tx	6-8h	1	28	1	6	5	2	4	30	0	23	0	24
1	Control	4	12/01/2009	Post Tx	24h	5	12	2	14	4	3	6	29	0	23	2	28
1	Control	4	12/01/2009	Post Tx	Post Thaw	8	9	5	5	13	0	3	21	3	33	0	41
1	Control	5	20/01/2009	Post Tx	6-8h	2	25	2	14	8	2	0	42	2	3	0	5
1	Control	5	20/01/2009	Post Tx	24h	4	19	1	7	7	1	1	20	1	39	0	43

1	Control	5	20/01/2009	Post Tx	Post Thaw	3	11	1	5	3	4	5	22	2	44	0	47
1	Control	6	28/01/2009	Post Tx	6-8h	11	19	1	7	5	0	0	47	1	9	0	20
1	Control	6	28/01/2009	Post Tx	24h	6	15	0	5	1	3	1	19	1	49	0	55
1	Control	6	28/01/2009	Post Tx	Post Thaw	0	12	2	3	3	0	2	21	0	57	0	57
2	Control	4	14/01/2009	Post Tx	6-8h	1	3	3	10	14	1	3	52	0	13	0	14
2	Control	4	14/01/2009	Post Tx	24h	1	9	1	12	12	9	0	30	0	26	0	27
2	Control	4	14/01/2009	Post Tx	Post Thaw	1	1	2	9	6	5	1	26	0	48	1	49
2	Control	5	28/01/2009	Post Tx	6-8h	1	20	0	21	13	1	2	31	1	8	2	9
2	Control	5	28/01/2009	Post Tx	24h	2	4	0	7	19	6	0	5	0	57	0	59
2	Control	5	28/01/2009	Post Tx	Post Thaw	1	8	2	1	15	13	5	12	0	38	5	39
2	Control	6	30/01/2009	Post Tx	6-8h	4	26	1	13	13	1	0	33	2	7	0	11
2	Control	6	30/01/2009	Post Tx	24h	5	15	0	12	8	1	0	40	0	19	0	24
2	Control	6	30/01/2009	Post Tx	Post Thaw	3	13	8	4	6	5	2	26	0	31	2	34
3	Control	4	14/01/2009	Post Tx	6-8h	2	20	1	3	9	2	1	51	1	10	0	12
3	Control	4	14/01/2009	Post Tx	24h	2	3	1	16	8	1	0	48	2	18	1	20
3	Control	4	14/01/2009	Post Tx	Post Thaw	0	6	1	1	6	7	6	27	1	44	1	44
3	Control	5	28/01/2009	Post Tx	6-8h	3	31	0	6	16	0	0	34	0	10	0	13
3	Control	5	28/01/2009	Post Tx	24h	0	5	0	5	7	7	2	21	0	52	1	52
3	Control	5	28/01/2009	Post Tx	Post Thaw	0	15	5	2	3	4	3	25	1	42	0	42
3	Control	6	30/01/2009	Post Tx	6-8h	4	16	2	24	15	0	0	28	3	6	2	10
3	Control	6	30/01/2009	Post Tx	24h	1	9	1	7	8	3	0	28	0	43	0	44
3	Control	6	30/01/2009	Post Tx	Post Thaw	4	8	3	0	8	8	3	17	0	49	0	53
4	Min & Vit	4	12/01/2009	Post Tx	6-8h	6	15	0	17	22	1	1	26	2	10	0	16
4	Min & Vit	4	12/01/2009	Post Tx	24h	0	13	1	13	14	6	0	24	0	29	0	29
4	Min & Vit	4	12/01/2009	Post Tx	Post Thaw	4	10	0	10	5	3	0	32	0	35	1	39
4	Min & Vit	5	20/01/2009	Post Tx	6-8h	2	15	2	17	25	1	1	23	1	12	1	14
4	Min & Vit	5	20/01/2009	Post Tx	24h	4	11	0	3	19	5	0	21	1	35	1	39
4	Min & Vit	5	20/01/2009	Post Tx	Post Thaw	1	7	2	3	5	7	12	17	2	44	0	45
4	Min & Vit	6	26/01/2009	Post Tx	6-8h	1	7	0	19	21	12	5	21	0	14	0	15
4	Min & Vit	6	26/01/2009	Post Tx	24h	6	11	3	18	10	0	0	33	0	19	0	25
4	Min & Vit	6	26/01/2009	Post Tx	Post Thaw	2	10	3	5	21	3	1	17	1	36	1	38
5	Min & Vit	4	14/01/2009	Post Tx	6-8h	0	9	6	8	9	2	2	49	0	15	0	15
5	Min & Vit	4	14/01/2009	Post Tx	24h	2	22	2	20	4	0	0	31	0	18	1	20
5	Min & Vit	4	14/01/2009	Post Tx	Post Thaw	1	9	4	1	3	6	1	26	1	47	1	48

5	Min & Vit	4	28/01/2009	Post Tx	6-8h	5	10	5	21	13	6	0	25	1	14	0	19
5	Min & Vit	5	28/01/2009	Post Tx	24h	1	12	0	13	7	15	1	7	0	44	0	45
5	Min & Vit	5	28/01/2009	Post Tx	Post Thaw	3	10	8	7	5	7	1	13	2	42	2	45
5	Min & Vit	6	30/01/2009	Post Tx	6-8h	4	38	3	14	16	4	2	8	1	10	0	14
5	Min & Vit	6	30/01/2009	Post Tx	24h	2	2	0	16	11	2	1	23	0	43	0	45
5	Min & Vit	6	30/01/2009	Post Tx	Post Thaw	6	8	8	29	13	0	1	19	0	16	0	22
6	Min & Vit	4	6/01/2009	Post Tx	6-8h	3	11	1	4	13	3	2	44	0	19	0	22
6	Min & Vit	4	6/01/2009	Post Tx	24h	4	16	3	30	4	1	0	21	0	20	1	24
6	Min & Vit	4	6/01/2009	Post Tx	Post Thaw	6	7	0	2	6	4	3	25	0	45	2	51
6	Min & Vit	5	14/01/2009	Post Tx	6-8h	0	10	0	10	3	1	1	56	1	18	0	18
6	Min & Vit	5	14/01/2009	Post Tx	24h	6	10	2	13	9	0	0	39	0	21	0	27
6	Min & Vit	5	14/01/2009	Post Tx	Post Thaw	3	7	1	4	8	5	1	12	0	58	1	61
6	Min & Vit	6	28/01/2009	Post Tx	6-8h	0	10	2	16	6	2	1	50	4	9	0	9
6	Min & Vit	6	28/01/2009	Post Tx	24h	2	6	1	2	13	2	1	17	0	56	0	58
6	Min & Vit	6	28/01/2009	Post Tx	Post Thaw	2	6	6	4	3	6	10	17	0	45	1	47
6	Min & Vit	7	30/01/2009	Post Tx	6-8h	4	32	0	3	12	5	0	23	0	21	0	25
6	Min & Vit	7	30/01/2009	Post Tx	24h	8	6	1	7	12	3	0	15	0	48	0	56
6	Min & Vit	7	30/01/2009	Post Tx	Post Thaw	1	4	6	0	4	4	6	43	0	32	0	33
7	Oil	4	14/01/2009	Post Tx	6-8h	12	7	3	12	19	1	0	30	0	0	0	28
7	Oil	4	14/01/2009	Post Tx	24h	0	19	1	7	12	0	0	41	0	20	0	20
7	Oil	4	14/01/2009	Post Tx	Post Thaw	1	24	5	5	16	8	2	10	0	29	0	30
7	Oil	5	28/01/2009	Post Tx	6-8h	1	17	8	10	11	1	0	34	1	17	0	18
7	Oil	5	28/01/2009	Post Tx	24h	0	20	3	5	19	4	0	24	0	24	1	24
7	Oil	5	28/01/2009	Post Tx	Post Thaw	3	23	2	8	17	3	0	11	1	32	0	35
7	Oil	6	30/01/2009	Post Tx	6-8h	3	34	19	6	9	0	1	24	0	3	3	7
7	Oil	6	30/01/2009	Post Tx	24h	9	21	3	5	8	6	0	22	0	26	0	35
7	Oil	6	30/01/2009	Post Tx	Post Thaw	2	17	8	4	14	7	2	30	1	16	0	18
8	Oil	4	6/01/2009	Post Tx	6-8h	5	11	1	19	17	2	1	28	1	15	0	20
8	Oil	4	6/01/2009	Post Tx	24h	0	12	0	20	14	1	0	33	0	20	0	20
8	Oil	4	6/01/2009	Post Tx	Post Thaw	2	8	2	7	11	2	1	42	1	24	0	26
8	Oil	5	14/01/2009	Post Tx	6-8h	0	23	2	19	10	1	1	37	0	6	1	6
8	Oil	5	14/01/2009	Post Tx	24h	0	23	0	7	8	3	1	32	0	26	0	26
8	Oil	5	14/01/2009	Post Tx	Post Thaw	5	9	4	4	7	1	3	22	2	43	0	48
8	Oil	6	28/01/2009	Post Tx	6-8h	4	24	1	6	15	0	0	36	0	14	0	18

8	Oil	6	28/01/2009	Post Tx	24h	2	21	1	8	8	1	2	16	2	39	0	41
8	Oil	6	28/01/2009	Post Tx	Post Thaw	3	11	4	7	8	2	4	18	1	42	0	45
8	Oil	7	30/01/2009	Post Tx	6-8h	1	47	1	7	8	1	0	27	2	6	0	7
8	Oil	7	30/01/2009	Post Tx	24h	0	8	1	11	14	1	1	38	2	23	1	23
8	Oil	7	30/01/2009	Post Tx	Post Thaw	1	8	3	10	9	3	2	33	2	29	0	30
9	1/2 Control	4	22/01/2009	Post Tx	6-8h	3	21	9	20	20	2	0	19	1	5	0	8
9	1/2 Control	4	22/01/2009	Post Tx	24h	1	14	0	4	23	16	4	7	2	29	0	30
9	1/2 Control	4	22/01/2009	Post Tx	Post Thaw	0	18	2	9	6	12	2	9	1	41	0	41
9	1/2 Control	5	30/01/2009	Post Tx	6-8h	1	24	2	11	27	1	0	20	1	13	0	14
9	1/2 Control	5	30/01/2009	Post Tx	24h	0	12	0	13	34	9	1	4	2	25	0	25
9	1/2 Control	5	30/01/2009	Post Tx	Post Thaw	2	9	5	6	6	11	2	34	1	25	0	26
10	1/2 Control	4	16/01/2009	Post Tx	6-8h	1	16	1	4	15	11	19	29	0	4	0	5
10	1/2 Control	4	16/01/2009	Post Tx	24h	1	10	1	3	3	24	11	25	1	21	0	22
10	1/2 Control	4	16/01/2009	Post Tx	Post Thaw	1	10	1	8	11	9	3	21	0	36	0	37
10	1/2 Control	5	20/01/2009	Post Tx	6-8h	2	17	2	17	12	0	0	44	1	5	0	7
10	1/2 Control	5	20/01/2009	Post Tx	24h	0	4	2	15	12	0	0	36	2	29	0	29
10	1/2 Control	5	20/01/2009	Post Tx	Post Thaw	3	8	3	5	6	8	4	19	1	42	1	45
10	1/2 Control	6	28/01/2009	Post Tx	6-8h	2	32	1	17	11	3	1	21	3	9	0	11
10	1/2 Control	6	28/01/2009	Post Tx	24h	3	10	0	6	7	13	3	22	1	34	1	37
10	1/2 Control	6	28/01/2009	Post Tx	Post Thaw	2	9	3	10	1	8	8	24	1	34	0	36
11	1/2 Min & Vit	4	16/01/2009	Post Tx	6-8h	4	4	1	10	28	6	0	35	0	12	0	16
11	1/2 Min & Vit	4	16/01/2009	Post Tx	24h	5	6	1	6	14	12	1	8	1	46	0	51
11	1/2 Min & Vit	4	16/01/2009	Post Tx	Post Thaw	2	5	4	0	11	10	0	21	5	41	1	43
11	1/2 Min & Vit	5	20/01/2009	Post Tx	6-8h	2	10	1	16	31	1	0	31	1	7	0	9
11	1/2 Min & Vit	5	20/01/2009	Post Tx	24h	2	7	5	6	22	5	0	20	1	32	0	34
11	1/2 Min & Vit	5	20/01/2009	Post Tx	Post Thaw	5	4	2	6	9	7	1	19	0	47	0	52
11	1/2 Min & Vit	6	26/01/2009	Post Tx	6-8h	4	13	1	13	14	7	0	39	3	6	0	10
11	1/2 Min & Vit	6	26/01/2009	Post Tx	24h	6	10	0	6	12	10	2	6	4	43	1	49
11	1/2 Min & Vit	6	26/01/2009	Post Tx	Post Thaw	2	5	1	8	9	8	2	15	1	49	0	51
11	1/2 Min & Vit	7	28/01/2009	Post Tx	6-8h	12	17	1	13	25	4	0	14	0	14	0	26
11	1/2 Min & Vit	7	28/01/2009	Post Tx	24h	3	11	1	8	11	12	3	8	2	41	0	44
11	1/2 Min & Vit	7	28/01/2009	Post Tx	Post Thaw	2	10	6	5	17	9	4	12	3	30	2	32
12	1/2 Min & Vit	4	24/01/2009	Post Tx	6-8h	0	29	5	9	13	4	2	34	2	2	0	2
12	1/2 Min & Vit	4	24/01/2009	Post Tx	24h	0	18	2	12	13	0	0	36	3	16	0	16

12	1/2 Min & Vit	4	24/01/2009	Post Tx	Post Thaw	1	17	4	15	9	9	5	14	6	20	0	21
12	1/2 Min & Vit	5	26/01/2009	Post Tx	6-8h	2	22	6	28	17	2	0	17	2	3	1	5
12	1/2 Min & Vit	5	26/01/2009	Post Tx	24h	0	15	3	5	12	21	0	17	1	26	0	26
12	1/2 Min & Vit	5	26/01/2009	Post Tx	Post Thaw	4	6	20	11	7	3	1	18	2	28	0	32
12	1/2 Min & Vit	6	28/01/2009	Post Tx	6-8h	3	42	3	25	7	0	1	13	2	4	0	7
12	1/2 Min & Vit	6	28/01/2009	Post Tx	24h	1	15	3	6	18	7	1	8	2	39	0	40
12	1/2 Min & Vit	6	28/01/2009	Post Tx	Post Thaw	1	16	9	6	13	4	2	12	1	36	0	37
12	1/2 Min & Vit	7	1/02/2009	Post Tx	6-8h	4	19	4	12	11	7	1	27	1	14	0	18
12	1/2 Min & Vit	7	1/02/2009	Post Tx	24h	0	17	1	20	18	7	3	11	0	23	0	23
12	1/2 Min & Vit	7	1/02/2009	Post Tx	Post Thaw	1	16	11	9	9	13	3	13	4	21	0	22
13	1/2 Oil	4	24/01/2009	Post Tx	6-8h	2	31	0	9	18	2	1	23	2	12	0	14
13	1/2 Oil	4	24/01/2009	Post Tx	24h	2	20	3	8	2	4	6	25	1	29	0	31
13	1/2 Oil	4	24/01/2009	Post Tx	Post Thaw	0	22	3	5	3	2	0	28	1	36	0	36
13	1/2 Oil	5	28/01/2009	Post Tx	6-8h	0	20	9	17	13	5	1	28	3	4	0	4
13	1/2 Oil	5	28/01/2009	Post Tx	24h	2	20	0	8	16	11	3	20	0	20	0	22
13	1/2 Oil	5	28/01/2009	Post Tx	Post Thaw	7	11	0	4	6	6	1	36	1	27	1	34
13	1/2 Oil	6	30/01/2009	Post Tx	6-8h	1	26	4	16	14	5	1	28	0	5	0	6
13	1/2 Oil	6	30/01/2009	Post Tx	24h	2	13	0	11	9	11	3	20	0	31	0	33
13	1/2 Oil	6	30/01/2009	Post Tx	Post Thaw	2	15	3	8	9	2	1	35	2	23	0	25
14	1/2 Oil	4	16/01/2009	Post Tx	6-8h	0	20	7	16	16	2	0	27	1	11	0	11
14	1/2 Oil	4	16/01/2009	Post Tx	24h	4	34	7	5	6	5	2	8	1	28	1	32
14	1/2 Oil	4	16/01/2009	Post Tx	Post Thaw	2	16	8	6	7	6	5	17	2	31	0	33
14	1/2 Oil	5	22/01/2009	Post Tx	6-8h	4	23	3	11	9	2	1	36	1	10	0	14
14	1/2 Oil	5	22/01/2009	Post Tx	24h	7	22	2	5	9	3	2	8	0	41	1	48
14	1/2 Oil	5	22/01/2009	Post Tx	Post Thaw	0	23	16	3	7	6	5	16	5	18	1	18
14	1/2 Oil	6	26/01/2009	Post Tx	6-8h	1	34	7	18	7	2	1	27	1	2	0	3
14	1/2 Oil	6	26/01/2009	Post Tx	24h	2	18	2	4	10	2	4	17	3	38	0	40
14	1/2 Oil	6	26/01/2009	Post Tx	Post Thaw	1	25	3	7	5	5	1	17	1	35	0	36
14	1/2 Oil	7	28/01/2009	Post Tx	6-8h	5	27	3	7	10	1	0	23	1	23	0	28
14	1/2 Oil	7	28/01/2009	Post Tx	24h	1	28	1	7	6	8	3	9	1	36	0	37
14	1/2 Oil	7	28/01/2009	Post Tx	Post Thaw	0	12	10	4	9	5	4	22	1	33	0	33

Table A6. 3 Raw Acrosome Data. Tx= Treatment, Assesst= Assessment, ES= Equatorial Segment. Values are in percentages (%).

Stallion ID	Treatment Diet	Sample	Date	Tx Period	Assesst time	% Intact	% Patchy	% 75% reacted	% Reacted	%ES
1	Control	1	15/09/2008	Pre Tx	Post Thaw	13	76	4	7	0
1	Control	2	22/09/2008	Pre Tx	Post Thaw	4	68	22	6	0
1	Control	3	30/09/2008	Pre Tx	Post Thaw	52	39	2	7	0
2	Control	1	26/09/2008	Pre Tx	Post Thaw	19	62	13	2	4
2	Control	2	6/10/2008	Pre Tx	Post Thaw	7	62	11	12	8
2	Control	3	6/01/2009	Pre Tx	Post Thaw	42	25	21	7	5
3	Control	1	26/09/2008	Pre Tx	Post Thaw	5	26	62	3	4
3	Control	2	6/10/2008	Pre Tx	Post Thaw	0	68	18	14	0
3	Control	3	6/01/2009	Pre Tx	Post Thaw	29	53	8	7	3
4	Min & Vit	1	15/09/2008	Pre Tx	Post Thaw	5	62	27	6	0
4	Min & Vit	2	22/09/2008	Pre Tx	Post Thaw	26	67	7	0	0
4	Min & Vit	3	30/09/2008	Pre Tx	Post Thaw	69	25	2	4	0
5	Min & Vit	1	26/09/2008	Pre Tx	Post Thaw	31	62	1	5	1
5	Min & Vit	2	6/10/2008	Pre Tx	Post Thaw	4	60	16	19	1
5	Min & Vit	3	7/11/2008	Pre Tx	Post Thaw	18	60	20	2	0
6	Min & Vit	1	26/09/2008	Pre Tx	Post Thaw	31	58	10	1	0
6	Min & Vit	2	6/10/2008	Pre Tx	Post Thaw	53	41	3	3	0
6	Min & Vit	3	7/11/2008	Pre Tx	Post Thaw	54	32	5	3	7
7	Oil	1	6/10/2008	Pre Tx	Post Thaw	22	58	19	1	0
7	Oil	2	7/11/2008	Pre Tx	Post Thaw	26	48	10	6	10
7	Oil	3	6/01/2009	Pre Tx	Post Thaw	9	67	16	7	1
8	Oil	1	26/09/2008	Pre Tx	Post Thaw	25	47	16	11	1
8	Oil	2	6/10/2008	Pre Tx	Post Thaw	35	40	14	10	0
8	Oil	3	7/11/2008	Pre Tx	Post Thaw	5	73	17	5	0
9	1/2 Control	1	15/09/2008	Pre Tx	Post Thaw	0	66	18	11	5
9	1/2 Control	2	22/09/2008	Pre Tx	Post Thaw	7	60	26	7	0
9	1/2 Control	3	30/09/2008	Pre Tx	Post Thaw	0	62	28	10	0
10	1/2 Control	1	15/09/2008	Pre Tx	Post Thaw	15	57	16	9	3
10	1/2 Control	2	22/09/2008	Pre Tx	Post Thaw	16	60	14	10	0

10	1/2 Control	3	30/09/2008	Pre Tx	Post Thaw	27	52	15	6	0
11	1/2 Min & Vit	1	15/09/2008	Pre Tx	Post Thaw	42	38	17	3	0
11	1/2 Min & Vit	2	22/09/2008	Pre Tx	Post Thaw	26	70	2	2	0
11	1/2 Min & Vit	3	30/09/2008	Pre Tx	Post Thaw	22	50	22	5	1
12	1/2 Min & Vit	1	15/09/2008	Pre Tx	Post Thaw	2	66	16	16	0
12	1/2 Min & Vit	2	24/09/2008	Pre Tx	Post Thaw	8	70	15	5	2
12	1/2 Min & Vit	3	30/09/2008	Pre Tx	Post Thaw	2	80	7	10	1
13	1/2 Oil	1	15/09/2008	Pre Tx	Post Thaw	12	53	26	9	0
13	1/2 Oil	2	22/09/2008	Pre Tx	Post Thaw	22	48	25	3	2
13	1/2 Oil	3	2/10/2008	Pre Tx	Post Thaw	29	45	11	10	5
14	1/2 Oil	1	15/09/2008	Pre Tx	Post Thaw	0	22	19	59	0
14	1/2 Oil	2	22/09/2008	Pre Tx	Post Thaw	1	24	11	64	0
14	1/2 Oil	3	30/09/2008	Pre Tx	Post Thaw	0	43	12	45	0
1	Control	4	12/01/2009	Post Tx	Post Thaw	26	51	22	0	1
1	Control	5	20/01/2009	Post Tx	Post Thaw	54	41	3	1	1
1	Control	6	28/01/2009	Post Tx	Post Thaw	2	61	26	10	1
2	Control	4	14/01/2009	Post Tx	Post Thaw	10	65	25	0	0
2	Control	5	28/01/2009	Post Tx	Post Thaw	20	63	9	1	0
2	Control	6	30/01/2009	Post Tx	Post Thaw	18	29	41	12	0
3	Control	4	14/01/2009	Post Tx	Post Thaw	0	33	39	28	0
3	Control	5	28/01/2009	Post Tx	Post Thaw	1	50	42	7	0
3	Control	6	30/01/2009	Post Tx	Post Thaw	27	63	8	2	0
4	Min & Vit	4	12/01/2009	Post Tx	Post Thaw	46	46	9	0	0
4	Min & Vit	5	20/01/2009	Post Tx	Post Thaw	6	72	21	1	0
4	Min & Vit	6	26/01/2009	Post Tx	Post Thaw	1	46	45	8	0
5	Min & Vit	4	14/01/2009	Post Tx	Post Thaw	6	56	28	10	0
5	Min & Vit	5	28/01/2009	Post Tx	Post Thaw	20	55	18	6	1
5	Min & Vit	6	30/01/2009	Post Tx	Post Thaw	1	52	43	1	3
6	Min & Vit	4	6/01/2009	Post Tx	Post Thaw	2	52	38	8	0
6	Min & Vit	5	14/01/2009	Post Tx	Post Thaw	32	63	2	1	2
6	Min & Vit	6	28/01/2009	Post Tx	Post Thaw	44	50	14	1	0
6	Min & Vit	7	30/01/2009	Post Tx	Post Thaw	0	82	15	3	0
7	Oil	4	14/01/2009	Post Tx	Post Thaw	27	33	16	23	1
7	Oil	5	28/01/2009	Post Tx	Post Thaw	51	36	9	2	2

7	Oil	6	30/01/2009	Post Tx	Post Thaw	29	58	8	4	1
8	Oil	4	6/01/2009	Post Tx	Post Thaw	44	53	2	1	0
8	Oil	5	14/01/2009	Post Tx	Post Thaw	16	61	17	6	0
8	Oil	6	28/01/2009	Post Tx	Post Thaw	5	77	16	2	0
8	Oil	7	30/01/2009	Post Tx	Post Thaw	12	47	30	11	0
9	1/2 Control	4	22/01/2009	Post Tx	Post Thaw	0	71	19	10	0
9	1/2 Control	5	30/01/2009	Post Tx	Post Thaw	36	49	15	0	0
10	1/2 Control	4	16/01/2009	Post Tx	Post Thaw	12	62	25	1	0
10	1/2 Control	5	20/01/2009	Post Tx	Post Thaw	3	77	12	6	2
10	1/2 Control	6	28/01/2009	Post Tx	Post Thaw	6	63	22	8	1
11	1/2 Min & Vit	4	16/01/2009	Post Tx	Post Thaw	10	74	12	4	0
11	1/2 Min & Vit	5	20/01/2009	Post Tx	Post Thaw	69	28	1	1	1
11	1/2 Min & Vit	6	26/01/2009	Post Tx	Post Thaw	70	18	4	3	5
11	1/2 Min & Vit	7	28/01/2009	Post Tx	Post Thaw	26	69	5	0	0
12	1/2 Min & Vit	4	24/01/2009	Post Tx	Post Thaw	11	77	4	8	0
12	1/2 Min & Vit	5	26/01/2009	Post Tx	Post Thaw	37	43	7	5	8
12	1/2 Min & Vit	6	28/01/2009	Post Tx	Post Thaw	14	74	7	1	4
12	1/2 Min & Vit	7	1/02/2009	Post Tx	Post Thaw	1	79	17	2	1
13	1/2 Oil	4	24/01/2009	Post Tx	Post Thaw	0	4	69	27	0
13	1/2 Oil	5	28/01/2009	Post Tx	Post Thaw	0	12	84	4	0
13	1/2 Oil	6	30/01/2009	Post Tx	Post Thaw	39	44	11	4	2
14	1/2 Oil	4	16/01/2009	Post Tx	Post Thaw	17	58	7	18	0
14	1/2 Oil	5	22/01/2009	Post Tx	Post Thaw	35	57	5	1	2
14	1/2 Oil	6	26/01/2009	Post Tx	Post Thaw	8	68	11	11	2
14	1/2 Oil	7	28/01/2009	Post Tx	Post Thaw	34	61	12	2	0

Table A6. 4 Per-cycle Pregnancy data.

Stallion ID	Period	No. Of Mares	No. Of Cycles	No. Of Inseminat ion	No of Pregnancies at 15 days	Total No of Mares	Pre Cycle Preg Rate (%)
1	Last Breeding Season	-	-	-	-		
1	15/9/08-15/11/08	44	48	72	36		75.0
1	16/11/08-14/1/09	37	47	67	28		59.6
1	15/01/2009-15/3/09	6	6	6	4	73	66.7
4	Last Breeding Season	171	224	185	145		64.7
4	15/9/08-15/11/08	29	32	48	22		68.8
4	16/11/08-14/1/09	20	28	40	12		42.9
4	15/01/2009-15/3/09	0	0	0	0	42	
7	Last Breeding Season						
7	15/9/08-15/11/08	4			4		
7	16/11/08-14/1/09	5			9		
7	15/01/2009-15/3/09	1			5	17	
8	Last Breeding Season						
8	15/9/08-15/11/08	38			37		
8	16/11/08-14/1/09	52			47		
8	15/01/2009-15/3/09	18			6	112	
2	Last Breeding Season						
2	15/9/08-15/11/08	54			51		
2	16/11/08-14/1/09	28			24		
2	15/01/2009-15/3/09	18			12	120	
3	Last Breeding Season						
3	15/9/08-15/11/08	41			35		
3	16/11/08-14/1/09	49			35		
3	15/01/2009-15/3/09	7			2	119	
5	Last Breeding Season						
5	15/9/08-15/11/08	25			24		
5	16/11/08-14/1/09	21			17		
5	15/01/2009-15/3/09	2			1	65	
6	Last Breeding Season						
6	15/9/08-15/11/08	5			5		
6	16/11/08-14/1/09	11			13		
6	15/01/2009-15/3/09	2			2	25	
13	Last Breeding Season	69			54		
13	15/9/08-15/11/08	3	3	3	2		66.7
13	16/11/08-14/1/09	4	5	5	1		20.0
13	15/01/2009-15/3/09	2	2	2	2	6	100.0
9	Last Breeding Season	100			81		
9	15/9/08-15/11/08	12	16	18	7		43.8
9	16/11/08-14/1/09	13	18	26	6		33.3
9	15/01/2009-15/3/09	3	4	5	1	19	25.0
11	Last Breeding Season	189			160		
11	15/9/08-15/11/08	70	72	89	46		63.9
11	16/11/08-14/1/09	98	124	144	63		50.8
11	15/01/2009-15/3/09	23	28	40	16	153	57.1
12	Last Breeding Season						
12	15/9/08-15/11/08	59	69	79	38		55.1
12	16/11/08-14/1/09	70	93	142	50		53.8
12	15/01/2009-15/3/09	9	9	10	5	111	55.6
14	Last Breeding Season	84			65		
14	15/9/08-15/11/08	19	26	28	12		46.2
14	16/11/08-14/1/09	29	37	47	11		29.7
14	15/01/2009-15/3/09	7	7	9	7	40	100.0
10	Last Breeding Season						
10	15/9/08-15/11/08	21	23	25	18		78.3
10	16/11/08-14/1/09	27	35	40	16		45.7
10	15/01/2009-15/3/09	15	15	21	13	53	86.7