ABSTRACT

LOVERCAMP, KYLE WAYNE. Factors Affecting the Presence of Reactive Oxygen Species in the Fresh and Extended Porcine Ejaculate. (Under the direction of Dr. William L. Flowers).

Experiment 1 examined changes in ejaculate characteristics, semen quality and membrane lipid peroxidation over time in boars maintained under a 3 times or 1 time per week collection frequency and determined the effects of semen extender and storage time on semen quality and sperm membrane lipid peroxidation. In general, extender and storage time affected sperm quality. Sperm stored in a commercially available 3-day extender were lower for sperm quality and higher for lipid peroxidation after 7 days of storage post-collection compared to a commercially available 5-day extender.

Experiment 2 used density gradient centrifugation to separate extended boar sperm into sub-populations for analysis of sperm quality, plasma membrane lipid peroxidation and sperm cell fatty acid composition over a 7 day storage period post-collection. Three ejaculates were collected and analyzed following exposure to three consecutive collection periods. The first ejaculate was collected from boars that had previously been maintained on a 1 time per week frequency. The second ejaculate was collected following a period of five collections in four days (fifth collection analyzed). The third ejaculate was a collected after a period of three days of rest following the collection of the second ejaculate. Collection period affected sperm motility over the storage period post-collection. Collection period, density layer and day of storage post-collection affected the separation patterns of sperm cells using density gradient centrifugation. These results suggest that changes in sperm separation seem to be primarily affected by collection period and day of storage post-collection and to a lesser extent, sperm motility, but not plasma membrane lipid peroxidation.

Experiment 3 evaluated the effect of dietary selenium on sperm production and sperm quality. The dietary treatments were a non-supplemented negative control basal diet or the basal diet supplemented at 0.3 ppm with either organic selenium or inorganic selenium. A secondary objective was to examine changes in sperm quality over a six day storage period post-collection. Boars were fed the dietary treatments beginning at the time of weaning. Dietary treatment affected the level of selenium in the blood plasma but not the semen. Dietary treatment did not affect volume, concentration or total sperm in the ejaculate, nor did dietary treatment affect sperm motility, progressive motility, morphology, membrane lipid peroxidation and glutathione peroxidase activity over the 6 day storage period post-collection. Following density gradient centrifugation, sperm motility, progressive motility, morphology and the percentage of sperm recovered were higher in the 90% gradient compared to the 45% gradient on day 1 but not day 6 of storage post-collection. These results indicate that dietary treatment affected selenium levels in the blood, but did not affect sperm production or quality. Boar sperm cells decrease in progressive motility and buoyant density over a six day storage period which appears to affect the sperm motility, progressive motility, morphology and the percentage of sperm recovered in the high and low density layers following density gradient centrifugation, however these changes do not appear to be affected by lipid peroxidation.

Factors Affecting the Presence of Reactive Oxygen Species in the Fresh and Extended Porcine Ejaculate

by
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BIOGRAPHY

Kyle Wayne Lovercamp was born and raised in Alma, Missouri, a small agriculturally based town located in west-central Missouri. Kyle's interest in agriculture and animal science stems from his early involvement with the family farm. He was active in 4-H at an early age and continued with the organization, as well as FFA, throughout high school. After graduation from Santa Fe High School in Alma, Kyle attended Central Missouri State University (now the University of Central Missouri) in Warrensburg, MO. Kyle majored in Agriculture Technology – Animal Science in college. It was during college that Kyle became interested in livestock reproductive physiology and began considering a career as a post-secondary educator. Through the encouragement of mentors at Central Missouri State University and the University of Missouri – Columbia, Kyle decided to pursue a Masters degree in animal science at the University of Missouri – Columbia under the guidance of Drs. Tim Safranski and Peter Sutovsky. The focus of Kyle's research was evaluating the proteins Arachidonate 15-Lipoxygenase and Ubiquitin as potential fertility markers in boars. Following the completion of his M.S. degree, Kyle elected to continue graduate studies towards a doctorate of philosophy in animal science under the direction of Dr. Billy Flowers at North Carolina State University. Kyle chose to continue research into spermatozoa physiology by examining factors affecting the presence of reactive oxygen species in the fresh and extended porcine ejaculate. Following the completion of his doctoral degree, Kyle looks forward to finding an academic position with teaching, extension and/or research appointments, with an emphasis on teaching.

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Introduction

History

Interest in the factors affecting the viability of the male gamete has been pursued since the identification of motile spermatozoa in the human ejaculate by Antonius Leeuwenhoek in 1677. However, experimentation to elucidate the effects of reactive oxygen species (ROS) on sperm metabolism and viability was not pursued until the 1940s with the observation by MacLeod that in human sperm, under high oxygen tensions, motility of sperm cells was depressed (MacLeod, 1943). In this classical study, MacLeod determined that motility failure could be prevented with the antioxidant enzyme catalase, indicating that the mechanism by which motility was decreased likely involved hydrogen peroxide and other ROS. Further experimentation by Tosic and Walton in the late 1940s confirmed in bull, boar and ram semen that hydrogen peroxide was a potent inhibitor of spermatozoa respiration and motility (Tosic and Walton, 1950). In fact, mammalian spermatozoa were the first cell type used to demonstrate cellular generation of hydrogen peroxide (reviewed in Aitken, 1995). These two early studies demonstrated the susceptibility of the male gamete to the ROS hydrogen peroxide. Although the means by which these early observations were made were primitive by today's standards, these researchers realized that mechanisms at the molecular level were likely driving the reduced viability observed in the sperm cell.

The research of Jones and Mann in the 1970s determined that the action of hydrogen peroxide and other ROS was taking place at the membrane level through the process of lipid peroxidation. Experiments using ram semen in the presence of Fe^{2+} demonstrated "that lipid

peroxidation may be a cause of the decline in respiratory rate and motility of moribund spermatozoa." In addition, the researchers determined that a component from the damaged sperm cell is released into the external medium (Jones and Mann, 1973). They speculated that this component may be a lipid and possible substrate for the peroxidation reaction. This observation may explain not only how lipid peroxidation occurs, but more importantly how lipid peroxidation acts as a cascading event in the lipids of the sperm plasma membrane that results in damage to the plasma membrane of the sperm cells. Further discussion of the lipid peroxidation cascade can be found later in this review.

The above investigations and other studies helped to lay the foundation for the current understanding of the impact that oxidative stress can have on the mammalian sperm cell. Specifically, these early studies identified that ROS 1) were associated with the sperm cell (MacLeod, 1943; Tosic and Walton, 1950); 2) were able to reduce the viability of the sperm cell (MacLeod, 1943; Tosic and Walton, 1950); and 3) mediated this action on the sperm cell through the lipid peroxidation cascade (Jones and Mann, 1973). Interest in the effect of ROS on sperm viability is largely due to the possibility that ROS are responsible for cases of male-mediated infertility or idiopathic infertility found in both humans and livestock species.

There are two main reasons that spermatozoa are susceptible to ROS-mediated lipid peroxidation. First, sperm cells are a uniquely designed cell, undergoing modifications during spermatogenesis for delivery of the DNA to the oocyte during fertilization. To accomplish this delivery, the spermatozoa are streamlined by removal of a large proportion of cytoplasm and condensation of the DNA into the sperm head, which is believed to render the mature sperm cell transcriptionally inactive. Since the sperm cell is devoid of cytoplasm and

transcriptionally inactive, it is unable to defend itself against oxidative attack. The second reason that sperm cells are susceptible to ROS and lipid peroxidation is that the plasma membrane contains a large proportion of polyunsaturated fatty acids, which are a substrate for ROS attack and propagation of the lipid peroxidation cascade. As a result, molecular investigations into the effects of ROS on the spermatozoa in the ejaculate have been pursued.

This review will focus on understanding how ROS negatively and positively influence mammalian sperm cell viability by exploring factors such as collection frequency, age of semen (i.e. storage length post-collection) and sperm morphology which have been shown to affect the presence and activity of ROS in the ejaculate from sperm and non-sperm sources. Insight into the relationships between ROS and mammalian spermatozoa will help researchers better understand sperm cell physiology. This information, in turn, should help livestock producers improve the reproductive efficiency of their operation by managing males for production of optimum sperm quality and using extenders designed to neutralize effects of ROS during storage and prior to insemination.

Background

The majority of the work characterizing the interactions of ROS with mammalian sperm has been done in humans and mice. The human male produces some of the poorest quality semen of all mammalian species (reviewed by Aitken and Sawyer, 2003). Therefore, ROS production and activity in the human male may be due to factors not common within other mammalian species. With this in mind, careful interpretation of results should be used, especially when attempting to apply this area of research to livestock species.

Reactive oxygen species

Reactive oxygen species (ROS), also known as free radicals, are ubiquitous chemical intermediates that contain one or more unpaired electrons. The term ROS includes partially reduced forms of atmospheric oxygen (O_2; oxygen ions, free radicals, and peroxides, both inorganic and organic) and their reaction products with other molecules (Ford, 2004, Drevet, 2006). Free radicals typically result from the transfer of one, two or three electrons to O_2 to form a superoxide anion (O2•−), hydrogen peroxide (H_2O_2) or a hydroxyl radical (OH•) (Alvarez and Storey, 1989; Aitken, 1995; Aitken and Baker, 2002; Chen et al., 2003; Sanocka and Kurpisz, 2004). Reactive nitrogen species (RNS) are also free radicals. Similar to ROS, RNS are produced as by-products of aerobic metabolism or in response to stresses. Although RNS are primarily found in plants, the RNS nitric oxide ($NO^{\cdot}$), and nitrogen dioxide ($NO_2^{\cdot}$) are also produced in mammals. In addition, ROS and RNS can interact, as is seen in the reaction of nitric oxide (NO) with superoxide anion to form peroxynitrite (ONOO−). The majority of research investigating the action of free radicals in the mammalian ejaculate has evaluated the action of ROS, and therefore, this review will also focus on ROS, and not RNS. The presence of unpaired electrons allows ROS to be highly chemically reactive and both positively and negatively affect the chemical structure of the lipids, amino acids, carbohydrates and nucleic acids of sperm cells through oxidation (Sanocka and Kurpisz, 2004). Although oxidation can be beneficial to the sperm cell by modulating cell signaling cascades and secondary messenger systems, in most situations oxidation is detrimental to the functionality of macromolecules for homeostatic cellular processes, eventually leading to cell death.

The ROS in the ejaculate originate through the formation of superoxide anion. The creation of superoxide anion is accomplished through a one-electron reduction of oxygen. The newly formed superoxide ion is considered a free radical because it has one unpaired electron. The exact mechanism by which superoxide anion is created in the sperm is not entirely understood, although evidence suggests that mitochondrial respiration, membrane bound NADPH oxidases, precursor germ cells, leukocytes or defective spermatozoa may be responsible (Aitken et al., 1995, Sanocka and Kurpisz, 2004). The superoxide anion produced can dismutate either spontaneously or through the actions of the antioxidant, superoxide dismutase to form hydrogen peroxide and oxygen. Early research in human sperm suggested that superoxide dismutase alone was responsible for the dismutation of superoxide anion, however, this has not held true (Alvarez et al., 1987). The superoxide dismutase enzyme is limited in its ability to dismutate superoxide anion only by the rate at which superoxide anion is able to diffuse into the reactive site of superoxide dismutase. In other words, the superoxide dismutase enzyme is extremely efficient at catalyzing the dismutation of superoxide anion to hydrogen peroxide (reviewed in Aitken, 1995). Surprisingly, superoxide dismutase actually acts as a quasi-oxidant in this case by mediating the formation of hydrogen peroxide. Although hydrogen peroxide is not a free radical since it does not possess unpaired electrons, it does have strong oxidizing properties, and unlike superoxide anion, can easily move across cell plasma membranes (reviewed in Mruk et al., 2002). As previously mentioned, hydrogen peroxide has been shown to have deleterious effects on sperm motility. Further development of ROS occurs through the interaction of superoxide anion and hydrogen peroxide to generate the hydroxyl radical. This reaction occurs in the

presence of the transition metals iron and/or copper. The hydroxyl radical is an extremely reactive free radical which makes it dangerous to cellular physiology. Unlike superoxide anion and hydrogen peroxide, there are no enzymes specifically designed for eliminating the hydroxyl radical. One reason for this might be due to the extremely short half life of the hydroxyl radical. Elimination of the hydroxyl radical would require its diffusion to the enzyme's active site, but since the diffusion is slower than the half-life (1 ns) of the molecule, the hydroxyl radical will react with any oxidizable compound in its vicinity before elimination by a specific scavenging enzyme could occur (reviewed in Brouwers et al., 2005).

Methods for detecting reactive oxygen species and lipid peroxidation

Although it is widely accepted that ROS actively interact with mammalian sperm, the methods for measuring ROS activity vary (Ford, 2004). The short half life of ROS makes detection difficult without the use of highly sensitive equipment. The only technique that can detect ROS directly is electron spin resonance, although even this method is too insensitive to detect directly the superoxide anion and the hydroxyl radical *in vivo*. Detection of these ROS is generally accomplished *in vitro* using spin trapping, in which a radical is allowed to react with a trap molecule to generate a stable, measurable product (Halliwell and Gutteridge, 1999). Other methods of measuring ROS use light emission via luminescence or fluorescence. These techniques measure light emitted by ROS-generating systems *in vitro*; for example, peroxidizing lipids. The most common method for generating light via ROS is adding luminol or lucigenin to sperm cell suspensions. Luminol is suspected to detect oxidation by the hydroxyl radical, whereas lucigenin is more specific for superoxide anion,

although specificity can not be assumed using these light-emission enhancing compounds (Halliwell and Gutteridge, 1999; Ford, 2004).

Indirect methods of evaluating ROS activity involve 'fingerprinting' methods of lipid peroxidation. Lipid peroxidation is a complex process that involves the oxidation and breakdown of lipids in the sperm plasma membrane from the activity of ROS, primarily through the indirect action of superoxide anion and hydrogen peroxide, and the direct action of the hydroxyl radical via the transition metals Fe and Cu (Aitken, 1995). The most abundant and common breakdown product measured is malondialdehyde (MDA), a reactive carbonyl compound by-product of lipid peroxidation. The measurement of MDA is accomplished through binding of compounds such as thiobarbituric acid (TBA) or N-methyl-2-phenylindole (NMPI) to the MDA molecule. Binding of these compounds to MDA produces a colormetric reaction that can be measured on a spectrophotometer at a wavelength of 532nm or 586 nm, respectively. The TBA assay, which measures thiobarbituric acid reactive substances (TBARS), is the most common assay used for measuring MDA. The TBA assay has been used to detect lipid peroxidation of sperm and reproductive tissues in humans (Aitken et al., 1993a), mice (Vernet et al., 1999), cattle (Slaweta et al., 1988; Laskowska-Klita and Szymanska, 1989) swine (Smutná and Synek, 1979; Cerolini et al., 2000; Strzezek et al., 2000) sheep (Christova et al., 2004), equids (Kankofer et al., 2005) and chickens (Surai et al., 1998). Although the TBA assay is popular due to ease of use for determining lipid peroxidation, it is often criticized due to its non-specificity and ability to react with other lipid peroxidation products such as 4-hydroxyalkenals and 4-hydroxynonenal (Halliwell and Gutteridge, 1999; Young and McEneny, 2001).

Lipid peroxidation can occur either spontaneously or be induced by the inclusion of the transition metals Fe or Cu in the reaction mixture (generally $FeSO_4$ and/or Na ascorbate). The purpose of adding Fe or Cu salts is that spontaneous lipid peroxidation gives a substantially lower color yield using the TBA assay. Inclusion of these transition metals to act as oxidizing agents accelerates the lipid peroxidation reaction (Halliwell and Chirico, 1993). Research comparing spontaneous and induced lipid peroxidation with the TBA assay has demonstrated that MDA levels with induced lipid peroxidation are approximately 3-10 fold higher compared to spontaneous lipid peroxidation (Mennella and Jones, 1980; Vernet et al., 1999). New methods of determining lipid peroxidation have shown increased specificity for MDA using the chromogenic reagent N-methyl-2-phenylindole (NMPI) compared to 4-hydroxyalkenals (Gerard-Monnier et al., 1998) and compared to the TBA assay (Long and Kramer, 2003). Other novel methods for detecting ROS activity and plasma membrane lipid peroxidation involve the use of flow cytometry, which is a technique for counting, examining, and sorting sperm (or any cell) cells based on characteristics of single cells when passed by optical detection apparatus via a stream of fluid. Different chemical probes and dyes can be incubated with sperm cells to determine ROS. For example, hydroethidine can be oxidized to ethidium to indicate the presence of ROS (Bucana et al., 1986; Guthrie and Welch, 2006). Similarly, lipid peroxidation activity can be observed by the oxidation of a membrane bound fatty acid conjugate, C11-BODIPY$^{581/591}$ (Brouwers and Gadella, 2003). The membrane probe C11-BODIPY$^{581/591}$ is able to incorporate into the plasma membrane of sperm cells and irreversibly fluoresce from red to green upon exposure to ROS during the early stages of lipid peroxidation. This allows the researcher the ability to both evaluate the

level and location of membrane lipid peroxidation. The C11-BODIPY$^{581/591}$ probe has been used for determination of lipid peroxidation in the bull (Brouwers et al., 2005), stallion (Ball and Vo, 2002) and boar (Brouwers et al., 2005; Guthrie and Welch, 2007).

Use of the C11-BODIPY$^{581/591}$ probe is warranted due to its specificity for lipid peroxidation activity at the membrane level; however, research has shown that the enzyme phospholipase A_2 can cleave lipid peroxides from the plasma membrane. The free floating lipids could then be scavenged by the antioxidants, or if the antioxidant system has been exhausted, could breakdown into MDA (Bennet et al., 1987; Aitken, et al., 1993; Halliwell and Gutteridge, 1999). As a result, there would be very little evidence of lipid peroxidation with the sperm cell itself since the lipid peroxides would have been cleaved from the sperm cell plasma membrane. It is not known whether the membrane bound fluorescent fatty acid conjugate is a substrate for phospholipase A_2 since the degree of 'preference' for oxidized fatty acids is uncertain (Halliwell and Gutteridge, 1999). Since the C11-BODIPY$^{581/591}$ probe measures the beginning stages of lipid peroxidation, and the TBA or NMPI assays measure the end stages of lipid peroxidation (i.e. TBARS and MDA), a side-by-side comparison of the two lipid peroxidation techniques is warranted.

Antioxidants

To counteract the effects of ROS, the male reproductive tract produces intra and extra cellular enzymatic and non-enzymatic low molecular weight antioxidants. The purpose of the antioxidants is to scavenge free radicals and neutralize them. The main antioxidant enzymes are superoxide dismutase, indolamine dioxygenase, glutathione peroxidase (GPX) and catalase (Sanocka and Kurpisz, 2004; Vernet et al., 2004). These antioxidants are found

either associated with sperm cells in the ejaculate or in the seminal plasma. Studies have identified antioxidant enzymes to be present in all regions of the epididymis and the ejaculate (reviewed by Vernet et al., 2004). Reactive oxygen species activity has been shown to be highest in the caput, but decrease towards the cauda epididymis in the mouse, indicating that the activity of antioxidants is variable in the epididymis (Aitken and Vernet, 1998). However, other studies have found that caudal sperm are also capable of producing high amounts of hydrogen peroxide (Fisher and Aitken, 1997) and the initial contact of the sperm cells with antioxidants begins during spermatogenesis in the testis (Matsuki and Ishii, 2003).

As previously discussed, superoxide anion can be neutralized either through spontaneous dismutation or through the actions of the metalloprotein superoxide dismutase to form hydrogen peroxide. Three isozymes of superoxide dismutase have been identified in mammals to counteract superoxide anion. They are classified based on the transition metal found at their active site. The first isozyme, superoxide dismutase 1, contains copper (Cu) and zinc (Zn) as the metal cofactors in the catalytic core and is mostly cytosolic (Matsuki and Ishii, 2003). The second isozyme, superoxide dismutase 2, contains manganese as the metal cofactor and is associated with the mitochondria. These superoxide dismutase isozymes have not been shown to affect male reproduction, although the Cu/Zn superoxide dismutase isozyme female knockout mice have been shown to be infertile (Matzuk et al., 1998). The third isozyme also contains Cu and Zn as its metal cofactors and is structurally similar to superoxide dismutase 1, except it is located extracellularly. Research has shown that extracellular superoxide dismutase is present in the mammalian testis. Mruk and colleagues (1998) detected extracellular superoxide dismutase at high levels in the epididymis compared

to other tissues in the rat using semi-quantitative RT-PCR. Identification of extracellular superoxide dismutase in the nucleus of developing spermatogonia was accomplished by Ookawara et al. (2002) using immunohistochemical analysis of tesis sections. Research has also shown that superoxide anion can be neutralized by indolamine dioxygenase into the harmless product, kynurenine, which can be rapidly and easily removed from cells (reviewed by Vernet et al., 2004).

The antioxidant defense systems for hydrogen peroxide include GPX and catalase, both of which act to rapidly convert hydrogen peroxide to water. Catalase is a heme protein located predominantly in the inner mitochondrial membrane. Although the ability of catalase to preserve sperm viability is widely accepted, research has demonstrated that catalase neutralizes hydrogen peroxide only when the concentration of hydrogen peroxide is above physiological levels of 10^{-6} M (Cohen and Hochstein, 1963), such as when the xanthine oxidase ROS generating system is used in experiments (Aitken et al., 1992) or when oxidative bursts in cells occur in response to stress (reviewed in Drevet, 2006). Therefore, the role of catalase may be secondary to that of the GPX family of enzymes. This appears to be the case in erythrocytes (Cohen and Hochstein, 1963). Catalase has not been detected in porcine (Strzezek, 2002) or bovine (Bilodeau et al., 2000) ejaculates. These observations seem to indicate that removal of hydrogen peroxide at physiological levels is accomplished only by GPX.

The GPX enzymes, GPX 1, 2, 3, 4 and 5, are a family of cytosolic enzymes that contain a post-transcriptionally incorporated selenocysteine residue (Chambers et al., 1986). The members of the GPX family that have been identified in the male reproductive tract of

the human and mouse are GPX3, GPX4 and GPX 5 (Schwaab et al., 1998; Cooper et al., 2003; Drevet, 2006). The GPX3 is a tetrameric extracellular GPX and has been identified in the epididymis, vas deferens and seminal vesicles. The GPX4 is also called phospholipid hydroperoxide glutathione peroxidase (PHGPX) and is a monomeric membrane-associated GPX localized to the testis and sperm. The GPX5 is a dimeric enzyme that has been shown to be highly expressed in the caput epididymis and associated with sperm (reviewed in Drevet, 2006). Surprisingly, the GPX5 enzyme does not contain selenium, but instead has a cysteine residue in the place of the selenocysteine residue (Ghyselinck et al., 1991). The activity of these GPX enzymes appears to be most important in the epididymis and through interactions with the sperm cells. Both GPX3 and 5 are expressed throughout the epididymis, with GPX5 demonstrating the highest degree of variability. The GPX5 can be found in the cytoplasm of the principal cells as well as within the epididymal lumen. Epididymal antioxidants are important because the epididymis is a site of multiple maturational events for the spermatozoa, including chromatin condensation of the DNA, acquisition of potential for progressive motility and modifications to the acrosome and surface of the plasma membrane. Both GPX 4 and 5 have been identified on the sperm cell. Glutathione peroxidase 4 has been localized in the sperm nucleus of the human and is suspected to facilitate sperm nuclear condensation in the caput (Aitken and Vernet, 1998). GPX4 is also found in the rat sperm midpiece, where it makes up ~50% of the total protein content (Ursini et al., 1999). Interestingly, enzymatic activity of GPX4 with the midpiece appears to occur primarily during spermatogenesis during modification of the sperm cell. Following spermatogenesis, GPX4 loses is solubility and enzymatic properties, and instead functions as a structural

component of the sperm midpiece (Ursini et al., 1999). The GPX5 is present on the sperm plasma membrane, likely through interactions between the maturing spermatozoa in the epididymis and lipid-rich vesicles called epididymosomes, which are released from the principal cells (Jimenez et al., 1992). These secretory membrane-enclosed vesicles fuse with the sperm and plasma membrane and transfer the GPX onto the spermatozoa. How the GPX 3, 4 and 5 enzymes interact with the male reproductive tract and subsequently in the ejaculate is not well understood. The apparent loss of enzymatic activity of GPX4 in the sperm midpiece suggests that GPX 3 and 5 are solely responsible for combating ROS. It may be possible that some enzymatic activity of GPX4 is retained and a synchronized effort of GPX 3, 4 and 5 and low molecular weight antioxidants minimize oxidative stress.

The main non-enzymatic low molecular weight antioxidants found in mammalian and avian species are glutathione, ascorbic acid (vitamin C), α-tocopherol (vitamin E), and to a lesser extent, thioredoxin (Strzezeck, 2002; Brèque et al., 2003; Matsuki and Ishii, 2003). Low molecular weight antioxidants are classified into two divisions, water-soluble and lipid-soluble. Water-soluble antioxidants neutralize ROS in the cytoplasm of the cell, whereas lipid-soluble antioxidants act within the cell plasma membrane to prevent lipid peroxidation. Glutathione and ascorbic acid are important water soluble cellular scavengers that provide reducing power for several reactions, including reduction of hydrogen peroxide and regeneration of antioxidant vitamins. Similarly, α-tocopherol is a lipid soluble antioxidant that is effective for breaking the lipid peroxidation cascade (Dumaswala et. al., 1999). Of the low-molecular weight antioxidants mentioned, glutathione may be the most important due to its role in the glutathione peroxidase/glutathione reductase reduction-oxidation (redox) cycle

and its ability to act independently as a reducing agent for other compounds (Irvine, 1996). Glutathione is a non-essential tripeptide composed of L-cysteine, L-glutamic acid and glycine that can be identified in cellular systems in the reduced or oxidized state. Glutathione exists primarily in the reduced state in physiological systems so as to provide a reducing equivalent to other unstable molecules, such as ROS. The reducing equivalent is provided by the thiol group of cysteine. Therefore, glutathione is a reducing agent because it becomes oxidized. As stated previously, glutathione plays a critical role as an electron donor or acceptor for redox cycling between GPX and glutathione reductase. In this cycle, GPX catalyzes the donation of an electron from each of two reduced forms of glutathione to yield two oxidized forms of glutathione that quickly react to form glutathione disulfide and two molecules of water. The newly created glutathione disulfide is then recycled by glutathione reductase back to two molecules of glutathione using NADPH and a hydrogen as the electron donors. Similar to GPX, glutathione reductase has been identified in the male reproductive tract of rats. A similar recycling situation occurs for the thioredoxin redox protein and its ability to donate an electron for ribonucleotide reductase (Matsuki and Ishii, 2003). Glutathione has been identified throughout the male reproductive tract of many mammalian species. High concentrations of glutathione have been found in the murine testis compared to the epididymis (Calvin and Turner, 1982). The source of this glutathione was thought to be primarily from the sertoli cells, which have been show to supply glutathione to the developing spermatocytes through both de novo synthesis and GPX/glutathione reductase recycling systems (Matsuki and Ishii, 2003). Investigations in the bull by Agrawal and Vanha-Perttula (1988) demonstrated that glutathione was prevalent in secretions of the

reproductive tract and also associated with the sperm cell. However, the lowest amounts of glutathione were found in the seminal plasma and in ejaculated sperm, suggesting that the protective mechanisms of glutathione are not as important once ejaculation occurs. Investigations in the boar are contradictory. Research by Li (1975) was not able to detect glutathione in the boar ejaculate. Conversely, Strezek (2002) was able to detect 5.7 mg/100ml in the boar ejaculate. The presence of glutathione reported by Strezek likely arose from the fact that the glutathione was precipitated out of the seminal plasma prior to analysis. Precipitating the glutathione out of the seminal plasma likely resulted in a concentrated sample of glutathione, thereby making detection of glutathione permissible. L-ascorbic acid has been detected in the boar seminal plasma at 2.9 mg/100ml (Strezek, 2002).

With natural insemination standpoint, the importance of glutathione is questionable because semen should be exposed to glutathione continuously. It quickly moves from one source provided by the male reproductive tract to another source provided by the female reproductive tract. However, in artificial insemination strategies, supplementation of glutathione in the extending or freezing media may be beneficial. A study by Brezezińska-Slebodzińska and colleagues (1995) demonstrated that supplementing the extending media with reduced glutathione inhibited lipid peroxidation by 57%. Supplementation of a freezing extender with reduced glutathione by Gadea et al. (2005) improved motility, viability and membrane stability. Somewhat surprising was that a 1 mM concentration of reduced glutathione was more protective of sperm properties than 5 mM. This suggests that a concentration of reduced glutathione above a specific threshold in the freezing media may be detrimental to the sperm cell viability.

Ascorbic acid (vitamin C) and α-tocopherol (vitamin E) are both recognized antioxidants capable of neutralizing free radicals and breaking the lipid peroxidation cascade. Ascorbic acid is an essential water soluble vitamin that can be oxidized by ROS to yield the unreactive dehydroascorbate and water. Conversely, α-tocopherol represents a group of eight essential lipid soluble tocopherols that have antioxidant properties. Of the eight, α-tocopherol has been the most studied due to its bioavailability. The chain-breaking properties of α-tocopherol are due to its ability to integrate in to the plasma membrane of sperm cells and neutralize lipid radicals produced in the lipid peroxidation chain reaction. This is accomplished by acting as a reducing agent to yield an oxidized α-tocopheroxyl radical. Ascorbic acid and other antioxidants, including glutathione, can then recycle the α-tocopheroxyl radical to the reduced form (Agarwal et al., 2004). Supplementation of liquid and freezing extenders with α-tocopherol has shown positive effects on sperm quality. Cerolini and co-workers (2000) found that supplementing Beltsville Thawing Solution (BTS) with 0.2 mg/ml of α-tocopherol resulted in a significantly higher number of live sperm cells (80% vs. 60%) compared to the control (non-supplemented BTS) following five days of storage at 19ºC. The researchers speculate that the role of α-tocopherol was due to its uptake by the sperm cell through incorporation into the sperm plasma membrane since the α-tocopherol content of sperm cells from the treated group was significantly higher than the control by day five of storage. Supplementation of a freezing extender with 200, 500 or 1000 µg/ml α-tocopherol also showed beneficial effects on sperm cell motility and prevention of sperm membrane lipid peroxidation following thawing compared to the non-supplemented control. Similar to supplementation with reduced glutathione, the lower concentration of α-

tocopherol was more beneficial than the higher concentrations of α-tocopherol, suggesting that a maximum threshold concentration must be present for the beneficial effects to be seen (Breininger et al., 2005). Supplementing the extending media with vitamin E analog (TROLOX) caused a 62% reduction in lipid peroxidation in boar sperm (Brezezińska-Slebodzińska et al., 1995).

Sources of reactive oxygen species in the ejaculate

It has been speculated that the ROS which affect sperm are produced either by non-sperm components in the ejaculate or by the sperm themselves (Ford, 2004).

Sperm

Cells can produce ROS through intracellular oxidases and peroxidases or through other enzymes such as cytochrome P_{450} or nitric oxide synthase (Halliwell and Gutteridge, 1999). However, the accidental loss of electrons from the electron transport chain is the most common source of ROS in cells (Han et al., 2001). Researchers have speculated that ROS production by the sperm cell occurs through two main sources: 1) through electron leakage via the electron transport chain from the mitochondria during cellular respiration and 2) through an intracellular NADPH oxidase attached to the plasma membrane (Aitken, 1995). In either of these situations it has not been established whether normal, actively respiring cells or damaged/dead sperm cells are the main source of ROS.

Cellular respiration in the mitochondria produces adenosine triphosphate (ATP) through the process of oxidative phosphorylation. During this process, electrons are passed along through the electron transport chain through series of redox reactions. Normally, the final destination for an electron in this system is an oxygen molecule, which is then reduced

to generate water. However, the oxygen can be incompletely reduced to produce superoxide anion (Halliwell and Gutteridge, 1999). The superoxide anion that is produced can be released into the mitochondrial matrix where it can dismutate to hydrogen peroxide either spontaneously or with the assistance of superoxide dismutase. The hydrogen peroxide that is produced can then move across the mitochondrial outer membrane and into the cytosol where it can react with other molecules (Han et al., 2001). Although the production of ROS is generally thought to be negative in the context of the detrimental effects of oxidative stress, ROS production is used for apoptosis as a means by which to monitor cell regulation in tissues.

Evidence of ROS production by human sperm cells has come under question in recent years due to the possibility of contamination by leukocytes in the human ejaculate (Ford, 2004). Production of ROS by leukocytes occurs via the respiratory burst that is used to destroy bacteria, fungi and other foreign bodies. Production of ROS by leukocytes will be discussed subsequently. Upon removal of contaminating leukocytes, however, Krausz et al. (1994) were still able to demonstrate the ability of human sperm to generate ROS with the addition of the phorbol ester, phorbol-12-myristate-13-acetate (PMA). The question arises as to what sperm cell mechanism is driving the formation of ROS. There is a consensus that loss of electrons through the electron transport chain are a source, however, the majority of production of ROS by human sperm is not thought to occur in this manner as the mitochondria in human sperm are low in number and may be less metabolically active compared to other mammalian sperm (Ford and Rees, 1990).

The second option proposed for ROS generation in sperm is an intracellular, membrane bound NADPH oxidase (Aitken et al., 1997). Proponents for this mechanism suggest that the membrane bound oxidase is essential for facilitating proper chromatin compaction during epididymal maturation by providing hydrogen peroxide as a hydrogen acceptor for the oxidation of thiol groups associated with sperm protamines (Aitken and Vernet, 1998). If this NADPH oxidase is not regulated correctly by antioxidants following ejaculation, it may be a source of ROS from sperm in the ejaculate. Investigations toward characterizing this proposed oxidase have not been successful, as monoclonal antibodies raised against the NADPH oxidase in leukocytes have not shown cross-reactivity with protein extracts on western blots (Aiken et al., 1995). Specifically investigators were unable to identify cytochrome b558, which is a component of the gp9lphox a subunit that makes up the NADPH oxidase enyme (Ford, 2004). Although the exact mechanism by which sperm cells are able to generate ROS is unknown, it is widely accepted that the plasma membrane of sperm do contain multiple unidentified redox enzymes that could produce ROS.

Non-sperm

Potential sources of ROS from non-sperm components are attached and unattached cytoplasmic droplets (Thundathil et al., 2001; Brouwers et al., 2005), excess residual cytoplasm in human sperm (Rao et al., 1989; Aitken and Sawyer, 2003; Aziz et al., 2004), leukocytes (Aitken et al., 1994b) and bacterial contamination (Ochsendorf, 1999).

Opinions differ regarding the proper nomenclature for the presence of residual cytoplasm retained by mammalian sperm cells following spermatogenesis. The terms in question are the ‘cytoplasmic droplet’ and ‘excess residual cytoplasm’. Normally, following

spermatogenesis, there is a small amount of circular cytoplasm retained on the sperm cell just behind the sperm head. When the residual cytoplasm is found in this manner, it is referred to as a cytoplasmic droplet. When attached to the spermatozoon, the cytoplasmic droplet is associated with the sperm tail midpiece in one of two locations: 1) at the proximal end of the midpiece, i.e. next to the sperm head (proximal cytoplasmic droplet) or 2) located at the distal end of the midpiece at the junction of the midpiece and principle piece of the sperm tail (distal cytoplasmic droplet). The distal cytoplasmic droplet can also be associated with the sperm tail abnormality known as the distal midpiece reflex, which is characterized as a 180° bend of the sperm tail around the distal cytoplasmic droplet at the midpiece. However, in human sperm the residual cytoplasm can be found irregular in shape and covering the entire length of the midpiece. This occurrence of residual cytoplasm has generally been called 'excess residual cytoplasm'. Cooper (2005) proposed that the term cytoplasmic droplet refer to the normal remnant of cytoplasm on sperm produced by a normal testis, and that excess residual cytoplasm refer to the large amounts of cytoplasm on sperm that are irregular in form. These are typically found on human sperm. When appropriate in this review, the term excess residual cytoplasm will be used for observations of human sperm, and the term cytoplasmic droplet will be used for sperm from livestock and other animals. The majority of work suggesting a relationship between excess residual cytoplasm has been performed in human sperm; however, there exists a possibility for a relationship between cytoplasmic droplets and ROS production. Aitken et al. (1994a) found that human spermatozoa with excess cytoplasm around the midpiece contained glucose-6-phosphate dehydrogenase. This enzyme may be able to stimulate production ROS by the previously discussed putative

NADPH oxidase enzyme, or some other enzyme capable of redox reactions present on the cytoplasmic face of the plasma membrane. Gomez and co-workers (1996) found a significant correlation between human sperm with excess residual cytoplasm and PMA induced ROS generation ($r = 0.48$). Similarly, a study by Aziz and colleagues (2004) found positive correlations between ROS production and human sperm with cytoplasmic droplets ($r = 0.28$) and midpiece defects ($r = 0.45$). Visual evidence for the activity ROS and cytoplasmic droplets in membrane oxidation has been provided using a membrane bound fatty acid conjugate C11-BODIPY$^{581/591}$ (Brouwers and Gadella, 2003). Studies by Brouwers and colleagues (2003; 2005) have demonstrated that ROS attack the membrane primarily in the midpiece and tail region of the sperm cell and to a lesser extent in the head of bovine and porcine sperm cells. In particular, the cytoplasmic droplet of the immature sperm cell displays a high degree of fluorescence indicating a high level of ROS exposure (Brouwers et al., 2005). This indicates that the cytoplasmic droplet is both susceptible to ROS attack for both initiation and propagation of the lipid peroxidation cascade. Therefore, the presence of cytoplasmic droplets in livestock species or excess residual cytoplasm on the human spermatozoa may serve to produce ROS by generating free radicals in the plasma or organelle membrane(s) for the free radicals to attack. Although there is no current evidence for the presence of NADPH oxidase or G-6-PDH in animal cytoplasmic droplets, it is known that the cytoplasmic droplet of rats contains a large amount of Golgi-derived structures (Oko et al., 1993). These structures may possess the G-6-PDH that would be required for both ROS production and metabolism within the cytoplasmic droplet. With this in mind, excessive ROS could then be released from cytoplasmic droplets by either cytoplasmic droplet

breakdown/rupture (Dott and Dingle, 1968; Kaplan et al., 1984; Hermo et al., 1988) or via secretory granules (Cortadellas and Durfort, 1994) into the epididymal lumen or semen. Excessive ROS generation by cytoplasmic droplets could overwhelm the antioxidant systems present in either the epididymis or the seminal plasma and cause damage to the entire sperm population. Release and subsequent contamination of ROS could explain how spermatozoa without an attached cytoplasmic droplet still show reduced fertility simply by being present in an ejaculate with a high amount of attached cytoplasmic droplets (Amann et al., 2000; Petrunkina et al., 2001; Thundathil et al., 2001).

Leukocytes produce ROS in order to destroy bacteria, fungi and other invading pathogens. Following ingestion of the foreign pathogen, the phagocytotic cells destroy the pathogen though a 'respiratory burst' of ROS, primarily superoxide anion and hydrogen peroxide. The ROS that are released damage essential proteins and lipids of the pathogen and help to degrade the ingested pathogen. Leukocyte contamination in the ejaculate can be beneficial for destroying foreign pathogens, but can also harm the surrounding spermatozoa by causing an increase ROS and thereby inducing the lipid peroxidation cascade in the sperm plasma membrane. Therefore, indirectly, leukocyte contamination can cause decreased sperm motility and fertility (Ochsendorf, 1999). The seminal plasma contains antioxidants that will combat the ROS produced by the leukocytes; however, the resulting increase in ROS from the 'respiratory burst' can compromise the surrounding antioxidant systems. As a result, malondialdehyde, a reactive carbonyl compound by-product of lipid peroxidation, can form harmful intermediaries with proteins and the DNA of the surrounding spermatozoa (Marnett, 1999).

The presence of leukocytes in the human ejaculate is a common occurrence, with the most prevalent leukocyte species being the polymorph and granulocyte (Aitken et al., 1995). Studies have shown a relationship between leukocytes and chemiluminescent ROS levels when measured in unprocessed human ejaculates. The ROS generated from leukocytes do not seem to impair sperm function as long as the protective action of the antioxidants in the seminal plasma is present. However, if the seminal plasma is removed using centrifugation and the sperm pellet is re-suspended in another solution or if the seminal plasma is diluted using an extender, then the detrimental effect of ROS from leukocytes on the sperm cell is seen through decreased motility and reduced sperm/oocyte fusion (Aitken et al., 1994a). Leukocytes can be removed from washed sperm suspensions using magnetic beads coated with a monoclonal antibody against a leukocyte antigen.

Leukocyte contamination in non-human mammalian species is not as prevalent and therefore ROS production by leukocytes in these species is not of great concern (Ford, 2004). The more likely cause of ROS generation in animal ejaculates is from mitochondrial activity, though data proving this hypothesis is lacking (Ford, 2004). However, in modern livestock insemination programs, the ejaculate is often diluted in order to extend the ejaculate so that more services can be performed from a single ejaculate. Considering that the antioxidants present in the seminal plasma are essential for combating endogenous ROS, it is warranted to speculate that dilution of the seminal plasma with extender solutions may make these antioxidants non-effective and allow for oxidative stress of the sperm cells to occur. A study by Smutná and Synek (1979) demonstrated that boar seminal plasma was effective in reducing lipid peroxidation. These researchers found that undiluted seminal plasma inhibited

lipid peroxidation 92% compared to the sperm samples assayed without the presence of seminal plasma. Further dilutions of seminal plasma from 1:10 to 1:100 inhibited lipid peroxidation from 52% to 12%, respectively.

Effects of reactive oxygen species on sperm

Beneficial effects for sperm cellular signaling

It is important to realize that low levels of ROS play an important role for some physiological processes, including DNA condensation during maturation in the male reproductive tract and capacitation in the female tract (Aitken and Vernet, 1998; Kim and Parthasarathv, 1998; Lapointe et al., 1998; Ford, 2004; Silva et al., 2007). These physiologically critical levels of ROS are carefully regulated by the previously described antioxidant systems.

Sperm are immature and incapable of fertilization of an oocyte when they are released from the testis following spermatogenesis. Maturation of the sperm cell occurs during transit through the caput, corpus and cauda of the epididymis. An important event that occurs in the caput is the process of sperm chromatin condensation. The methods proposed to accomplish this task involve the GPX4 enzyme that is tightly bound to the mitochondria and the chromatin of sperm. The function of GPX4 is to cross-link the protamines present in the sperm nucleus through the oxidation of protmine thiol groups using a ROS as an electron acceptor. The putative ROS available are hydrogen peroxide, a fatty acid hydroperoxide or a phospholipids hydroperoxide (Aitken and Vernet, 1998). The chromatin compaction produced by this redox activity helps protect the male genome from oxidative stress.

Capacitation is a multi-step process that sperm cells must undergo in order to be capable of oocyte recognition and binding (Rodríguez-Martínez et al., 2005; O'Flaherty et al., 2006). During capacitation, intracellular signaling molecules mediate lipid redistribution. The purpose of this activity is to destabilize the plasma membrane and allow for oocyte binding and acrosomal exocytosis of the sperm cell. It has been hypothesized that low level ROS production by the sperm cell, either through a proposed membrane bound NADPH oxidase or electron leakage from the mitochondria, trigger the signaling cascade for modulation of capacitation (O'Flaherty et al., 2006). In this model, ROS produced at the membrane level stimulates membrane-bound adenylyl cyclase, which results in the formation of cyclic AMP. The increased levels of cAMP lead to activation of protein kinase A (PKA), which in turn phosphorylates specific PKA substrates in the MEK and ERK pathways. These pathways lead to phosphorylation of proteins in the tail and equatorial segment of sperm cells, thereby inducing capacitation. Research has demonstrated such phosphorylation patterns in permeabilized sperm cells that had undergone capacitation (O'Flaherty et al., 2006). Evidence for the role of ROS in these phosphorylation events and capacitation were prevented by inclusion of antioxidants (O'Flaherty et al., 2004) and inhibitors of NADPH oxidase (Aitken et al., 1998) into the sperm culture media. These results suggest that low-level ROS production that is carefully regulated by antioxidants is important for capacitation.

Detrimental effects on sperm macromolecules

Chronic, excessive ROS levels that deplete the endogenous antioxidant system may cause harm to the male gamete, including apoptosis, decreased mitochondrial membrane potential, loss of motility and DNA injury in the sperm nucleus (Lopes et al., 1998; Sanocka

and Kurpisz, 2004). Perhaps the most serious consequence is loss of membrane fluidity and integrity since this sperm property is required for membrane fusion events associated with capacitation and fertilization in the human (Aitken et al., 1989).

Most often, ROS attack manifests in the form of lipid peroxidation of the sperm plasma membrane. The process of lipid peroxidation is the breakdown of polyunsaturated fatty acids (PUFAs) that make up a large constituent of the plasma membrane that surrounds cells and cell organelles. The PUFAs are mainly located in the phospholipids of the sperm plasma membrane and are highly susceptible to attack by ROS (Drevet, 2006). In addition to lipids, extrinsic proteins loosely attached to the membrane and intrinsic proteins tightly attached to the membrane are another component of the membrane susceptible to lipid peroxidation (Halliwell and Gutteridge, 1999). The PUFA moieties of phospholipids and cholesterol are preferred substrates for the hydroxyl radical generated in membranes (Brouwers et al., 2005).

Attack of ROS on the lipid plasma membrane can initiate a 'lipid peroxidation cascade'. The lipid peroxidation cascade consists of three parts: 1) initiation; 2) propagation and 3) termination. The sperm plasma membrane is susceptible to lipid peroxidation due to its high PUFA content. The PUFA lipids contain one or more methylene groups positioned between cis double bonds (Marnett, 1999). These methylene groups are highly susceptible to oxidizing agents, such as the previously discussed hydroxyl radical in the presence of the transition metals Fe or Cu. The ROS superoxide anion and hydrogen peroxide are less likely to initiate lipid peroxidation due to low reactivity and/or the inability to enter the hydrophobic interior of the membranes (Halliwell and Gutteridge, 1999). During the

initiation state of lipid peroxidation, a hydrogen is removed from a methylene group by a free radical to form a carbon centered radical which can then react with O_2 to for a peroxyl radical. The newly formed peroxyl radicals undergo molecular rearrangement to form a conjugated diene, which is a compound containing two double bonds between carbon atoms. The conjugated dienes are relatively stable regardless of their location in the carbon chain in the absence of the transition metals iron or copper (Marnett, 1999). Propagation of the lipid peroxidation cascade occurs, however, since the absence of such transition metals is rare and as such the transition metals rapidly reduce all fatty acid hydroperoxides by one electron to alkoxyl radicals. Similarly, propagation of the cascade commences in phospholipids membranes by another fatty acid reducing the peroxyl radicals to lipid hydroperoxides. In doing so, a new carbon-centered radical is formed, which continues the fatty acid oxidation of the lipid peroxidation cascade. There are three ways in which termination of the cascade occurs. First, when the concentration of radical species is high enough for two radicals to react and produce a non-radical species. This occurs by each radical contributing its unpaired electron to form a covalent bond linking the two. Second, when there is no longer any substrate available, and third, when an antioxidant breaks the cascade. The most common chain breaking antioxidant is α-tocopherol. Vitamin E breaks the radical chain by reducing peroxyl radicals, which slows the rate of lipid peroxidation (Marnett, 1999). The resulting α-tocopheroxyl radical causes lipid peroxidation cascade chain termination by forming a compound with very low reactivity (Young and McEneny, 2001). The lipid peroxidation cascade is unique in that it is initiated by ROS, but also produces ROS in the form of the peroxyl radical. Therefore, the cascade demonstrates that sperm are susceptible to ROS, but

also can generate free radicals. Ultimately, the loss of membrane fluidity and integrity is perhaps the most serious consequence of ROS, since this sperm property is essential for the gamete membrane fusion events required for fertilization in the human (Aitken et al., 1989).

In research studies evaluating lipid peroxidation, the effects of sperm membrane lipid peroxidation are observed as either spontaneous (Alvarez et al., 1987; Alvarez and Storey, 1989) or induced through the addition of a ferrous ion promoter or use of the xanthine-xanthine oxidase system (Jones and Mann, 1976; Aitken et al., 1992). Spontaneous lipid peroxidation has been shown to be slower than the induced lipid peroxidation (Alvarez and Storey, 1984; Storey, 2008). Since these two methods of evaluating lipid peroxidation differ, the question becomes which is more accurate. One school of thought suggests that spontaneous lipid peroxidation should be considered the more physiological relevant method of evaluating lipid peroxidation since this is primarily what is observed in *in vivo* situations. However, research by Alvarez and Storey (1984; 1995) determined that the same detrimental effects of membrane lipid peroxidation are observed either spontaneously or through use of inducers of lipid peroxidation. Both methods of detecting sperm membrane lipid peroxidation are used in research. Spontaneous lipid peroxidation measures what is actually happening to the sperm membrane, while induced lipid peroxidation measures what could happen to the sperm membrane.

As with most animal cells, sperm cells are highly susceptible to lipid peroxidation because of the high content of PUFAs in the lipid membrane. The dominant lipids in plasma membranes are phospholipids and sphingolipids. The most predominant sterol in the plasma membrane is cholesterol. The most common phospholipid in animal cell plasma membranes

is phosphatidylcholine (lecithin). Conversely, organelle membranes rarely contain sphingolipid or cholesterol; however, the mitochondria do contain large amounts of the phospholipids cardiolipin (Halliwell and Gutteridge, 1999). The phospholipids and sphingolipids are important for the lipid bilayer because the saturated and PUFA side chains of the lipids help with stability, fluidity and movement of polar solutes and hydrophobic molecules through passive diffusion across the plasma membrane.

The plasma membrane of mammalian sperm is mainly composed of neutral fatty acids, cholesterol, phospholipids and glycolipids (Lenzi et al., 1996). The main phospholipids are lecithin, cephalin and sphingomyelin. As stated above, phospholipids are the main component of the sperm plasma membrane lipid fraction, with phosphatidylcholine and phosphatidylethanolamine being the classes of phospholipids most represented in the human (Mann and Lutwak-Mann, 1981). Studies investigating the lipid composition of ejaculated porcine sperm have also found that the major phospholipids components are phosphatidylcholine and phosphatidylethanolamine. Evans and co-workers (1980) determined that the choline and ethanolamine glycerophospholipids represented 49.9 % and 28.2%, respectively of the total phospholipids of ejaculated washed porcine sperm. Similarly, Parks and Lynch (1992) also demonstrated that choline (~50%) and ethanolamine (~30%) glycerophospholipids represented the two major phosphotides in the boar ejaculate. In agreement, Johnson and co-workers (1969a) found the same major phosphatides in the boar ejaculate, however, no differences existed for a daily collection interval compared to a 3-day collection interval for the phospholipid components. The predominant phospholipids in the bull, stallion and rooster are choline and ethanolamine glycerophospholipids, along with

sphingomyelin (Parks and Lynch, 1992). Sphingomyelin is a unique phospholipid that employs an amide linkage to bind the fatty acid rather than the typical glycerol backbone.

The fatty acid profile of ejaculated porcine sperm was analyzed by Johnson et al. (1969b). These researchers determined that the serine phosphatides were composed of mainly saturated fatty acids (palmitic; 16:0, stearic; 18:0, and myristic; 14:0), whereas choline and ethanolamine phosphatides primarily contained unsaturated fatty acids, of which docosapentaenoic acid; 22:5ω-6 and docosahexaenoic acid; 22:6 were the major unsaturated acids, 38% and 25%, respectively. These results agree with other research that has found palimitic acid, docosapentaenoic acid and docosahexaenoic acid to be the major fatty acids of the boar plasma membrane (Johnson et al., 1972; Nikolopoulou et al., 1985; Parks and Lynch, 1992). Cholesterol has also been found to be the major sterol component of the plasma membrane in rooster, stallion bull and boar (Parks and Lynch, 1992). Cholesterol is thought to be especially important for membrane stabilization during cooling of sperm.

The seminal plasma of the boar ejaculate has also been shown to contain both saturated and PUFAs. Johnson and co-workers (1972) found that the seminal plasma contained primarily six saturated fatty acids, with palmitic being the most prevalent. Surprisingly, the PUFAs, docosapentaenoic acid and docosahexaenoic acid, represented a much lower percentage of the total fatty acids (5-6%) found in the seminal plasma compared to the spermatozoa. At present, the role of the PUFAs in the seminal plasma is not known. Research has not demonstrated that PUFAs in the seminal plasma have an effect on the PUFA content in the sperm plasma membrane, and vice versa.

The phospholipid composition of epididymal sperm is similar to ejaculated sperm. Nikolopoulou et al. (1985) showed that choline and ethanolamine glycerophospholipids represented approximately 70% of the phospholipids in epididymal sperm. However, there were detectable changes in the sperm plasma membrane during migration from the caput to the cauda. Most noticeable was a decrease in ethanolamine, serine and inositol phospholipids and an increase in choline, sphingomyelin and inositides. It is not known why these levels of choline and ethanolamine change during epididymal transit. A logical assumption is that the changes observed play a role in proper sperm cell maturation at both the membrane level and also perhaps the acrosome level in preparation for capacitation, hyperactivation, chemotactic oocyte recognition and oocyte binding and penetration during fertilization. The major fatty acid types seen in epididymal sperm were similar to ejaculated porcine sperm with palmitic being the major saturated fatty acid and docosapentaenoic and docosahexaenoic acids representing the major unsaturated fatty acids. Similar results for the phospholipids classes and major fatty acids classes of epididymal sperm were seen by Johnson et al. (1972) analyzing porcine sperm from the epididymis and in the ejaculate.

Secretions from the seminal vesicle glands make up a portion of the total ejaculate in livestock species. In particular, a large portion of the seminal plasma found in the boar ejaculate comes from the seminal vesicle secretions. Interestingly, Johnson el al. (1972) discovered that the phospholipids secreted by the seminal vesicle glands primarily consisted of almost equal amounts of three 18 carbon fatty acids (18:0, 18:1 and 18:2). These 18 carbon fatty acids accounted for approximately 80% of all the fatty acids evaluated in the seminal vesicle gland secretions. It is not known why there is such a discrepancy between the

fatty acid profiles seen in the seminal plasma of the ejaculate and the seminal vesicle gland secretions. The 18 carbon fatty acids might possibly be modified through fatty acid metabolism during or quickly after ejaculation. Another possibility is that the fatty acids may interact with proteins that are secreted from the seminal vesicle glands that bind to phospholipids of the plasma membrane. Molecular investigations of these proteins indicate that the proteins bind to the phosphatidylcholine, phophatidylcholine plasmalogen and sphingomyelin lipids that make up the sperm plasma membrane. A plasmalogen is a specialized phospholipid found on sperm and thought to play a role in cell signal transduction. The phospholipid binding proteins may be important for capacitation since research in the bovine has shown that these proteins stimulate cholesterol and phospholipid efflux from the sperm membrane (Manjunath and Thérien, 2002). Research in the boar and stallion has identified similar phospholipids binding proteins in the seminal vesicle gland secretions (Calvete et al., 1997).

Factors affecting reactive oxygen species activity

Collection frequency

The time required for the boar to produce a mature sperm cell capable of fertilization is approximately 6-8 weeks. This time period in roughly broken into two parts: the time required in the testis for spermatogenesis and the time required for maturation in the epididymis (reviewed in Senger, 2003). The former requires around 39 days and the latter takes between 9 and 14 days. Inadequate maturation in the epididymis can be detrimental for sperm quality and fertility. As previously discussed, sperm in the epididymis are subjected to redox events necessary for maturation. Typically boars are collected for use in artificial

insemination (AI) programs approximately one to one and a half times per week with an average rest period of 5-7 days in between collections (Knox et al., 2008). Increasing this collection frequency could cause the sperm cells to move through the epididymis at a faster rate, thereby reducing the amount of time for maturation. In theory, an insufficient amount of time in the epididymis may make the sperm cells more susceptible to the detrimental actions of ROS in the ejaculate and result in lower sperm cell viability.

Research suggests that the frequency at which a boar's semen is collected can affect the amount and quality of the sperm in the ejaculate. Bonet et al. (1991) compared a twice-a-week collection against a two-day interval (every-other-day) collection in six mature boars (3 per collection frequency treatment) from the age of 10 months to 22 months. The researchers found that sperm from the two-day interval collection frequency had lower concentration (210 vs 300 x 10^6 sperm/mL). Similar results have been shown for a 24 hour collection frequency compared to a 72 hour collection frequency as volume (161 vs 195 mLs) and concentration (99 vs 221 x 10^6 sperm/mL) both were reduced (Swierstra and Dyck, 1976). Five adult boars collected at 2-day intervals for 10 collections also demonstrated a reduction in volume (120 vs 86 mLs) and concentration (35 vs 16 x 10^6 sperm/mL) when the first two ejaculates were compared to the last two ejaculates (Schilling and Vengust, 1987). Reductions in sperm motility due to increased collection frequency have also been demonstrated. Bonet et al. (1991) detected a 40% reduction (35% vs 76%), whereas Schilling and Vengust (1987) and Frangež and co-workers (2004) both detected an approximately 10-15% reduction in motility (78% vs 63%; 78% vs 70%, respectively).

Generally speaking, boars subjected to an increased collection frequency of greater than 1 time per week produce more morphologically abnormal sperm, including proximal cytoplasmic droplets, distal cytoplasmic droplets, abnormal heads and tails and abnormal apical ridge (Schilling and Vengust, 1987; Bonet et al., 1991; Frangež et al., 2004; Pruneda et al., 2005). The exception to this was by Bonet et al. (1991) who observed a reduction in the percent of distal cytoplasmic droplets (25% vs 1%).

Collection frequency has been shown to increase the level of lipid peroxidation. A study by Strzezek et al. (2000) demonstrated that a multiple collections per day regimen over a 10 day period increased the level of lipid peroxidation in the sperm collected on days 4-10 compared to the sperm collected on days 1-3. This experiment was performed in the summer and winter, with both seasons showing an increase in lipid peroxidation for days 4-10 compared to days 1-3.

Fertility has also been shown to decline for boars on high collection frequencies. Bonet and colleagues (1991) artificially inseminated 75 multiparous females per boar on each collection frequency at 22 months of age (no mention was made to control for parity effects). There was a lower fertility for the boars on the two-day interval frequency (7% vs 73%). Similar results have been found by Frangež et al. (2004) who found a 13% lower farrowing rate (87% vs 74%) when comparing a 1 time per week against a 7 times per week collection frequency. Conversely, Swierstra and Dyck, (1976) detected a higher pregnancy rate in sows inseminated with semen collected on a 24 hour interval compared to semen collected on a 72 hour interval collection schedule. In this study the boars were paired by testis size and motile sperm in the ejaculate prior to initiation of the study. One boar from each pair was randomly

assigned to either a collection frequency of 24 hour intervals or 72 hour intervals for 12 days. After the initial 12 day frequency, the boars were switched to the opposite collection frequency. The authors noted that some boars had better fertility when on the 24 hour collection interval compared to the 72 hour collection interval. Therefore, the authors speculated that the higher pregnancy rates seen for the 24 hour collection interval may be due more to boar variation than the collection frequency.

Components of semen extenders

Liquid semen extenders are designed to prolong the life of sperm following collection. Flowers (2004) proposed that the semen extender must accomplish five basic functions: 1) provide nutrients for sperm metabolism; 2) neutralize metabolic waste products; 3) stabilize sperm membranes and prevent capacitation; 4) maintain an osmotic equilibrium; and 5) retard bacterial growth during storage. In order to accomplish these tasks, four main components are added to semen extenders: 1) an energy source, 2) a pH buffering system, 3) a plasma membrane stabilizer and 4) an antibiotic to prevent bacterial growth (Gadea, 2003; Flowers, 2004). Extenders are generally classified as short- (3 day) or long-(5-7 day) term based on their ability to maintain sperm viability, and it is generally assumed that the different components added to address each function are what determine how long a liquid extender is able to maintain viability of spermatozoa.

The extender components important to ROS activity are the plasma membrane stabilizers, which include macromolecules and chelating agents to prevent capacitation and promote membrane stability. Of particular interest is the chelating agent, ethylenediamietetraacetic acid (EDTA) and the macromolecule, bovine serum albumin

(BSA), since these two components have been implicated in ROS activity. The suppressive effect of EDTA on lipid peroxidation is likely due to its ability to act as an iron chelator and restrict the ability of iron to abstract hydrogen from lipid hydroperoxides to induce lipid peroxidation (Aitken et al., 1993a). Research by Guthrie and Welch (2007), however, was mixed for EDTA. These researchers found that the presence of EDTA in the extending media did not confer any additional protection for suppressing spontaneous oxidation of a membrane bound fluorescent fatty acid conjugate, C11-BODIPY$^{581/591}$, over a five day storage period for boar sperm. However, when sperm were induced for lipid peroxidation with a combination of FeSO4 and Na ascorbate, inclusion of EDTA in the incubation media did prevent oxidation of C11-BODIPY$^{581/591}$ compared to incubation media that did not contain EDTA. These results suggest that EDTA suppresses lipid peroxidation of sperm cells when lipid peroxidation is induced through the addition of $FeSO_4$ and Na ascorbate during incubation at 37ºC for 120 minutes. In the absence of $FeSO_4$ and Na ascorbate, however, spontaneous lipid peroxidation is low during hypothermic liquid storage, indicating that the presence of EDTA to bind Fe and prevent lipid peroxidation is not needed. Surprisingly, Aitken et al. (1993b) demonstrated that, although EDTA did prevent lipid peroxidation, it did not prevent the formation of the hydroxyl radical in the culture media. The hydroxyl radical was monitored by its ability to hydroxylate salicylate using 2,3-dihydroxybenzoate as a standard. This suggests that hydroxyl radical may not play as large a role in initiating lipid peroxidation as previously suspected. Rather, the presence of Fe or Cu may be more important for first chain initiation of lipid peroxidation by acting as oxidizing agents. Another possibility is that lipid peroxides may build up in the sperm plasma membrane

during storage post-collection, but do not initiate the lipid peroxidation cascade due to the presence of the antioxidant system from the seminal plasma or from components in the extender. However, depletion of the antioxidant capacity of the seminal plasma or the extender during storage over time may allow the lipid peroxides in the sperm plasma membrane to form alkoxyl radicals or peroxyl radicals, thereby initiating the lipid peroxidation cascade (Aitken et al., 1993b). Even though the seminal plasma antioxidants are diluted when the semen is extended, there may be enough antioxidant activity to suppress lipid peroxidation. This may explain why BTS without EDTA did not show spontaneous oxidation of C11-BODIPY$^{581/591}$. EDTA is included in both short and long term extenders.

Bovine serum albumin is a macromolecule protein isolated from bovine plasma. Bovine serum albumin is added to sperm extender to act as a membrane stabilizer. Studies have shown that BSA is able to bind to the membrane of rabbit sperm (Blank et al., 1976). Interestingly, the authors observed that BSA bound to the sperm membrane so that roughly half the sperm membrane was coated while the other half was uncoated. They speculated that this binding pattern may be important for physical properties of the sperm required for binding to the oocyte, such as membrane thickness, elasticity or formation of intramolecular links within the membrane. The addition of BSA to sperm cell culture media has been shown to maintain motility and prevent lipid peroxidation in rabbit sperm (Alvarez and Storey, 1983). Although the exact mechanisms for how BSA prevents lipid peroxidation are not known, the authors speculate that since BSA is too large to traverse the plasma membrane, it may protect against lipid peroxidation by attaching to the extracellular side of the plasma membrane. This hypothesis by is supported by the ability of BSA to form intramolecular

links with the sperm plasma membrane, as noted in Blank et al. (1976). Bovine serum albumin is a common additive in the long term extenders such as Androhep (Flowers, 2004).

Age of semen

Sperm are viable in the female reproductive tract for approximately 24 hours following artificial or natural insemination (Hunter, 2004). Physiologically speaking, the sperm cell begins to die once it has been released from the cauda epididymis. Therefore, the purpose of liquid semen extenders used in artificial insemination programs is to provide an environment that slows down the aging process of ejaculated sperm. Generally speaking, most liquid semen extenders can maintain the viability of sperm for a maximum of 7-10 days post collection. The specific reasons for the decline in viability of sperm cells diluted in semen extender are not exactly known. It has been proposed that the oxidative and osmotic stresses encountered by sperm cells following ejaculation and dilution in liquid semen extenders alter sperm metabolism and damage membrane proteins, lipids and chromatin (Ball, 2008). Buffering systems for maintaining pH and osmolarity are present in liquid semen extenders to neutralize the metabolic waste that accumulates from sperm metabolism. As previously discussed, the seminal plasma and sperm possess antioxidants that protect the sperm cell from oxidative stress. Theoretically if these oxidative and pH/osmotic buffering systems are exhausted during the storage period, then the sperm cells will likely die due to the stress.

Sperm viability and fertility have been shown to be reduced in sperm stored in liquid extenders. Research by Waberski et al. (1994) involving hypothermic liquid extending medium demonstrated that sperm stored in a short-term (BTS) extender for 48-87 hours

showed reduced fertility compared to sperm stored for 0-48 hours even when the insemination occurred within 12 hours of ovulation. Evidence for differences in ability of extenders to preserve sperm was shown by a reduction in fertility for BTS at 24-48 hours of storage compared to 48-72 hours of storage for Androhep, both being compared to sperm stored for 0-24 hours (Waberski et al., 1994). Similarly, even long term extenders can differ in their ability to maintain sperm viability and fertility. Research by Kuster and Althouse (1999) looked at the effect of sperm storage in Androhep and X-Cell, two long-term (7 day) extenders, on fertility of 1,431 gilts. These researchers found a significant drop in farrowing rate using sperm stored in Androhep for 5-6 days, whereas there was not a significant decrease for sperm stored in X-Cell. Similarly, there was also a decrease in litter size after 3-4 days of storage in Androhep, while X-Cell did not have a reduced litter size. These results suggest that differences in the extender components can potentially have a large effect on sperm quality and viability. Therefore, it is possible to speculate that as semen ages in a liquid extender the sperm may lose their viability and fertilizing ability due to the exhaustion of the extender components and seminal plasma components, especially if the seminal plasma was heavily diluted due to a high concentration of sperm. A likely scenario is that as the sperm cells age they may become more susceptible to oxidative and osmotic stresses.

Sperm quality

The quality of the sperm cell may be related to its ability to produce ROS and undergo lipid peroxidation. Sperm quality can refer to the morphology of the sperm cell, that is, the overall structure and form of the sperm cell as evaluated by microscopy. Sperm quality can also refer to the viability of the sperm cell, which in this discussion refers to its capability

for demonstrating activity and movement when evaluated by microscopy. Both sperm morphology and viability have shown relationships with ROS activity and lipid peroxidation. Morphologically abnormal human sperm with midpiece abnormalities have shown positive correlations with lipid peroxidation (Rao et al., 1989; Aitken and Sawyer, 2003; Aziz et al., 2004). Non-viable bovine sperm have shown the ability to produce ROS through an aromatic amino acid oxidase (Shannon and Curson, 1982).

The theory that morphologically abnormal and/or non-viable sperm generate ROS at higher levels compared to morphologically normal and/or viable sperm can be tested by separating the sperm into two populations using density gradient centrifugation (Garbers et al., 1970; Lessley and Garner, 1983; Aitken et al., 1989; Grant et al., 1994). Density gradient centrifugation systems operate by using solutions to create layers of differing densities. The gradient is then formed in a test tube, beginning with the high density layers and progressing to the low density layers. Prior to centrifugation, the sperm cells are placed on top of the low density layer. Research has demonstrated sperm cells that are able to separate to the high density layers have better morphology and viability as measured by motility compared to the sperm cells that are retained in the low density layers (Lessley and Garner, 1983; Ball et al., 2001). After centrifugation, the two sperm populations can then be analyzed. Research with human sperm has shown that separation of the ejaculate into different density layers using a two-step discontinuous gradient with 80% and 40% percoll resulted in higher levels of lipid peroxidation in sperm from the 40% layer compared to the 80% layer (Aitken et al., 1993a). However, the specific components of the 40% layer that led to the higher lipid peroxidation were not identified. The authors speculated that the increase in lipid peroxidation may be due

to the increased presence of sperm with midpiece abnormalities since a correlation was found between midpiece abnormalities and lipid peroxidation from sperm in both layers. Furthermore, it is not known if the components of the 40% layer caused the lipid peroxidation or were more susceptible to lipid peroxidation. Additionally, sperm from the 40% layer had lower motility characteristics compared to sperm from the 80% layer.

Similar results using a 40:80% discontinous percoll gradient separation system have been demonstrated by Ball and co-workers (2001) using equine sperm. The authors found that sperm from the 40% layer had significantly greater production of hydrogen peroxide compared to sperm from the 80% layer after incubation for 60 minutes at 38ºC under aerobic conditions. The authors speculate that the higher production of hydrogen peroxide in the 40% layer was due to the increased percentage of morphologically abnormal sperm. Since morphologically normal, viable sperm cells are not separated from morphologically abnormal, non-viable sperm cells prior to dilution in a semen extender, it is tempting to speculate that ROS generation by morphologically abnormal and/or non-viable sperm may compromise the quality of viable sperm over the storage period. However, this scenario is likely dependent on the degree to which morphologically abnormal and/or non-viable sperm are present in the ejaculate (Shannon and Curson, 1982).

A common practice in the swine industry is to pool ejaculates from multiple boars during dilution with a semen extender (Knox et al., 2008). This practice presents an interesting situation regarding how the ROS and antioxidants present in one boar's ejaculate may affect the ROS and antioxidant activity from another boar's ejaculate. In a negative context, pooling of ejaculates may expose the sperm in the pool to an ejaculate with high

levels of ROS and low levels of antioxidants, thereby resulting in a potential increase in ROS activity and lipid peroxidation of the sperm in the pool. In this case, the overall quality of the pooled insemination dose may be compromised by the ROS producing ejaculate. Conversely, the opposite may be true. An ejaculate with low levels of ROS activity and high levels of antioxidants may alleviate ROS activity and lipid peroxidation. Evidence for a beneficial effect of semen components, specifically the seminal plasma, on fertility have been demonstrated (Flowers, 1997). In this study, when the seminal plasma from a high fertility boar was mixed with the sperm from a low fertility boar, the low fertility boar sired a higher proportion of piglets compared to the same sperm mixed with his own seminal plasma. This indicates that at least part of fertility is affected by the seminal plasma, perhaps through the antioxidant system.

Selenium in the diet

Research has shown that selenium is important for fertility and that a selenium deficiency is related to male infertility (Hansen and Deguchi, 1996; Maiorino and Flohe, 1999). The reason for this may be that selenium appears to play an important role in mammalian sperm production, morphology, viability and fertility. Research by Marin-Guzman et al. (1997; 2000) fed boars a basal diet supplemented with 0.5 ppm of inorganic selenium from sodium selenite from the time of weaning until approximately 18 months of age. The non-supplemented basal diet had a level of 0.063 ppm selenium. At 18 months of age, there were higher levels of selenium in the whole ejaculate and spermatozoa, but not the seminal plasma of boars fed the supplemented selenium diet compared to boars fed the non-supplemented basal diet. These results indicate that selenium is readily absorbed from the

diet and incorporated into the ejaculate. However, the beneficial effects of selenium would appear to be associated with the sperm cell rather than the seminal plasma. This observation is supported by the fact that the boars consuming the non-supplemented basal diet produced ejaculates that contained a lower percentage of morphologically normal sperm, a higher percentage of sperm with cytoplasmic droplets and abnormal mitochondria surrounding the midpiece. In addition, the boars on the non-supplemented basal diet had a lower fertilization rate of oocytes compared to boars that were supplemented with 0.5 ppm of selenium. The beneficial effects on sperm fertility from the supplemented boars may have been due an increased activity level of the GPX enzyme in the semen, sperm cells and seminal plasma.

Similar research in chickens has also demonstrated the benefits of supplementing selenium. A study by Surai and co-workers (1998) found that 7 month old cockerels fed a diet supplemented at either 20 or 200 ppm of vitamin E and 0.3 ppm of selenium had significantly greater GPX activity in the seminal plasma, sperm and testes compared to the non-supplemented selenium control diet and diets containing only 20 or 200 ppm of vitamin E.

Collectively, these results suggest that the beneficial effects of selenium may come from its association with the antioxidant GPX enzymes, and therefore may be an important trace mineral for counteracting the effects of ROS during sperm maturation in the testis and epididymis and for preserving sperm function in the in the ejaculate (Roveri et al., 2001). Selenium is an integral part of the catalytic site for all the members of the GPX antioxidant enzyme family (GPX1-4), except for GPX5. With GPX5, the selenocysteine residue is replaced with a cysteine residue. Of the GPX family members, GPX3, 4 and 5 are found

associated with the male reproductive tract and sperm cells. Although it is widely accepted that selenium is required for male fertility, the importance of GPX5, which lacks selenium in the catalytic core, for assisting in antioxidant activities has been raised. Research by Vernet et al. (1999) demonstrated that selenium deficient mice were not adversely affected by oxidative stress in the epididymis. In fact, there was an increase in GPX5 mRNA and protein levels, suggesting that GPX5 was up regulated to account for the loss of the selenium dependent GPX enzymes. The authors suggest that GPX5 plays a backup role for GPX 3 and 4; however, it may be more likely that the three isoforms of GPX work in synchronicity. A similar strategy may be present in the boar, since GPX5 has been identified in swine (Drevet, 2006).

The source of selenium may also be important for its effect on semen quality. Selenium is mainly absorbed from the duodenum in the pig since there is no evidence for absorption in the stomach. Once selenium is absorbed from the gastrointestinal tract, its water solubility allows it to travel with the blood through the portal vein to the liver, where it is then distributed to tissues throughout the body. Selenium in the diet can be absorbed from either a natural selenium-containing feedstuff or as inorganic selenite (Pond et al., 1995). A study by Jacyno and Kawecka (2002) fed boars diets supplemented at 0.2 ppm of selenium from either an organic or inorganic source beginning 70 days of age. The authors found that boars fed the organic selenium diet had a higher sperm concentration and total number of sperm in the ejaculate and a lower percentage of morphologically abnormal sperm compared to the boars fed the inorganic selenium diet. Interestingly, there were no differences detected between the diets for level of selenium measured in the seminal plasma. In addition, the level

of GPX activity in the seminal plasma was actually lower for boar on the organic selenium diet compared to boars on the inorganic selenium diet. Previous research had indicated that an organic source of selenium from selenium-enriched yeast (Mahan and Parrett, 1996) was more 'bio-available' than an inorganic source of selenium due to results showing a higher level of selenium in loins of growing and finishing pigs. However, the results of this study and previous studies indicate the improved 'bio-availability' of organic selenium over inorganic selenium demonstrated for muscle tissue may not be applicable to blood or to components of the boar ejaculate.

Based on these results, it appears that, compared to a non-supplemented control, boars fed diets supplemented with inorganic selenium have better sperm quality possibly due to higher levels of the selenium and GPX activity in the ejaculate. Similarly, boars fed a diet containing organic selenium compared to boars fed a diet containing inorganic selenium have better sperm production and sperm morphology. However, the reasons behind the improved sperm production and sperm morphology for boars fed the organic selenium diet are not readily apparent since the levels of selenium in the seminal plasma were not different, and since the GPX activity in the seminal plasma was lower compared to boars fed the inorganic selenium. It may be that the beneficial effects of the organic selenium were more associated with the sperm cell, and not the seminal plasma. Measuring selenium levels and GPX activity in the sperm cell, or the whole ejaculate, may reveal the source of the beneficial effects of the organic selenium.

Statement of the problem

Due to the widespread use of artificial insemination in the swine industry, it is essential that research is performed to investigate factors that influence the fertility of the male gamete (Knox et al., 2008). Specifically, it is important to understand what factors affect semen quality between the time the semen is collected, suspended in a semen extender and inseminated. Protection against ROS in the fresh boar ejaculate is provided by the reproductive tract in the form of antioxidant enzymes and low molecular weight antioxidants. However, these protective antioxidants of the seminal plasma are diluted when the ejaculate is extended. Therefore it is plausible that deterioration of semen quality could be due to the action of ROS through propagation of the lipid peroxidation cascade during the storage period post-collection within the extended insemination dose. Consequently, research investigating factors that affect the presence of ROS in the fresh and extended porcine ejaculate is warranted.

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CHAPTER 2

EFFECT OF COLLECTION FREQUENCY AND EXTENDER ON SPERM QUALITY PARAMETERS AND MEMBRANE LIPID PEROXIDATION IN BOARS

Abstract

The primary objective of this research was to examine changes in ejaculate characteristics, semen quality and membrane lipid peroxidation over time in boars maintained under high and normal collection frequencies. A secondary objective was to determine the effects of semen extender and storage time on semen quality and sperm membrane lipid peroxidation. Sexually mature boars were randomly assigned to a collection frequency of 1 (n=5) or 3 (n=5) times per week and collected over a 5 week period. On weeks 0, 2 and 5 the ejaculate was split, extended in a commercially available 3-day or 5-day extender and evaluated at 0, 1, 3, and 7 days following collection. Malondialdehyde (MDA) was used to evaluate membrane lipid peroxidation and was standardized by measuring activity from 375 x 10^6 sperm/mL. Ejaculate characteristics included volume, concentration and total sperm numbers. Semen quality parameters measured were percentage of motile and progressively motile sperm, morphologically normal sperm, abnormal sperm and sperm with normal acrosomes. A collection frequency x week interaction ($P < 0.01$) was present for the ejaculate characteristics. Concentration (322 vs. 219 x 10^6 sperm/mL, $P < 0.01$) and total sperm numbers (81 vs. 55 x 10^9 sperm, $P < 0.05$) decreased between weeks 0 and 5 for boars collected 3 times per week, while no changes over time were observed for boars collected 1 time per week ($P > 0.3$). A collection frequency x day interaction for motility ($P < 0.001$) was observed. Motility for sperm collected from boars maintained on the 3 times per week frequency was lower on day 7 ($P < 0.0001$) compared to day 0, although boars collected 1 time per week did not change ($P = 0.5$). Similar results existed for progressive motility with a collection frequency x day x week interaction ($P = 0.01$). Generally, the 3 times per week

treatment boars had greater ($P < 0.0001$) progressive motility at day 0 compared to the 1 time per week treatment boars. The 3 times per week treatment boars had lower ($P < 0.01$) progressive motility on day 7, while progressive motility of the 1 time per week treatment boars did not change ($P > 0.05$) compared to day 0. No differences ($P = 0.13$) in motility or progressive motility were found between semen extenders at any day of storage. An extender x storage time effect for MDA was present ($P = 0.03$). Sperm stored in the 3-day extender were significantly higher ($P = 0.0014$) for MDA at days 3 and 7 (2.23 and 2.24 μM, respectively) compared to day 0 (1.87 μM), while MDA did not change ($P > 0.9$) for sperm stored in the 5-day extender from day 0 to 7. An extender x storage time interaction was present for normal acrosomes ($P = 0.0001$). The percentage of sperm with normal acrosomes was lower ($P < 0.0001$) by day 1 for the 3-day extender, but not until day 7 for the 5-day extender ($P = 0.004$). A storage time effect was present for the percentage of morphologically normal sperm. The percentage of normal sperm significantly decreased between days 0 and 7 (70 vs. 67%, $P < 0.0001$). These results suggest that the 3 times per week collection frequency and storage time affected sperm motility, whereas extender and storage time altered membrane lipid peroxidation and the percentage of sperm with normal acrosomes.

Introduction

In the swine industry, the majority of semen used for artificial insemination is extended in a liquid solution designed to keep the sperm viable over a period of days post-collection. However, studies have shown that extender can affect sperm fertility over the time period following extending as evidenced by differences in farrowing rates and litter size of piglets (Waberski et al., 1994, Kuster and Althouse, 1999). Theoretically, the reason for the

decreased fecundity is that, over time, sperm quality deteriorates after being placed in the extending solution. However, the causative agents for the sperm quality decline are not known. One possible agent could be the activity of reactive oxygen species, which have been shown to affect sperm motility and plasma membrane integrity through lipid peroxidation of the plasma membrane (Kim and Parthasarathy, 1998; Silva et al., 2007). It is not well understood what factors affect the presence of reactive oxygen species in the boar ejaculate. A study by Strzezek et al. (2000) showed that collection frequency may have an effect, as multiple collections per day over a 10 day period resulted in increased the levels of lipid peroxidation in the ejaculates collected on days 4-10 compared to days 1-3. Another interesting result from this study was that the percentage of morphologically abnormal sperm also increased with the high collection frequency. Some researchers have speculated that abnormal sperm may be a source of reactive oxygen species in the ejaculate (Aitken et al., 1989). The sperm midpiece may be a source of reactive oxygen species production. Research on human sperm has speculated that retention of excess cytoplasm in humans (analogous to the cytoplasmic droplet) may be associated with the incidence of reactive oxygen species (Aitken and Sawyer, 2003; Aziz et al., 2004). If this is the case, then a collection frequency that increases the amount of abnormal sperm, including sperm with a retained cytoplasmic droplet, could be expected to also increase the generation of reactive oxygen species. Therefore, the purpose of this research was to determine the effects of collection frequency (3 times per week vs. 1 time per week), extender (3-day vs. 5-day) and day of storage (0, 1, 3, and 7 post-collection) on reactive oxygen species mediated lipid peroxidation and estimates of semen quality parameters in boars.

Materials and Methods

Animal Care and Use

All procedures that involved the use of animals were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC # 06-036-A). Ten crossbred boars (age: 2.03 ± 0.28 years; weight: 249.43 ± 6.68 kg) that were terminal crosses of Duroc, Landrace, Yorkshire, and Hampshire breeds were used in the study. Boars were provided ad libitum access to water and were fed a pelleted gestation corn–soybean meal diet that met or exceeded nutritional requirements for adult boars (NRC, 1998). Boar housing consisted of individual stalls (2 m x 0.7m) located in an environmentally controlled building using a curtain-sided, under slat ventilation system. Supplemental ventilation fans and a drip cooling system were set to activate when ambient temperatures reached 23ºC and 26ºC, respectively. Weekly high and low temperatures and humidity levels are reported in Table 2.1. Prior to beginning the study, boars were on a 1 time per week collection frequency for approximately two months.

Experimental design

Boars were randomly assigned to a collection frequency of either 1 time or 3 times per week for five consecutive weeks beginning in July and ending in August, 2006. Semen analyses were performed during weeks 0, 2 and 5. Boars collected 1 time per week were collected on Tuesdays and those collected 3 times per week were collected on Monday, Wednesday, and Friday. For the boars on the 3 times per week frequency, the ejaculate used for the laboratory semen analysis was collected on Monday. All boars were collected between 06:30 and 09:00 on collection days. The ejaculates collected on Wednesday and

Friday for the 3 times per week collection frequency and the ejaculates collected during weeks 1, 3 and 4 when semen analyses were not performed were measured for sperm concentration and volume and then discarded.

Ejaculates were collected using the gloved-hand technique (Almond et al., 1998) into a pre-weighted collection thermos containing a plastic bag (IMV International Corp., Maple Grove, MN) and warmed to 37ºC. The collection thermos was covered with a filter (IMV International, Eden Prairie, MN) to separate the fluid fraction (semen) of the ejaculate from the gel fraction. The volume of semen was estimated by weighing each ejaculate with a gravimetric electronic balance (V-300, Acculab, Edgewood, NY) and using the conversion of 1 g of semen equals 1 mL. The concentration of sperm cells in the semen was evaluated using a photometer (SpermaCue™, Minitube of America, Verona, WI). The total number of sperm cells in the semen was calculated by multiplying the volume by the concentration. Ejaculate volume, concentration and total sperm cell numbers were recorded following each collection. Following collection, ejaculates were stored in a water bath pre-warmed to 37ºC (Minitube of America, Verona, WI). Ejaculates were transferred to the lab approximately 1.5 hours after the first ejaculate had been collected.

During weeks 0, 2 and 5 the semen analysis was performed. Upon arrival at the laboratory, semen was placed into a water bath pre-warmed to 37ºC. Each ejaculate was split and extended to a concentration of 37.5×10^6 sperm/mL in 0.01 M phosphate buffered saline (PBS; pH 7.2), a commercially available 3-day extender (BTS; Minitube of America, Verona, WI) and 5-day extender (Androhep; Minitube of America, Verona, WI). Three, 15 mL aliquots containing 562.5×10^6 sperm in 15 mLs for each boar and extender combination

were prepared from a total 50 mL volume of extended semen and stored in a semen cooling unit (Minitube of America, Verona, WI) at 17-18°C until analysis. During storage the aliquots were gently inverted once per day to re-suspend the sperm cells into solution. Laboratory analyses for semen quality estimates were performed on days 0, 1, 3, and 7 after collection. The following semen quality estimates were performed for each sample: the percentage of motile and progressively motile sperm, the percentage of sperm with normal acrosome morphology, the percentage sperm classified with normal morphology or abnormal morphology including abnormal tail, abnormal head, and sperm possessing a cytoplasmic droplet in the proximal, distal or as part of the distal midpiece reflex. In addition, concentration of malondialdehyde (MDA) was measured for each sample.

Semen quality analysis

Motility analysis was performed using the Sperm Vision™ digital semen evaluation software (Minitube of America, Verona, WI) using images obtained by a digital camera attached to phase contrast microscope (Model BX41; Olympus Optical; Tokyo, Japan) with a 20x phase objective. Prior to analysis, a one mL aliquot from each sample was incubated at 37°C for 30 minutes. Four-chamber Leja slides (IMV International Corp., Maple Grove, MN) were pre-warmed to 37°C on a slide warmer (Minitube of America, Verona, WI) before motility analysis. Five microscopic fields were analyzed for each sample, in duplicate, for a total of ten fields analyzed per sample. Approximately 250-400 total cells were analyzed for the percentage of sperm exhibiting motility and progressive forward motility.

Acrosome and sperm morphology were performed using a phase contrast microscope (Zeiss; West Germany) using a 100x oil-immersion objective. Prior to analysis, a one mL

aliquot from each sample was fixed with 100 μL of 10% buffered formalin. For acrosome and sperm morphology, 10 μL of each sample was placed on a slide, covered with an 18mm x 18mm coverslip and then given at least 5 minutes for the sperm to settle before analysis. Acrosome and sperm morphology analysis consisted of evaluating all sperm in 15 evenly spaced fields across the slide. The average number of sperm evaluated per sample was 143.80 ± 2.35 (mean ± standard error). For acrosome morphology, the sperm were classified according to Pursel et. al. (1972). For sperm morphology, the sperm were classified according to Almond et. al. (1998) and Lovercamp et al. (2007). Sperm were classified into one of the following six morphological groups: normal, abnormal head, abnormal tail, proximal cytoplasmic droplet, distal cytoplasmic droplet and the distal midpiece reflex with a cytoplasmic droplet. Spermatozoa classified as a proximal cytoplasmic droplet possessed a cytoplasmic droplet located on the anterior half of the sperm tail midpiece proximal to the sperm head. Spermatozoa classified as distal cytoplasmic droplet possessed a cytoplasmic droplet located on the posterior half of the sperm midpiece. All spermatozoa classified as distal midpiece reflex possessed a cytoplasmic droplet in the 180° bend of the tail. Spermatozoa with tail defects observed without a distal cytoplasmic droplet in the bend of the tail were classified as an abnormal tail.

Spontaneous sperm lipid peroxidation activity was evaluated using the MDA-586 kit (OxisResearch, Portland, OR, USA). The assay detects the presence of a carbonyl compound, malondiadehyde (MDA), which is an end-product of the lipid peroxidation cascade resulting from the breakdown of lipid peroxides. For each day of the analysis, samples were prepared for MDA analysis using the following washing procedure. For each sample, ten mLs of

extended semen containing 37.5 x 10^6/mL sperm were washed twice in PBS at 3600 rpm for 5 minutes in a hinged rotor centrifuge (Model Marathon 3200; Fisher Scientific, Pittsburgh, PA) at room temperature to form a sperm pellet. After each wash the pellet was then gently re-suspended in 1 mL of PBS. The pellet was finally re-suspended in 1 mL of PBS to a final concentration of 375 x 10^6/mL for quantification of MDA. The MDA assay was performed to measure free MDA by incubating the samples at 45°C for 60 minutes. After incubation, the samples were centrifuged at 10,000 x g for 10 minutes to obtain a clear supernatant, as directed by the protocol. Samples were immediately plated and absorbances measured in a 96-well plate at a wavelength of 586 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT). Intra- and inter-assay coefficients of variation for twenty-three assay batches were 2.61 and 6.06%, respectively based on a known 2.5 μM amount of the MDA standard that was pre-made, stored frozen and measured in duplicate on each assay day. The MDA-586 assay kit was validated for boar sperm using a spike and recovery technique of known concentrations of the MDA standard into three samples of boar sperm stored in the 5-day extender for 2 days. The recoveries were (mean ± standard error) 77.0 ± 2.5%, 83.0 ± 3.0% and 89.5 ± 1.5% (Appendix A).

Statistical analysis

All statistical analyses were performed using the mixed procedure of SAS (SAS Ins., Cary, NC). The variance/covariance structure used for the repeated measures analyses was determined by finding the appropriate structure with the lowest fit statistics. Prior to analysis, all percentage data were normalized with an arcsine transformation (Snedecor and Cochran, 1989). Results for percentage data are reported as non-transformed arithmetic means ±

standard error of the mean. All other data are reported as least squares means ± standard error of the mean. Non-significant ($P > 0.05$) interactions were removed from all models. For all models, the Tukey-Kramer adjustment was used to determine differences among means of independent variables when significant effects were observed (Kaps and Lamberson, 2004).

Two additional cumulative variables were derived from the morphological analysis for each ejaculate and are included in the statistical analysis for sperm morphology: 1) a total attached cytoplasmic droplet group (proximal cytoplasmic droplet + distal cytoplasmic droplet + distal midpiece reflex); and 2) a total abnormality group (abnormal tail + abnormal head + total attached cytoplasmic droplets).

One boar from the 3 times per week collection frequency was removed midway through the trial because of injury to the rear legs and was not included in the week 5 analysis.

Data for ejaculate characteristics volume, concentration and total sperm numbers were analyzed as a complete randomized design using analysis of variance procedures for repeated measures (Gill and Hafs, 1971). The model included collection frequency (3 times or 1 time per week), week (0, 2 and 5) and the collection frequency x week interaction. The error term used to test for treatment effects was boar nested within collection frequency. Week was considered the repeated variable. A collection frequency x week interaction was present for concentration ($P = 0.007$) and total sperm numbers ($P = 0.005$). Therefore additional analyses were conducted to evaluate differences among weeks within collection frequency.

Data for the semen analysis were analyzed as a complete randomized design using analysis of variance procedures for repeated measures (Gill and Hafs, 1971). The model included collection frequency (3 times or 1 time per week), extender (3-day or 5-day), week (0, 2 and 5) and day of storage (0, 1, 3 and 7) and all appropriate interactions. The error term used to test for treatment effects was boar nested within the collection frequency x extender x week interaction. Day of storage was considered the repeated variable. A collection frequency x day of storage interaction was present for sperm motility ($P < 0.0001$), the percentage of sperm with normal acrosomes ($P = 0.02$) and the percentage of normal and abnormal sperm ($P = 0.04$). Therefore additional analyses were conducted to evaluate differences among day of storage within collection frequency. A collection frequency x day of storage x week interaction was present for sperm progressive motility ($P = 0.01$). Therefore additional analyses were conducted to evaluate differences among day of storage within collection frequency and week. An extender x day of storage interaction was present for the percentage of sperm with normal acrosomes ($P = 0.0001$) and MDA concentration ($P = 0.03$). Therefore additional analyses were conducted to evaluate differences among day of storage within extender.

Results

Ejaculate characteristics

Collection frequency or week did not have an effect on ejaculate volume ($P = 0.7$). A collection frequency x week interaction ($P = 0.0074$) was present for concentration (Figure 2.1.). No differences ($P > 0.05$) were found over the 5 week period for boars collected 1 time

per week. The concentration of sperm was lower ($P < 0.01$) in ejaculates collected from boars on the 3 times per week collection frequency during weeks 2 and 5 compared to week 0.

A collection frequency x week interaction ($P = 0.0052$) was present for the number of total sperm in the ejaculate (Figure 2.2.). No differences ($P > 0.05$) were found over the five week period for the 1 time per week collection frequency boars, however, the number of total sperm was lower ($P < 0.05$) in ejaculates collected from boars on the 3 times per week collection frequency during week 5 compared to week 0.

Motility

A collection frequency x day of storage interaction was present ($P < 0.0001$) for motility (Figure 2.3.). The 3 times per week collection frequency had lower ($P < 0.0001$) motility on days 1, 3 and 7 compared to day 0. There were no differences in motility between days 0, 1 and 7, however, day 3 had lower ($P < 0.0001$) motility compared to day 0 for the sperm from the 1 time per week collection frequency. There was no effect of extender or week ($P > 0.2$) on motility.

A collection frequency x day of storage x week interaction ($P = 0.01$) was present for progressive motility (Figure 2.4.). Generally, in each of the weeks there was a reduction ($P < 0.05$) in sperm progressive motility between day 0 and days 3 and 7 for the 3 times per week collection frequency, however, no reduction ($P > 0.05$) in progressive motility occurred between day 0 and days 1, 3 and 7 for sperm from the 1 time per week collection frequency. There was no effect of extender ($P = 0.1$) on progressive motility.

Sperm morphology

Data for the percentage of sperm with normal acrosomes are presented in Figures 2.5. and 2.6. There was a collection frequency x day of storage interaction ($P = 0.02$; Figure 2.5.). The 3 times per week collection frequency had a lower ($P < 0.05$) percentage of sperm with normal acrosomes on day 7 compared to days 0 and 1. In the 1 time per week collection frequency, a reduction ($P < 0.05$) in the percentage of sperm with normal acrosomes occurred on days 1, 3 and 7 compared to day 0.

An extender x day of storage interaction ($P = 0.0001$; Figure 2.6.) was also present for the percent of sperm with normal acrosomes. Both extenders had a lower ($P = 0.004$) percentage of sperm with normal acrosomes on day 7 compared to day 0, however, the sperm stored in the 3-day extender were also lower ($P < 0.03$) at days 1 and 3 compared to day 0. There was no effect of week ($P = 0.9$) on the percentage of normal acrosomes.

There were no effects of collection frequency, extender, day of storage or week for the percentage of sperm with an abnormal head or an abnormal tail ($P > 0.1$). A collection frequency x day of storage interaction ($P = 0.04$) was present for the percentage of morphologically normal sperm and total abnormal sperm (Table 2.2). The percentage of morphologically normal sperm were lower ($P < 0.01$) on days 3 and 7 compared to days 0 and 1 for sperm from the 3 times per week collection frequency. In the 1 time per week collection frequency, the percentage of morphologically normal sperm was lower ($P < 0.05$) on day 7 compared to days 0 and 1. However, the percentage of total abnormal sperm was higher ($P < 0.05$) on day 7 compared to days 0, 1 and 3. Extender or week ($P > 0.8$) did not

affect the percentage or morphologically normal sperm or the percentage of total abnormal sperm.

Table 2.3. contains the percentage of sperm possessing a proximal cytoplasmic, distal cytoplasmic droplet, distal midpiece reflex and total attached cytoplasmic droplets over the 7 day storage period. There were no differences for sperm with the distal cytoplasmic droplet during the storage period ($P = 0.07$). The percentage of sperm with a proximal cytoplasmic droplet was higher on days 3 ($P < 0.0001$) and 7 ($P = 0.002$) compared to day 0. The percentage of distal midpiece reflex sperm increased on days 3 ($P = 0.007$) and 7 ($P = 0.01$) compared to day 0. The percentage of total attached cytoplasmic droplet sperm were greater ($P < 0.0001$) on days 3 and 7 compared to days 0 and 1.

Collection frequency affected ($P < 0.05$) the percentage of normal sperm, sperm with a distal cytoplasmic droplet, distal midpiece reflex, total attached cytoplasmic droplets and total abnormal sperm (Table 2.4.). For each significant dependent variable, the percentage of sperm with the morphological abnormality was higher ($P < 0.05$) for boars from the 1 time a week collection frequency compared to boars from the 3 times per week collection frequency.

Collection frequency, extender and week did not affect ($P > 0.7$) the percentage of sperm with a proximal cytoplasmic droplet. The percentage of sperm with a distal cytoplasmic droplet, distal midpiece reflex and total attached cytoplasmic droplets were not affected by extender or week ($P > 0.1$).

Lipid peroxidation

An extender x day of storage interaction (P = 0.03; Figure 2.7.) was present for the concentration of MDA produced. The 3-day extender had higher (P = 0.001) levels of MDA on days 3 and 7 of storage post-collection compared to day 0. There was not a significant increase in the 5-day extender by day 7 of storage (P = 0.9). The concentration of MDA was not affected by collection frequency (P > 0.1).

Discussion

The primary purpose of this study was to determine if an elevated collection frequency of 3 times per week compared to a 1 time per week frequency would result in a higher percentage of morphologically abnormal sperm and increased plasma membrane lipid peroxidation from reactive oxygen species activity. The secondary objective was to evaluate the effects of a 3-day and 5-day extender on sperm morphology and protection against plasma membrane lipid peroxidation over a 7 day storage period post-collection.

The boars on the 3 times per week collection frequency produced ejaculates that had lower sperm concentration and total sperm numbers at week 5 compared to week 0. The boars on the 3 times per week collection frequency decreased approximately 31% for sperm concentration and for total sperm numbers from week 0 to 5. These results agree with other studies that have demonstrated a reduction in total sperm numbers of approximately 50% for boars collected over an elevated collection frequency period (Schilling and Vengust, 1987).

In the present study, there was a reduction in sperm motility and progressive motility over the 7 day storage period for boars on the 3 times per week collection frequency, but not for boars on the 1 time per week collection frequency. This observation is logical, since it

could be expected that the 3 times per week collection frequency would exacerbate the decline in sperm motility over the 7 day storage period post-collection. Other studies demonstrated that boars subjected to an elevated collection frequency produced sperm with reduced motility and progressive motility (Schilling and Vengust, 1987; Bonet et al., 1991; Frangež et al., 2004). However, these studies only determined motility on the day of collection and did not determine sperm motility over a post-collection storage period. Therefore, one might speculate that motility of sperm from the elevated collection frequency boars might have declined at a greater rate compared to the control collection frequency boars in these studies.

The likely cause for not seeing a drop in motility for the 1 time per week collection frequency may be due to the values on day 0 post-collection (motility: ~60%; progressive motility: ~18%). Although the day 0 motility value for the 1 time per week collection frequency was low at 60%, this value is acceptable for use of sperm in an artificial insemination program without expecting a reduction in fertility (Flowers, 1997). However, the low motility seen on day 0 for the 1 time per week frequency compared to the day 0 motility value for the 3 times per week frequency indicates that the population of boars may not have been the same prior to the onset of the study and that the randomization used for assigning the boars to treatments was ineffective. This situation may have been avoided by monitoring motility of the boars prior to the study for 2-3 weeks and then blocking boars on motility.

Previous research has demonstrated that boars subjected to an elevated collection frequency produce ejaculates containing a higher number of sperm abnormalities compared

to boars on a control collection frequency (Pruneda et al., 2005). In the present study, the 3 times per week collection frequency did not induce ejaculates with a higher percentage of abnormal sperm compared to the 1 time per week collection frequency. Rather, the opposite was true as the 1 time per week collection frequency had higher percentages of abnormal sperm. As discussed above, this indicates that the population of boars was different at the onset of the study and that randomly assigning the boars to treatments was not effective. Analysis of the data following the study showed that two boars on the normal collection frequency were greater than the mean for total abnormalities by approximately 1.5 standard deviations. Blocking boars based on prior sperm morphology may have allowed for better evaluation of the effect of collection frequency on the sperm morphological parameters.

The type of extender used to dilute the sperm for storage had an effect on the percent of sperm with normal acrosomes over the 7 day storage period post-collection. Sperm stored in the 3-day extender (BTS) decreased for the percentage of normal acrosomes on days 1, 3 and 7 of storage compared to day 0. The 5-day extender used (Androhep) was able to provide better protection of the acrosome, as the percentage of sperm with normal acrosomes was not lower until day 7 of storage post-collection. These results suggest that different components for membrane stabilization in the respective extenders may be responsible for the difference seen in protecting the acrosome over the 7 day storage period. The likely candidate component would be bovine serum albumin (BSA), a plasma protein that is added to Androhep but not BTS. The purpose of BSA in a semen extender is to provide membrane stabilization during the storage period. Studies have shown that BSA is capable of binding to the membrane of rabbit sperm and possibility modifying membrane permeability and

mechanical properties of the membrane by altering the membrane thickness or through formation of intramolecular links (Blank et al., 1976).

Changes in sperm morphology may be possible during the storage period post-collection due to normal degradation of the sperm cell exacerbated by the inability of the extender to maintain membrane integrity. If changes in sperm morphology do occur over the storage period, it may be expected that there would be a decrease in the percentage of sperm with normal morphology and a corresponding increase in the percentage of abnormal sperm. The results of this study support this hypothesis, as the percentage of sperm with normal morphology was lower on days 3 and 7 of storage while the percentage of sperm with abnormalities correspondingly increased. The drop in the percentage of normal sperm was not due to an increase in the percentage of sperm with head or tail structural abnormalities, but was due to an increase in the percentage of sperm with either a proximal cytoplasmic droplet or sperm with the distal midpiece reflex around the distal cytoplasmic droplet. To our knowledge, there is no evidence that the cytoplasmic droplet has the ability to move from the distal position back to the proximal position at any time following spermiation, as is suggested by the increase in the percentage of sperm with the proximal cytoplasmic droplet. Therefore, it is more likely that the increase in the percentage of sperm with a proximal cytoplasmic droplet was due to inherent inaccuracies that occur when a subjective morphological evaluation of sperm is performed, even with two trained technicians, as occurred in the present study. In the present study, the morphological evaluation consisted of evaluating all sperm in 15 evenly spaced fields across a slide. The average number of sperm evaluated per sample was 143.80 ± 2.35 (mean ± standard error).

Results of the current study are not consistent with previous research reporting an increase in the concentration of MDA due to lipid peroxidation following a high collection frequency in boars. When Strzezek and co-workers (2000) subjected 4-5 year old Polish Landrace boars to multiple daily collections for 10 days they found an increase in MDA from lipid peroxidation of sperm cells collected on days 4-10 compared to sperm cells collected on days 1-3 of the 10 day collection period. In the present study, boars were subjected to a less rigorous collection frequency of three collections per week, which may explain why an increase in MDA was not observed. The effect of multiple collections per day over multiple days may be required to sufficiently alter sperm maturation in the epididymis and, as a result, make the sperm cells susceptible to membrane lipid peroxidation, which would result in an increase of MDA. This scenario could possibly explain the results of Strzezek et al. (2000).

The type of extender used for dilution of sperm during storage post-collection may be important for preventing lipid peroxidation. The concentration of MDA increased approximately 19% in the 3-day extender on days 3 and 7 compared to day 0 of storage. A similar increase in MDA concentration over the 7 day storage period was not observed for sperm stored in the 5-day extender in the present study. The increase in MDA concentration over the 7 day storage period post-collection in the 3-day extender and lack of an increase in MDA concentration over the storage period in the 5-day extender is likely due to the different components of each extender. The 5-day extender contains BSA, which as previously discussed, is a membrane stabilizer that is capable of binding to the plasma membrane and possibility protecting the membrane by modification of membrane mechanical properties. The benefits of BSA appear to be important for protecting sperm

plasma membranes from reactive oxygen species attack and lipid peroxidation. Addition of BSA to the incubation media has been shown to preserve motility and prevent lipid peroxidation in rabbit sperm (Alvarez and Storey, 1983). The specific mechanism by which BSA protects against lipid peroxidation is not known. Since BSA is too large to traverse the plasma membrane, it may protect against lipid peroxidation by attaching to the extracellular side of the plasma membrane and potentially inhibit lipid peroxidation initiation by preventing the interaction of reactive oxygen species with the methylene groups of the polyunsaturated fatty acids found in the sperm plasma membrane (Alvarez and Storey, 1983; Marnett, 1999).

The findings of the present study which show an increase in lipid peroxidation of boar sperm over a 7 day storage period agree with the results of Ball and Vo (2002) that showed a slight increase of plasma membrane lipid peroxidation of equine sperm stored over a two day period post collection. Ball and Vo (2002) measured lipid peroxidation using a membrane bound fluorescent fatty acid conjugate, C11-BODIPY$^{581/591}$, which irreversibly fluoresces from red to green when exposure to reactive oxygen species occurs (Brouwers and Gadella, 2003). However, the present study contradicts the results of other studies that did not show an increase in lipid peroxidation of stored sperm cells post-collection. Cerolini et al. (2000) found a non-significant increase in MDA concentration from lipid peroxidation of boar sperm cells diluted in BTS following a five day storage period. Although there was an approximate 19-fold increase in the MDA concentration from day 1 to day 5, the lack of significance may have been due to the low number of boars (n = 3) used in the study. Guthrie and Welch (2007) also were unable to detect an increase in lipid peroxidation over a five day

storage period post-collection in boar sperm diluted in BTS. These researchers also evaluated membrane lipid peroxidation using a membrane bound fluorescent fatty acid conjugate, C11-BODIPY$^{581/591}$. In contrast, the present study evaluated membrane lipid peroxidation by measuring the presence of a carbonyl compound, MDA, which is a by-product of lipid peroxidation from the breakdown of lipid peroxides of the membrane. Although each method has been shown to be indicative of lipid peroxidation of the membrane, to our knowledge no research has directly compared the results of these assays side-by-side to determine the correlation between the two assays. This comparison warrants investigation.

It is difficult to determine why a significant increase in lipid peroxidation was not found using the membrane bound fatty acid conjugate in boar sperm. One possible reason is that the enzyme phospholipase A_2 can cleave lipid peroxides from the plasma membrane. The free floating lipids could then be scavenged by the antioxidants, or if the antioxidant system has been exhausted, could breakdown into MDA (Aitken, et al., 1993; Halliwell and Gutteridge, 1999). As a result, there would be very little evidence of lipid peroxidation within the membrane of the sperm cell itself since the lipid peroxides, the precursors to MDA, would have been cleaved from the sperm cell plasma membrane. This could explain why Guthrie and Welch (2007) observed negligible spontaneous lipid peroxidation of the membrane bound fluorescent fatty acid conjugate after five days of storage in BTS, whereas, in the present study, an increase in lipid peroxidation was observed. It is not know if the membrane bound fluorescent fatty acid conjugate is a substrate for phospholipase A_2 or not since the degree of preference for oxidized fatty acids is uncertain (Halliwell and Gutteridge,

1999). Further research is warranted to find a reliable marker of lipid peroxidation in the boar.

Conclusions

The results of this study indicate that a high collection frequency of 3 times per week exacerbates the decline in motility of sperm cells over a 7 day storage period post-collection, but does not affect sperm morphology or sperm plasma membrane lipid peroxidation compared to a normal collection frequency of 1 time per week. The ability of a 3-day extender to protect against spontaneous lipid peroxidation is lost after 3 days of storage post-collection. A 5-day extender was able to protect against lipid peroxidation over the 7 day storage period. To avoid the detrimental effects of lipid peroxidation, swine producers should use the appropriate extender for storage of sperm cells prior to use in an artificial insemination program.

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Table 2.1. Weekly high and low barn temperatures and humidity levels.

	Barn temperature, ºC		Barn humidity, %	
Week	High	Low	High	Low
1	30.56 ± 0.67	23.41 ± 0.33	90.43 ± 1.15	61.86 ± 2.91
2	33.10 ± 0.73	26.11 ± 0.24	86.14 ± 0.55	51.14 ± 2.42
3	29.76 ± 1.03	22.70 ± 0.61	85.86 ± 1.49	58.43 ± 3.77
4	30.16 ± 0.56	22.86 ± 0.48	87.29 ± 0.99	56.86 ± 2.14
5	29.84 ± 0.45	22.94 ± 0.34	88.71 ± 0.97	54.86 ± 5.65

Temperature and humidity levels were recorded daily at approximately 08:00

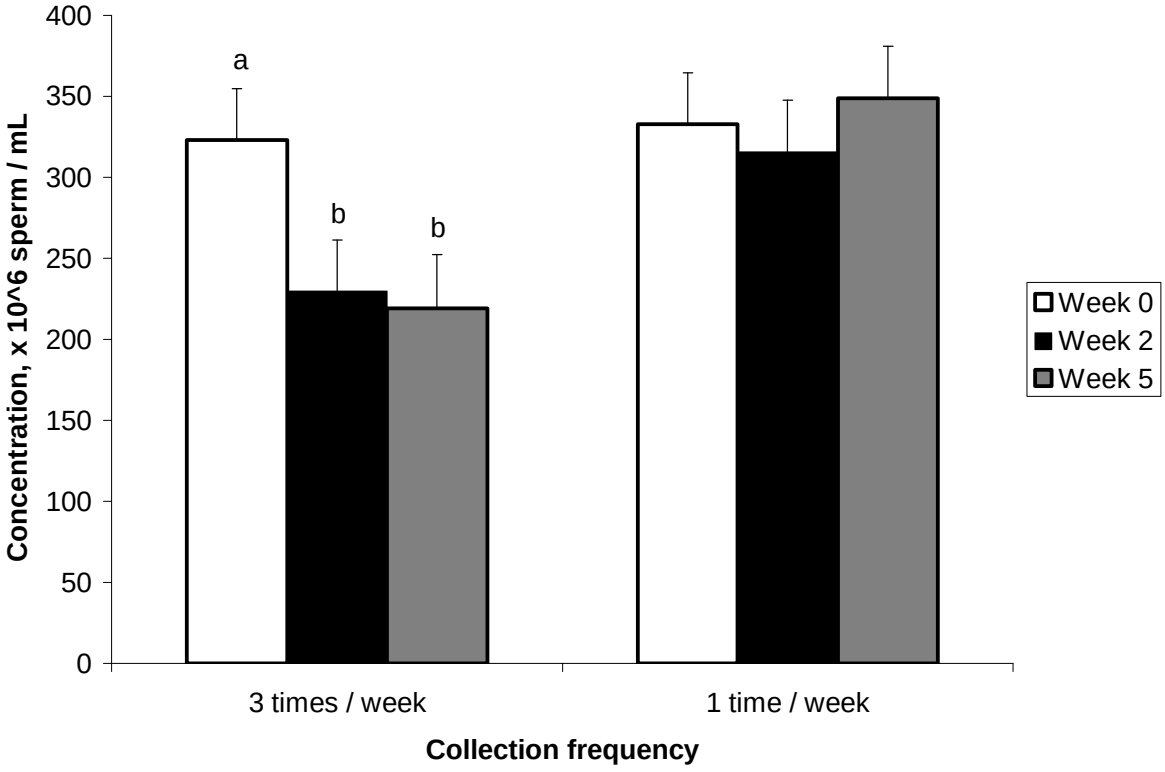


Figure 2.1. Effect of collection frequency and week on the concentration of sperm in the ejaculate.
[ab]Means with different superscripts within collection frequency are different ($P < 0.05$)

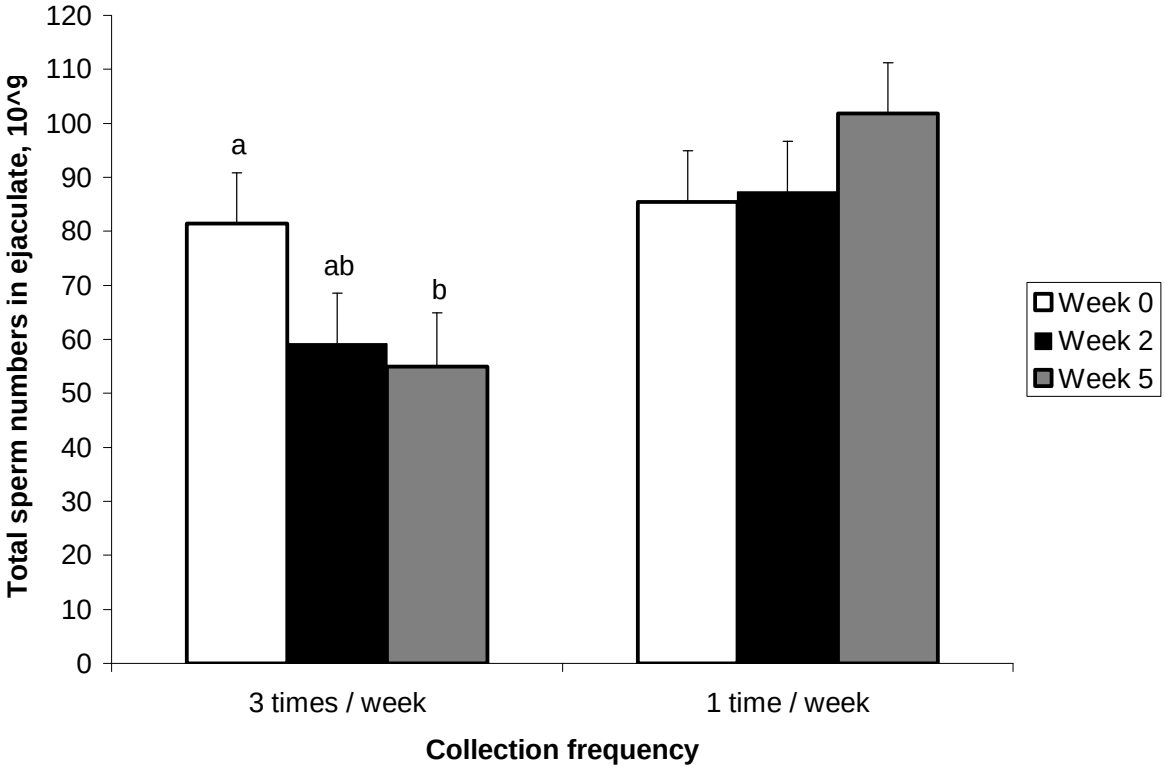


Figure 2.2. Effect of collection frequency and week on the total number of sperm in the ejaculate.
[ab]Means with different superscripts within collection frequency are different ($P < 0.05$)

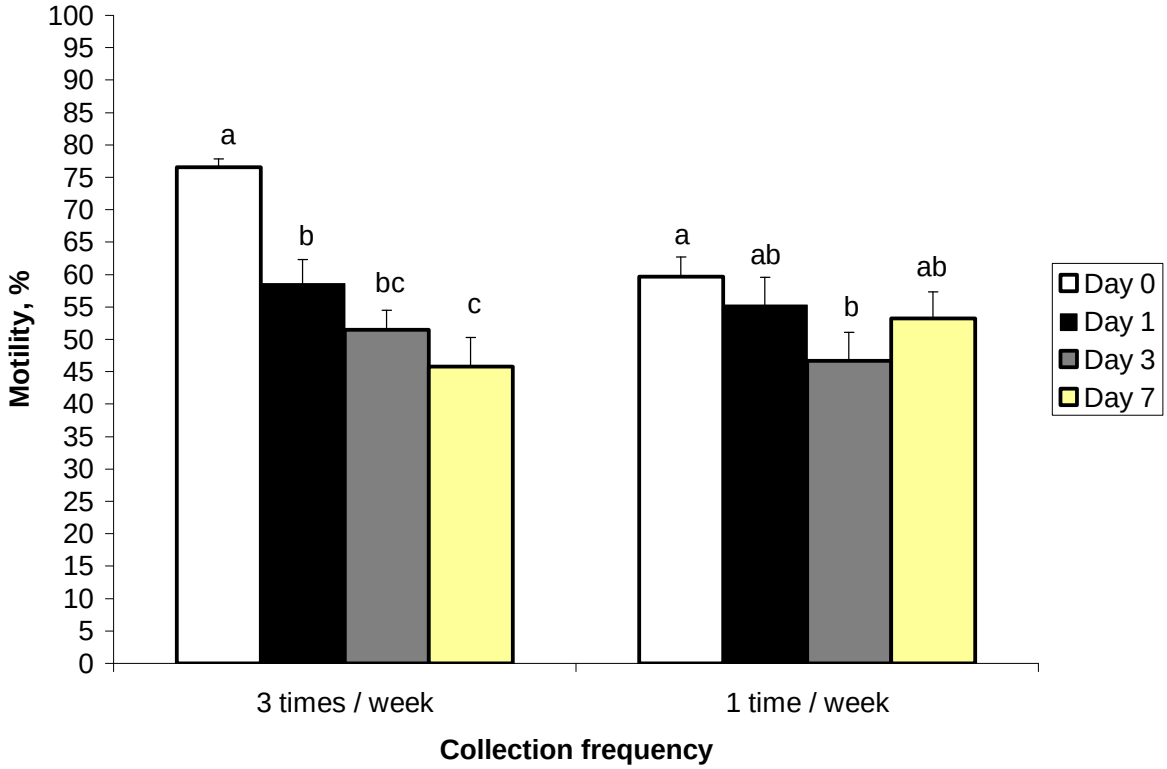
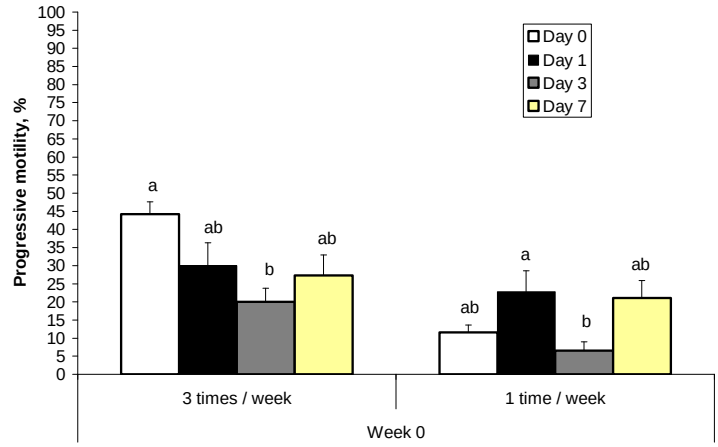
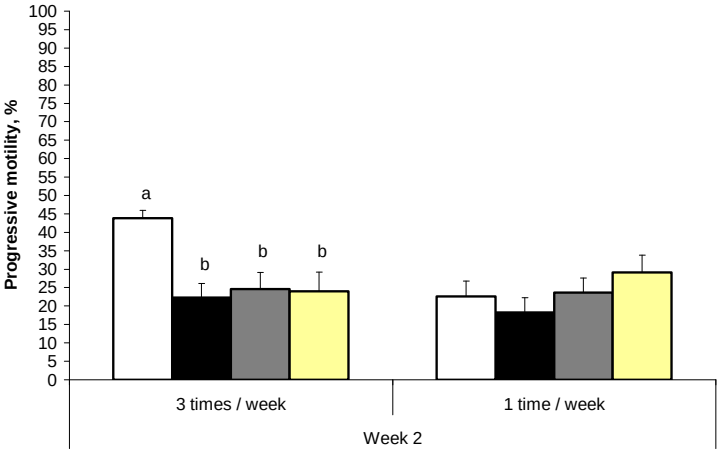
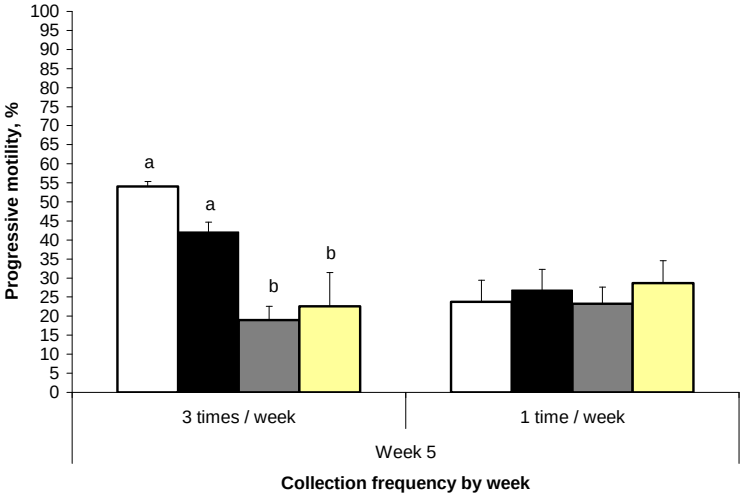


Figure 2.3. Effect of collection frequency and day of storage post-collection on the percentage of motile sperm in the extended ejaculate. The data were arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.
[abc]Means with different superscripts within collection frequency are different ($P < 0.05$)

Figure 2.4. Effect of collection frequency, week and day of storage post-collection on the percentage of progressively motile sperm in the extended ejaculate. The data were arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.
[ab]Means with different superscripts within collection frequency and week are different ($P < 0.05$)


Day 0
Day 1
Day 3
Day 7
Progressive motility, %
a
ab
b
ab
ab
a
b
ab
3 times / week
1 time / week
Week 0


Progressive motility, %
a
b
b
b
3 times / week
1 time / week
Week 2


Progressive motility, %
a
a
b
b
3 times / week
1 time / week
Week 5
Collection frequency by week

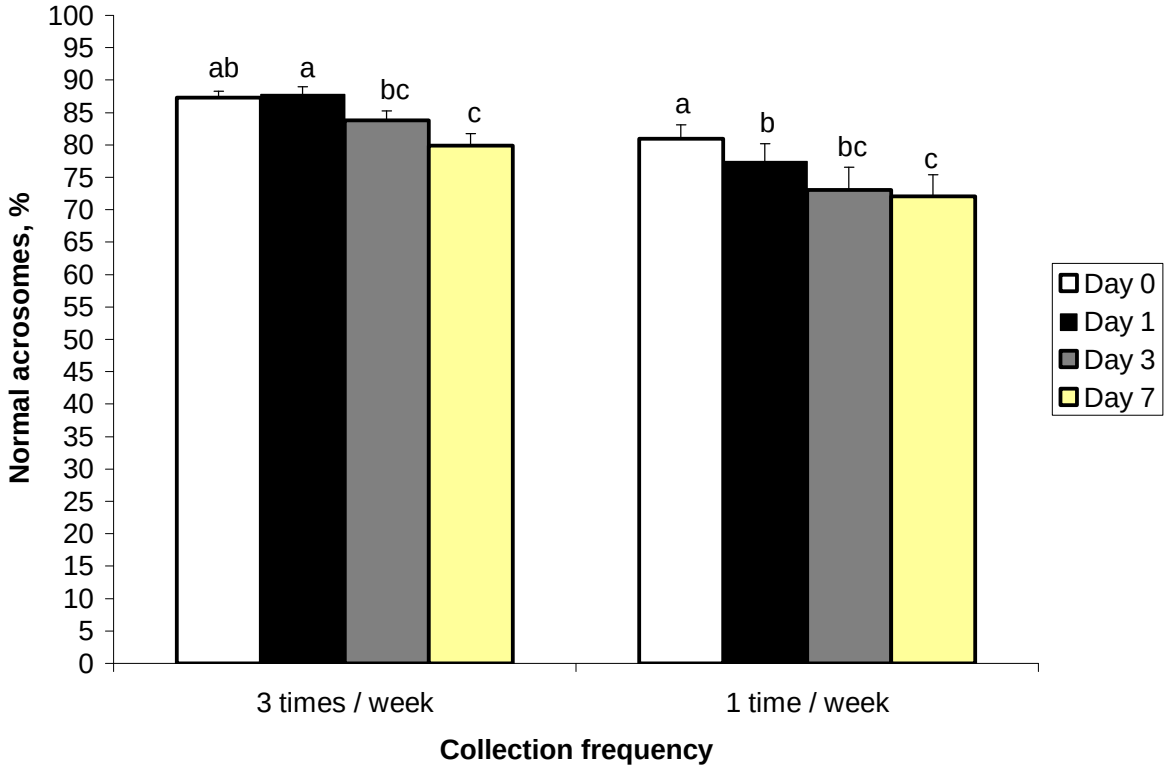


Figure 2.5. Effect of collection frequency and day of storage post-collection on the percentage of sperm with normal acrosomes in the extended ejaculate. The data were arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.

[abc]Means with different superscripts within collection frequency are different ($P < 0.05$)

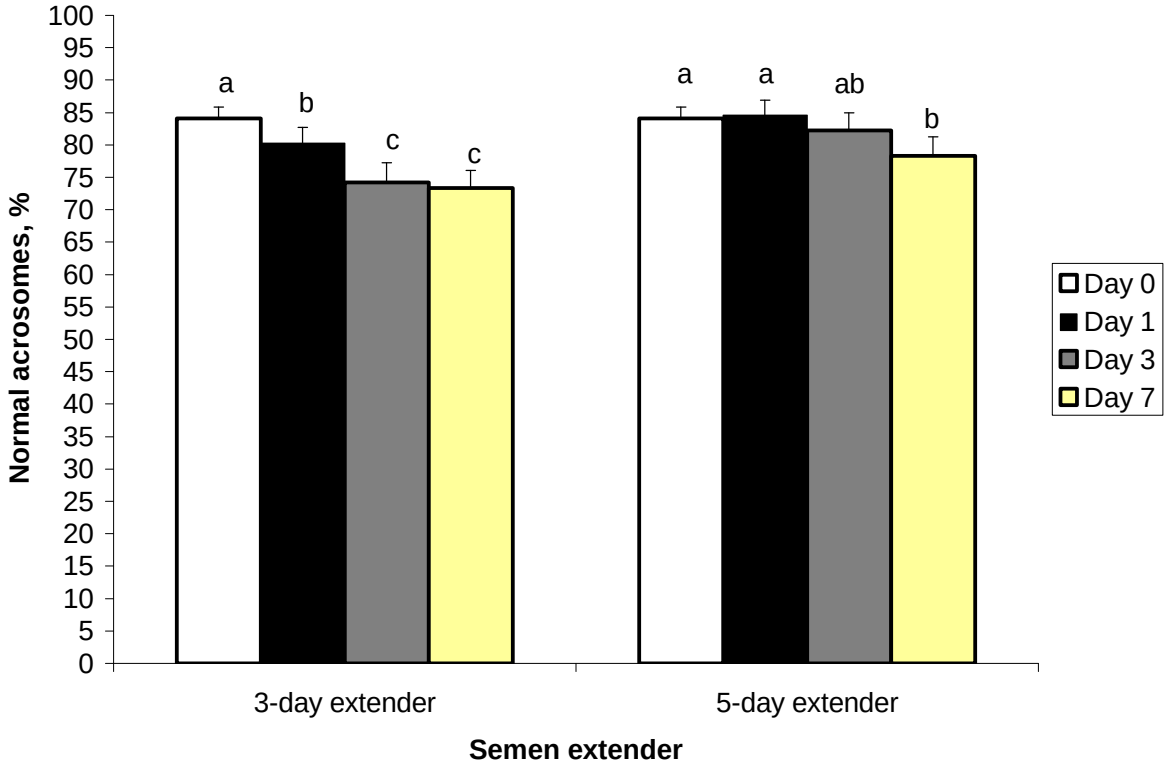


Figure 2.6. Effect of semen extender and day of storage post-collection on the percentage of sperm with normal acrosomes in the extended ejaculate. The data were arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.
[abc]Means with different superscripts within semen extender are different ($P < 0.05$)

Table 2.2. Effect of collection frequency and day of storage post-collection on the percentage of morphologically normal sperm and total abnormal sperm.

	Collection frequency							
	3 times per week				1 time per week			
Sperm morphological parameter[1], %	Day 0	Day 1	Day 3	Day 7	Day 0	Day 1	Day 3	Day 7
Normal	83.43 ± 3.53[a]	83.75 ± 3.42[a]	80.39 ± 3.50[b]	80.36 ± 3.82[b]	59.07 ± 6.00[a]	57.97 ± 5.70[a]	56.97 ± 5.68[ab]	54.63 ± 5.76[b]
Total abnormal	16.57 ± 3.53[a]	16.25 ± 3.42[a]	19.64 ± 3.50[b]	19.64 ± 3.82[b]	40.93 ± 6.00[a]	42.03 ± 5.70[ab]	43.03 ± 5.68[b]	45.43 ± 5.75[c]

The data was arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.

[1]Definitions: Total abnormal (total attached cytoplasmic droplets + abnormal tail + abnormal head)

[abc]Means within a row within collection frequency without common superscripts are different ($P < 0.05$)

Table 2.3. Effect of day of storage post-collection on the percentage of sperm with a proximal cytoplasmic droplet, distal cytoplasmic droplet, distal midpiece reflex with a cytoplasmic droplet and total attached cytoplasmic droplets.

	Day of storage post-collection			
Sperm morphological parameter[1], %	0	1	3	7
Proximal cytoplasmic droplet	4.07 ± 0.58[a]	5.02 ± 0.74[a]	6.10 ± 0.73[b]	6.19 ± 0.86[b]
Distal cytoplasmic droplet	16.00 ± 3.29	12.31 ± 2.34	12.34 ± 2.21	13.24 ± 2.34
Distal midpiece reflex[2]	4.69 ± 0.77[a]	8.03 ± 1.52[b]	8.60 ± 1.63[b]	8.72 ± 1.70[b]
Total attached cytoplasmic droplets	24.66 ± 3.85[a]	25.34 ± 3.72[a]	26.88 ± 3.65[b]	28.00 ± 3.77[b]

The data was arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.

[1]Definitions: Total attached cytoplasmic droplets (proximal + distal + distal midpiece reflex)

[2]The distal midpiece reflex sperm contained a cytoplasmic droplet in the midpiece reflex

[ab]Means within a row without common superscripts are different ($P < 0.05$)

Table 2.4. Effect of collection frequency on the percentage of normal sperm, sperm with a proximal cytoplasmic droplet, distal cytoplasmic droplet, distal midpiece reflex with a cytoplasmic droplet, total attached cytoplasmic droplets and total abnormal sperm.

	Collection frequency	
Sperm morphological parameter[1], %	3 times per week	1 time per week
Normal	81.98 ± 1.77[a]	57.16 ± 2.86[b]
Proximal cytoplasmic droplet	5.43 ± 0.51	5.27 ± 0.53
Distal cytoplasmic droplet	4.69 ± 0.81[a]	21.68 ± 2.11[b]
Distal midpiece reflex[2]	3.05 ± 0.45[a]	11.68 ± 1.23[b]
Total attached cytoplasmic droplets	13.11 ± 1.52[a]	38.46 ± 2.90[b]
Total abnormal	18.03 ± 1.77[a]	42.86 ± 2.86[b]

The data was arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.

[1]Definitions: Total attached cytoplasmic droplets (proximal + distal + distal midpiece reflex)
Total abnormal (tot. att. cyto. drop. + abnormal tail + abnormal head)

[2]The distal midpiece reflex sperm contained a cytoplasmic droplet in the midpiece reflex

[ab]Means within a row without common superscripts are different ($P < 0.05$)

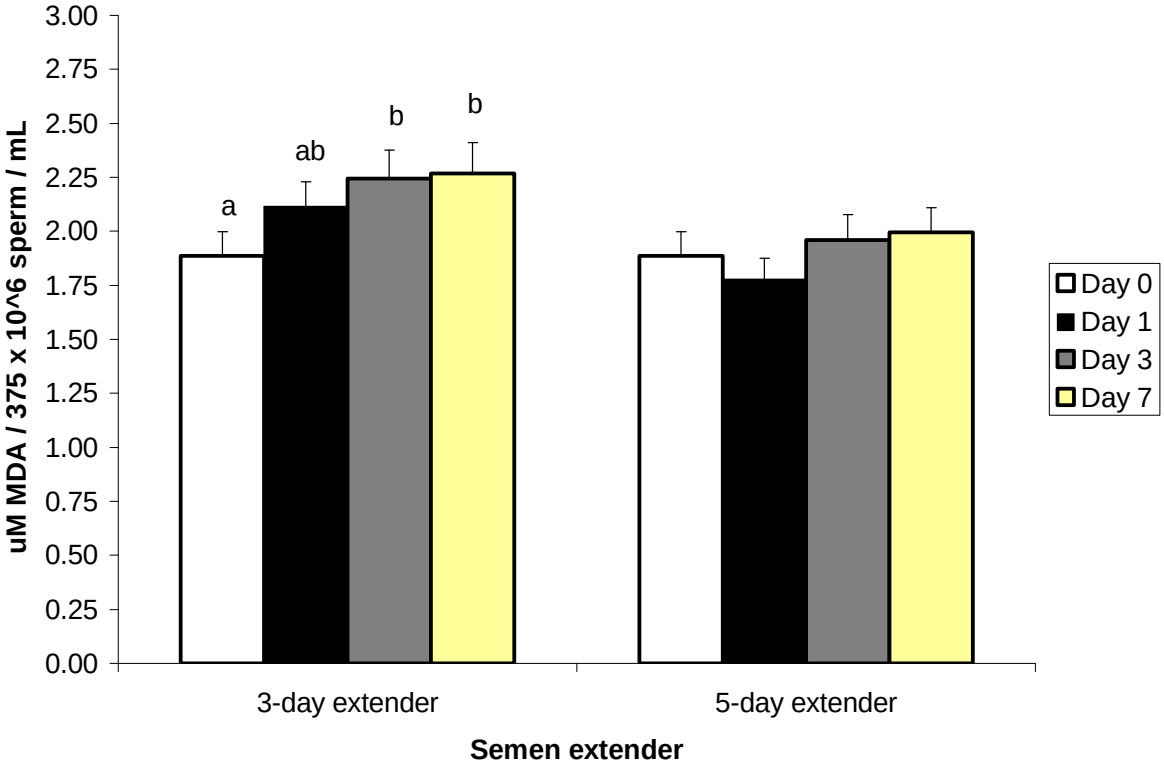


Figure 2.7. Effect of semen extender and day of storage post-collection on the concentration of MDA measured from sperm in the extended ejaculate.
[ab]Means with different superscripts within semen extender are different ($P < 0.05$)

CHAPTER 3

CHARACTERISTICS OF EXTENDED BOAR SEMEN FOLLOWING SEPARATION BY DISCONTINUOUS PERCOLL DENSITY GRADIENT CENTRIFUGATION

Abstract

The objective of this research was to use density gradient centrifugation to separate extended boar sperm into sub-populations for analysis of sperm quality, plasma membrane lipid peroxidation and sperm cell fatty acid composition over a 7 day storage period post-collection. Three ejaculates from 6 sexually mature boars were collected and analyzed following exposure to three consecutive collection periods. The first ejaculate was collected from boars that had previously been maintained on a 1 time per week frequency. The second ejaculate was collected following a period of five collections in four days (fifth collection analyzed). The third ejaculate was a collected after a period of 3 days of rest following the collection of the second ejaculate. Each ejaculate was extended in a commercially available 5-day extender and evaluated at 1, 4, and 7 days following collection. On each day of analysis, the samples were separated into low and high density layers using 2-step discontinuous density gradient centrifugation through isotonic percoll. Sperm motility, morphology and cellular particles were evaluated in the 45% layer, the 45/90% layer interface and the 90% layer. For lipid peroxidation and sperm cell fatty acid composition analysis, the 45% and 45/90% interface layers were combined. Concentration and total sperm numbers were lower ($P < 0.05$) for the ejaculate from the multiple collections period and the ejaculate from the 3 day rest period compared to the ejaculate from the 1 time per week period. Following density gradient centrifugation, a collection period x day of storage interaction was present for sperm motility ($P < 0.0001$) There were no differences ($P > 0.05$) in motility for the ejaculate from the 1 time per week period or the ejaculate from the 3 day rest period, however, sperm motility was lower ($P < 0.05$) on days 4 and 7 compared to day 1

for the ejaculate from the multiple collections period. A collection period x density layer x day of storage interaction ($P = 0.04$) was present for the percentage of sperm recovered from each density layer. For the ejaculate from the 1 time per week period, the percentage of sperm recovered from the 90% layer on day 7 was lower ($P = 0.04$) compared to day 1. In the ejaculate from the multiple collections period, the percentage of sperm recovered from the 45% layer on days 4 and 7 increased ($P = 0.02$) compared to day 1. Although a density layer x day of storage interaction ($P < 0.01$) was present for the percentage of normal sperm, total sperm with an attached cytoplasmic droplet and total abnormal sperm no pair-wise differences were detected. The number of cellular particles was higher ($P < 0.05$) in the 45% layer compared to the 45/90% and 90% layers. A collection period x density layer x day of storage interaction ($P < 0.05$) was present for the amount of malondialdehyde (MDA) produced per 200 x 10^6 sperm/mL, however, no pair-wise differences were detected. The ratio of polyunsaturated to saturated fatty acids in the sperm cells was lower ($P < 0.05$) for sperm cells from the 3 day rest period compared to the 1 time per week and multiple collections period. These results suggest that extended boar sperm decrease in their ability to penetrate density gradients of 45% and 90% isotonic percoll as measured by the percentage of total sperm recovered from each layer. The changes in sperm separation seem to be primarily affected by collection period and day of storage post-collection and to a lesser extent, sperm motility. Sperm separation does not appear to be affected by sperm morphology, lipid peroxidation or fatty acid composition.

Introduction

In the swine industry, sperm are stored at 17-18ºC in a liquid extender until used for artificial insemination. Research has shown that the fertilizing ability of the sperm decrease during this storage period when 3- and 5-day extenders are used (Waberski et al., 1994). Although the causes of this decrease are not known, the detrimental effects of reactive oxygen species via lipid peroxidation of the sperm plasma membrane may contribute to the loss of fertilizing ability. Oxidative stress from exposure to reactive oxygen species has been shown to be detrimental to sperm quality for a number of mammalian species (Aitken et al., 1993a; Silva et al., 2006; Guthrie and Welch, 2007).

The production of reactive oxygen species by sperm cells is not well understood. The mitochondria encapsulated within the mitochondrial helix that surrounds the sperm midpiece are considered to be a likely source of reactive oxygen species generation in livestock species (Ford, 2004). Research in human sperm has demonstrated positive correlations between lipid peroxidation levels and sperm with morphological abnormalities of the midpiece (Rao et al., 1989; Aziz et al., 2004). In addition, some researchers speculate that reactive oxygen species may be produced by non-viable and/or, morphologically abnormal sperm (Shannon and Curson, 1982; Aitken, 1995; Ball, 2008).

Density gradient centrifugation techniques can be used to separate sperm cells into sub-populations (Garbers et al., 1970; Lessley and Garner, 1983). Two specific sperm properties, progressive forward motility and buoyant density, are thought to be the factors affecting how sperm cells separate through the density gradients (Lessley and Garner, 1983; Grant et al., 1994; Popwell and Flowers, 2004). Using density gradient centrifugation

techniques, research has shown that high density layers consist primarily of viable, highly motile, morphologically normal sperm cells, while low density layers contain mostly non-viable, lowly motile, morphologically abnormal sperm cells (Aitken et al., 1993b; Ball et al., 2001; Popwell and Flowers, 2004). However, the majority of these results have been obtained using freshly collected sperm (within 24 hrs of collection). Consequently, it would be of interest to determine if the same motility and morphology characteristics seen with density gradient centrifugation of freshly collected sperm are also seen with sperm that has been stored for a given period of time. Separation of extended boar sperm into sub-populations using density gradient centrifugation would allow investigations into whether sperm cells from the low density layer have more reactive oxygen species activity compared to sperm from the high density layer, as has been demonstrated in humans (Aitken et al., 1993b) and equids (Ball et al., 2001). The objective of this research was to separate extended boar sperm using density gradient centrifugation techniques into sub-populations for analysis of sperm motility, progressive motility, morphology, plasma membrane lipid peroxidation and sperm cell fatty acid composition over a 7 day storage period post-collection.

Materials and Methods

Animal Care and Use

All procedures that involved the use of animals were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC # 06-035-A). Six crossbred boars (age: 1.21 ± 0.06 years; weight 227.43 ± 2.26 kg) of mixed genotypic frequencies (Yorkshire x Landrace x Large White sows bred to Hampshire x Duroc x Pietran boars) were used in the study. The boars were provided *ad libitum* access to water and were

fed a pelleted gestation corn–soybean meal diet that met or exceeded nutritional requirements for adult boars (NRC, 1998). Boar housing consisted of individual stalls (2m x 0.7m) located in an environmentally controlled building using a curtain-sided, under slat ventilation system. Supplemental ventilation fans and a drip cooling system were set to activate when ambient temperatures reached 23ºC and 26ºC, respectively.

Experimental design

The six boars chosen for the study were from a group of 12 boars that had previously been maintained on a 1 time per week collection frequency for approximately 6 months. The 12 boars were ranked by the percentage of normal sperm cells in the ejaculate using the five previous months of sperm morphology data. Six boars were selected for the study by choosing the top two, middle two and bottom two boars from the rankings.

Three ejaculates were collected and analyzed following exposure to three consecutive collection periods. The first ejaculate collected for semen analysis was collected while the boars were on the 1 time per week collection frequency. Approximately one week after the 1 time per week ejaculate was collected; the second ejaculate for semen analysis was collected following a period of five collections in four days. On the first day the boars were collected twice. The first collection occurred in the morning and the second collection occurred in the late afternoon. There was approximately eight hours between the collections. The fifth collection was used for analysis. The four ejaculates not used for analysis were measured for volume and concentration, and then discarded. The third ejaculate used for semen analysis was collected after a 3 day rest period following the collection of the second ejaculate.

Ejaculates were collected using the gloved-hand technique (Almond et al., 1998) into a pre-weighted collection thermos containing a plastic bag (IMV International Corp., Maple Grove, MN) and warmed to 37ºC. The collection thermos was covered with a filter (IMV International, Eden Prairie, MN) to separate the fluid fraction (semen) of the ejaculate from the gel fraction. The volume of semen was estimated by weighing each ejaculate with a gravimetric electronic balance (V-300, Acculab, Edgewood, NY) and using the conversion of 1 g of semen equals 1 mL. The concentration of sperm cells in the semen was evaluated using a photometer (SpermaCue™, Minitube of America, Verona, WI). The total number of sperm cells in the semen was calculated by multiplying the volume by the concentration. Ejaculate volume, concentration and total sperm cell numbers were recorded following each collection. Following collection, ejaculates were stored in a water bath pre-warmed to 37ºC (Minitube of America, Verona, WI). Ejaculates were transferred to the lab approximately 1.5 hours after the first ejaculate had been collected.

Upon arrival at the laboratory, the semen was placed into a water bath pre-warmed to 37ºC. The ejaculate from the 1 time per week period was extended to a concentration of 150 x 10^6 sperm/mL. The ejaculate from the multiple collections period and the ejaculate from the 3 day rest period were extended to a concentration of 100 x 10^6 sperm/mL. The reason for the different dilutions was due to the lower sperm concentrations of some boars following the multiple collections period and the 3 day rest period. All ejaculates were extended using a commercially available 5-day extender (Androhep; Minitube of America, Verona, WI) and stored in a semen cooling unit (Minitube of America, Verona, WI) at 17-18°C until analysis. A total of 45 mLs of extended semen were made for each ejaculate. Three 15 mL aliquots

were made for each day of semen analysis. The 15 mL aliquots for the 1 time per week collection period contained 2.2 x 10^9 sperm and the 15 mL aliquots for the multiple collections and 3 day rest periods contained 1.5 x 10^9 sperm. During storage post-collection the aliquots were gently inverted once per day to re-suspend the sperm cells into solution.

Laboratory analyses for semen quality estimates were performed on days 1, 4, and 7 after collection. Day 0 was considered the day of collection. The following semen quality estimates were performed for each sample: the percentage of motile and progressively motile sperm, the percentage sperm classified with normal morphology or abnormal morphology. Abnormal morphology included the following: abnormal tail, abnormal head, and sperm possessing a cytoplasmic droplet in the proximal, distal or as part of the distal midpiece reflex. In addition, the concentration of malondialdehyde (MDA) and sperm cell fatty acid composition was measured for each sample. Sperm were also subjected to density gradient centrifugation and sperm motility, progressive motility, morphology, cellular particles, the percent of sperm recovered from each density layer, lipid peroxidation and sperm cell fatty acid composition were evaluated.

Sperm quality analyses

Motility analysis. Motility analysis was performed using the Sperm Vision™ digital semen evaluation software (Minitube of America, Verona, WI) using images obtained by a digital camera attached to phase contrast microscope (Model BX41; Olympus Optical; Tokyo, Japan) with a 20x phase objective. Prior to analysis, a one mL aliquot from each sample was incubated at 37°C for 30 minutes. Four-chamber Leja slides (IMV International Corp., Maple Grove, MN) were pre-warmed to 37°C on a slide warmer (Minitube of

America, Verona, WI) before motility analysis. Five microscopic fields were analyzed for each sample, in duplicate, for a total of ten fields analyzed per sample. Approximately 250-400 total cells were analyzed for the percentage of sperm exhibiting motility and progressive forward motility.

Density gradient centrifugation. Sperm cells were separated into layers for evaluation of sperm cell motility, progressive motility, morphology, lipid peroxidation, sperm cell fatty acid analysis and the percent of sperm recovered from each layer. Separation of sperm cells was accomplished by centrifugation of extended sperm on each day of analysis using a percoll (P-1644, Aldrich, Milwaukee, WI) 2-step discontinuous gradient centrifugation procedure with 45% and 90% isotonic percoll solutions. The 45% and 90% percoll solutions were made from an iso-osmotic 100% percoll-saline solution prepared from a stock percoll suspension (1.130 g/mL; P-1644, Aldrich, Milwaukee, WI) and a 10-fold saline stock solution, according to Vincent and Nadeau (1984) as described in Harrison et al. (1992). The individual 45% (~1.055 g/mL) and 90% (~1.110 g/mL) percoll solutions were prepared the night before analysis and kept separate.

On each day of analysis, the discontinuous percoll gradient was prepared by the sequential layering of 3mls of the 90% percoll solution and 3 mls of the 45% percoll solution into a 15ml conical tube. Three percoll gradient tubes were prepared for each sample from ejaculates collected during the 1 time per week period. Likewise, four percoll gradient tubes were prepared for each sample from ejaculates collected during the multiple collections period and the 3 day rest period. For each sample, four mls of extended sperm were gently added on top of the 45% layer. After the addition of sperm, the percoll gradient tubes were

subjected to centrifugation (Model Marathon 3200; Fisher Scientific, Pittsburgh, PA) for 5 minutes at 200 x g followed by 15 minutes at 1000 x g. Following centrifugation, the original 45% (3mLs) and 90% (3 mLs) layers were removed in such a way to produce three individual layers containing sperm cells: 1) a 45% layer (2 mLs), 2) a 45/90% interface layer (2 mLs) and 3) a 90% layer (2 mLs). These three layers were formed by first removing and discarding the 4 mLs of extender. Next, the first two mLs of the percoll gradient were removed and designated as the 45% layer. The following two mLs were then removed and designated as the 45/90% interface layer. The remaining two mLs were then removed and designated as the 90% layer. A 100 μL sample from each layer for each sample was removed and placed into a separate bullet tube for motility and morphological analysis.

The 45% layers and 45/90% interface layers were combined (45-45/90% layer) in order to have enough sperm for the lipid peroxidation and sperm cell fatty acid assays. These layers came from the 3 percoll gradient tubes for the ejaculates collected during the 1 time per week period and the 4 percoll gradient tubes for the ejaculates collected during the multiple collections period and the 3 day rest period. Percoll was removed from the layers by washing two times with 0.01 M PBS. The concentration of spermatozoa in each layer was determined with a photometer (SpermaCue™, Minitube of America, Verona, WI, USA). The combined 45-45/90% layer and the 90% layer were then diluted to a concentration of 200 x 10^6 sperm/ml for the lipid peroxidation assay and sperm cell fatty acid assay.

Sperm morphological and cellular particles analysis. Evaluation of sperm morphology and cellular particles was performed using a phase contrast microscope (Zeiss; West Germany) using a 45x objective. Prior to analysis, a 50 μL aliquot from each layer for

each boar was fixed with 5 μLs of 10% buffered formalin. For sperm morphology analysis, the sample was diluted for evaluation and 10 μL of the diluted sample was placed on a hemocytometer, covered with an 18mm x 18mm coverslip and then given at least 5 minutes for the sperm and cellular particles to settle before analysis. The analysis consisted of classifying all intact sperm and cellular particles within the triple lines of the hemocytometer. The morphology and concentration of sperm in each layer (45%, 45/90% and 90%) was determined from four hemocytomer counts. The average number of sperm evaluated per sample was 242.47 ± 6.64 (mean ± standard error).

For sperm morphology, the sperm were classified according to Almond et. al. (1998) and Lovercamp et al. (2007). Intact sperm were classified into one of the following six morphological groups: normal, abnormal head, abnormal tail, proximal cytoplasmic droplet, distal cytoplasmic droplet and the distal midpiece reflex with a cytoplasmic droplet. Spermatozoa classified as a proximal cytoplasmic droplet possessed a cytoplasmic droplet located on the anterior half of the sperm tail midpiece proximal to the sperm head. Spermatozoa classified as distal cytoplasmic droplet possessed a cytoplasmic droplet located on the posterior half of the sperm midpiece. All spermatozoa classified as distal midpiece reflex possessed a cytoplasmic droplet in the 180° bend of the tail. Spermatozoa with tail defects observed without a distal cytoplasmic droplet in the bend of the tail were classified as an abnormal tail.

Cellular particles were evaluated using a slightly modified version of the analysis described in Lessley and Garner (1983). Briefly, the cellular particles evaluated were unattached cytoplasmic droplets and cellular debris. Cellular debris included sloughed

epithelial cells from the male reproductive tract, leukocytes, unidentifiable debris and broken heads and tails (Glover et al., 1990; Brunzel, 1994; Strasinger, 2001). The number of unattached cytoplasmic droplets and cellular debris were counted and the data are reported as the number of unattached cytoplasmic droplets or cellular debris quantified per 100 sperm cells.

Sperm lipid peroxidation. Spontaneous sperm lipid peroxidation activity was evaluated using the MDA-586 kit (OxisResearch, Portland, OR, USA). The assay detects the presence of a carbonyl compound, malondiadehyde (MDA), which is an end-product of the lipid peroxidation cascade resulting from the breakdown of lipid peroxides. For each day of the analysis, samples were prepared for MDA analysis as described previously. After washing, the pellet was re-suspended in PBS to a final concentration of 200 x 10^6 sperm/mL for quantification of MDA. The MDA assay was performed to measure free MDA by incubating the samples at 45°C for 60 minutes. After the incubation, the samples were centrifuged at 10,000 x g for 10 minutes to obtain a clear supernatant, as directed by the protocol. Samples were immediately plated and absorbances measured in a 96-well plate at a wavelength of 586 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT). Intra- and inter-assay coefficients of variation for nine assay batches were 3.59 and 18.63%, respectively based on a known 2.5 μM amount of the MDA standard that was pre-made, stored frozen and measured in duplicate on each assay day. The MDA-586 assay kit was validated for boar sperm using a spike and recovery technique of known concentrations of the MDA standard into three samples of boar sperm stored in the 5-day extender for 2 days.

The recoveries were (mean ± standard error) 77.0 ± 2.5%, 83.0 ± 3.0% and 89.5 ± 1.5% (Appendix A).

Sperm cell fatty acid analysis. Prior to analysis, the samples were centrifuged to form a sperm pellet to remove PBS. Samples were then diluted with methanol and stored at -80ºC until analysis. On the day of analysis, samples were thawed for 30 minutes at 4ºC. Chloroform was added to each sample to bring the ratio of chloroform:methanol to 2:1 for total lipid extraction from the sperm cell suspension. The samples were then sonicated for 30 minutes with a frequency of 40 kHz in a water bath at approximately 20ºC (Model 2510R-DTH, Bransonic Ultrasonics Corp., Danbury, CT). The amount of chloroform:methanol solution in each sample was determined and 20% of this amount of 0.9% NaCl was added to each sample. The samples were vortexed and then centrifuged for 10 minutes at 2000 rpm at room temperature. The organic phase was removed and then dried down under N_2 gas. The samples were then baked for two hours at 104ºC.

After baking, 50 µg of an internal standard (C17:0) was added to each sample and dried under N_2 gas. One milliliter of a reagent containing 3.75M NaOH dissolved in a 1:1 (vol/vol) methanol, distilled water mixture was added and the tubes were heated in a boiling water bath for 5 min, vortexed, and returned to the water bath for 25 min. The samples were then placed into cool water and 2 mL of a 1.7:1 (vol/vol) methyl alcohol and 6.0 N hydrochloric acid mixture was added. The samples were placed into the boiling water bath for 10 min and then immediately placed in cool water. Three milliliters of a 1:1 (vol/vol) methyl tert-butyl ether and hexane mixture was then added to the samples. Samples were vortexed and mixed continuously for 10 min until they were clear and the lower, aqueous

phase was discarded. Finally, 3 mL of 0.3 M NaOH was added to the remaining organic layer and the tubes were mixed and centrifuged. Two-thirds of the top, organic, layer was removed to a clean vial and dried under N_2 gas.

The fatty acid derivatives were separated on a HP-23 capillary column (cis/trans FAME CR), 30 m x 0.25 mm, film thickness 0.3 um (Agilent Technologies, Wilmington, DE). Mass spectrometric analysis was conducted by using an Agilent Technologies 5973N mass spectrometric detector equipped with a 6890 N model gas chromatograph (Agilent Technologies, Wimington, DE). For electron ionization (EI) analysis, the temperature was programmed from 50 to 100°C at 10°C/min, then to 200°C at 4°C/min, held for 2 min, and finally to 220°C at 4°C/min, held for 12 min. The average helium velocity was 36 cm/sec and the split ratio was 100:1. One uL of the methyl ester was manually injected into the GC and the total fatty acid amounts were determined by the areas of the total ions for each fatty acid.

Statistical analysis

All statistical analyses were performed using the mixed procedure of SAS (SAS Ins., Cary, NC). The variance/covariance structure used for the repeated measures analyses was determined by finding the appropriate structure with the lowest fit statistics. Prior to analysis, all percentage data were normalized with an arcsine transformation (Snedecor and Cochran, 1989). Results for percentage data are reported as non-transformed arithmetic means ± standard error of the mean. All other data are reported as least squares means ± standard error of the mean. Non-significant ($P > 0.05$) interactions were removed from all models. For all

models, the Tukey-Kramer adjustment was used to determine differences among means of independent variables when significant effects were observed (Kaps and Lamberson, 2004).

Two additional cumulative variables were derived from the morphological analysis for each ejaculate and are included in the statistical analysis for sperm morphology: 1) a total attached cytoplasmic droplet group (proximal cytoplasmic droplet + distal cytoplasmic droplet + distal midpiece reflex); and 2) a total abnormality group (abnormal tail + abnormal head + total attached cytoplasmic droplets).

One boar was removed from the study following the 1 time per week period because of death. A boar of nearly identical semen quality replaced this boar on the study for the multiple collections and 3 day rest periods.

The percentage of sperm recovered from each density layer was calculated by first determining the total number of sperm recovered from each layer. Next, the total number of sperm was calculated by summing the sperm from each layer. Finally, the percentage of sperm from each layer was calculated by dividing the total sperm from each layer by the total number of sperm recovered from all three layers, multiplied by 100.

Data for volume, concentration and total sperm numbers in each ejaculate were analyzed as a complete randomized design using analysis of variance procedures. The model included collection period (1 time per week period, multiple collections period, 3 day rest period).

Data for the semen analysis were analyzed as a complete randomized design using analysis of variance procedures for repeated measures (Gill and Hafs, 1971). The statistical model for sperm motility, progressive motility and sperm morphology included collection

period (1 time per week period, multiple collections period, 3 day rest period) and day of storage (1, 4 and 7) and the collection period x day of storage interaction. The error term used to for test treatment effects was boar nested within collection period. The day of storage was treated as the repeated variable. A collection period x day of storage interaction was present for motility ($P = 0.0003$) and progressive motility ($P < 0.0001$). Therefore additional analyses were conducted to evaluate differences among collection periods within day of storage. Furthermore, analyses were conducted to evaluate differences among days of storage within collection period. A collection period x day of storage interaction was present ($P = 0.01$) for the percentage of normal and total abnormal sperm. Additional analyses were conducted to evaluate differences among collection periods within day of storage.

Data for the semen analysis for density gradient centrifugation were analyzed as a complete randomized design using analysis of variance procedures for repeated measures (Gill and Hafs, 1971).The statistical model for the density gradient analysis of the 45%, 45/90% and 90% layers evaluated sperm motility, progressive motility, sperm morphology, cellular particles and percent of recovered sperm from each layer. The model included collection period (1 time per week period, multiple collections period, 3 day rest period), layer (45%, 45/90% and 90%), day of storage (1, 4 and 7) and all appropriate interactions. The error term used to for test treatment effects was boar nested within the collection period x layer interaction. The day of storage was treated as the repeated variable. A layer x day of storage interaction was present ($P <0.05$) for the percentage of normal, distal cytoplasmic droplet, total attached cytoplasmic droplet and total abnormal sperm and unattached cytoplasmic droplets. Therefore additional analyses were conducted to evaluate differences

among layers within day of storage. For the percentage of sperm recovered from each layer a collection period x layer x day of storage interaction was present ($P < 0.05$). Therefore additional analyses were conducted to evaluate differences among day of storage within collection period and layers.

The statistical model for the density gradient analysis of the 45-45/90% and 90% layers evaluated sperm lipid peroxidation and sperm cell fatty analysis. The model included collection period (1 time per week period, multiple collections period, 3 day rest period), density layer (45-45/90% and 90%), day of storage (1, 4 and 7) and all appropriate interactions. The error term used to for test treatment effects was boar nested within the collection period x layer interaction. The day of storage was treated as the repeated variable. For MDA and the percentage of PUFAs, a collection period x density layer x day of storage interaction was present ($P < 0.03$). For sperm lipid peroxidation, additional analyses were conducted to evaluate differences among collection periods. For the 1 time per week period, an effect ($P < 0.0001$) of day of storage was present, for the multiple collections period, a two-way interaction ($P = 0.02$) for day of storage and density layer was present and for the 3 day rest period, an effect ($P = 0.002$) of density layer was present. For the percentage of PUFAs, additional analyses were conducted to evaluate differences among layers within collection period and day of storage.

Results

Ejaculate characteristics

The ejaculate characteristics are presented in Table 3.1. There was no effect of collection period on ejaculate volume ($P = 0.72$). Collection period decreased ($P = 0.0008$)

the concentration of sperm cells in the ejaculates of the multiple collections period and 3 day rest period compared to the 1 time per week period. As a result, there was a greater ($P = 0.03$) number of total sperm in the ejaculates from the 1 time per week period compared to the ejaculates measured from the multiple collections period and 3 day rest period.

Motility

A collection period x day of storage interaction was present for the sperm motility ($P = 0.0003$) and progressive motility ($P < 0.0001$). The collection periods were not different ($P > 0.05$) on days 1 and 4. On day 7, the 1 time per week collection period had greater ($P < 0.03$) motility (Figure 3.1) and progressive motility (Figure 3.2.) compared to the multiple collections period. There was no difference ($P > 0.05$) in motility (Figure 3.3.) and progressive motility (Figure 3.4) over the 7 day storage period for the 1 time per week and 3 day rest periods. Motility and progressive motility were lower ($P < 0.05$) on days 4 and 7 compared to day 1 for the multiple collections period.

Morphology

A collection period x day of storage interaction ($P < 0.03$) was present for the percentage of normal sperm, sperm with a distal cytoplasmic droplet and total abnormal sperm (Table 3.2.). However, differences among means were not found. Collection period or day of storage did not affect ($P > 0.5$) the percentage of sperm with an abnormal tail, abnormal head, proximal cytoplasmic droplet, distal midpiece reflex or total attached cytoplasmic droplets.

Density gradient centrifugation analyses

Motility and progressive motility. There was no effect of collection period or day of storage on motility or progressive motility for the density layers ($P > 0.1$). No differences ($P > 0.05$) in sperm motility or progressive motility were detected between the density layers.

Percentage of sperm recovered. A collection period x density layer x day of storage interaction ($P = 0.04$) was present for the percentage of sperm recovered from the density layers (Figure 3.5.). For the the 1 time per week collection period, the percent of sperm recovered from the 90% layer on day 7 was lower ($P = 0.04$) compared to day 1. In the multiple collections period, the percentage of sperm recovered from the 45% layer on days 4 and 7 were increased ($P = 0.02$) compared to day 1. No differences ($P > 0.05$) were observed for the 3 day rest period.

Sperm morphology and cellular particles. A density layer x day of storage interaction ($P < 0.01$) was present for the percentage of normal, distal cytoplasmic droplets, total attached cytoplasmic droplets and total abnormal sperm. The percentage of sperm with a distal cytoplasmic droplet was higher in the 45% layer compared to the 90% layer on day 1. Differences among means were not found for the percentage of normal, total attached cytoplasmic droplets and total abnormal sperm (Table 3.3.). There was no effect of collection period on sperm morphology for the density layers ($P > 0.4$).

A density layer x day of storage interaction ($P < 0.0001$) was present for the number of unattached cytoplasmic droplets per 100 sperm cells (Figure 3.6.). The number of unattached cytoplasmic droplets per 100 sperm cells was lower in the 90% layer on days 1, 4

and 7 ($P < 0.04$) compared to the 45% layer, while the 45/90% layer was lower only on days 1 and 7 ($P < 0.0001$) compared to the 45% layer.

There was an effect ($P < 0.0001$) of density layer on the concentration of cellular debris (sloughed epithelial cells from the male reproductive tract, leukocytes, unidentifiable debris and broken heads and tails) per 100 sperm cells (Figure 3.7.). The 45% layer contained a higher ($P < 0.0001$) amount of debris compared to the 45/90% and 90% layers. There was no effect of collection period on cellular debris for the density layers ($P > 0.6$).

Sperm lipid peroxidation (MDA). A collection period x density layer x day of storage interaction ($P = 0.006$) was present for sperm lipid peroxidation as measured by the concentration of malondialdehyde (MDA) produced per 200 x 10^6 sperm / mL (Figure 3.8.). Therefore, additional analyses were conducted to evaluate differences among collection periods. For the 1 time per week period, there was an effect ($P < 0.0001$) of day of storage, where the concentration of MDA was lower ($P < 0.05$) on day 7 compared to days 1 and 4 of storage post-collection. For the multiple collections period, a two-way interaction ($P = 0.02$) for day of storage and density layer was present. The data were evaluated between density layers; however, no differences ($P > 0.05$) were found between the density layers. For the 3 day rest period, an effect ($P = 0.002$) of density layer was present. The 45-45/90% density layer had a higher ($P < 0.05$) concentration of MDA compared to the 90% density layer.

Sperm cell fatty acid analysis. A collection period x density layer x day of storage interaction ($P = 0.02$) was present for the percentage of PUFAs. However, differences were not found between layers (Figure 3.9.).

There was an effect of collection period ($P < 0.0001$; Figure 3.10.) and day of storage ($P = 0.04$; Figure 3.11.) on the ratio of polyunsaturated (PUFA) fatty acids to saturated fatty acids in the sperm cell. For collection period, the 3 day rest period had a lower ($P < 0.0001$) ratio of PUFA to saturated fatty acids present in the sperm cell compared to the 1 time per week and multiple collections periods. No differences among means were found between day of storage post-collection. Layer did not affect ($P > 0.1$) the ratio of PUFA to saturated fatty acids in the sperm cell.

Discussion

The purpose of this research was to examine differences in sperm quality, plasma membrane lipid peroxidation and sperm cell fatty acid composition from boar sperm separated into distinct sub-populations using density gradient centrifugation over a 7 day storage period post-collection. Previous research has indicated the sperm that separate to the low density layer are non-viable, lowly motile and of abnormal morphology, whereas the opposite is true for sperm cells that separate to the high density layer (Aitken et al., 1993b; Ball et al., 2001; Popwell and Flowers, 2004). In the present study, three different collection periods were imposed upon boars in an attempt to create conditions of varying sperm quality. The idea was that ejaculates with high quality and low quality sperm motility and morphology would result in greater levels of abnormal sperm in the low density layer and vice versa. In theory, higher levels of sperm membrane lipid peroxidation and decreased levels of sperm cell PUFAs would be expected from sperm present in the low density layer due to the higher percentage of abnormal sperm. Conversely, lower levels of lipid

peroxidation and higher levels of sperm PUFAs would be expected from sperm present in the high density layer.

Three collection periods were imposed upon the boars. The first period was the regular 1 time per week collection. This period would be expected to produce sperm of high quality since the sperm were allowed full maturation during epididymal transit and storage in the cauda epididymis. The second period was a multiple collection period of 5 collections in 4 days. This period was expected to remove all sperm reserves from the cauda epididymis and produce sperm of low quality since sperm were not allowed normal maturation in the epididymis. The third period was a collection after 3 days of rest following the multiple collections period. This period was expected to produce sperm of intermediate quality since this rest period was designed to allow a new pool of sperm cells to move into the cauda epididymis for maturation.

The decrease in ejaculate concentration and total sperm numbers for the multiple collections period compared to the 1 time per week and 3 day rest periods indicates that the multiple collections period frequency induced a physiological response. The results of the present study agree with other reports that have shown ejaculates from boars on a control collection frequency to have higher sperm cell concentrations compared to ejaculates from boars subjected to an elevated collection frequency (Schilling and Vengust, 1987; Bonet et al., 1991). Generally speaking, a new pool of sperm cells enters the cauda epididymis every three days. In the present study, a three day recovery period following the multiple collections period did not appear to replenish the sperm cell reserves in the cauda sufficiently to return the sperm cell numbers to levels observed during the 1 time per week period. This

indicates that the multiple collections period possibly altered the rate at which sperm cells moved through the epididymis. If the rate at which sperm cells moved through the epididymis was sufficiently modified, then the maturation of sperm cells may also be impaired due to the shorter epididymal transit time. This may affect quality and fertilizing ability of the sperm cells subjected to the increased epididymal transit rate (Franca et al., 2005).

The multiple collections period did not affect the motility or progressive motility of the extended sperm compared to the 1 time per week and 3 day rest periods until day 7 of storage. The decrease in sperm motility and progressive motility for sperm collected following the multiple collections period compared to the 1 time per week period is likely due to the altered epididymal maturation time of sperm from the multiple collections period. Compared to the 1 time per week period, the decline in sperm motility for the multiple collections period indicates that the higher intensity collection frequency was detrimental to the ability of the sperm cells to maintain motility over the 7 day storage period post-collection.

No differences in sperm morphology were detected between the collection periods or over the 7 day storage period post-collection. The lack of differences for sperm morphology for the collection periods do not agree with previous research which have shown higher levels of abnormal sperm in the ejaculates of boars subjected to a high frequency multiple collection period compared to a control collection frequency. Pruneda et al. (2005) subjected Pietrain boars at one year of age to a collection frequency of 8 collections in 4 days. The boars in this study had higher levels of abnormal sperm in the ejaculate compared to boars

maintained on a control frequency of 2 collections in four days. The discrepancy in results between this study and present study may be due to fewer collections during the 4 day collection period.

The main factors affecting how sperm cells separate into different density layers using density gradient centrifugation are thought to be the sperm cell's progressive motility and buoyant density. In the present study, sperm motility was not different between the 45%, 45/90% interface and 90% layers following separation of the sperm cells. This data contradicts the results of similar experiments using density gradient separation techniques in humans (Aitken et al., 1993b), equids (Ball et al., 2001) and swine (Popwell and Flowers, 2004) which found that sperm separated into the high density layer displayed higher motility compared to sperm separated into the low density layer.

The discrepancy in motility results may be due to the age of the sperm at centrifugation. In the previous studies, centrifugation of the sperm through the density gradients was performed within approximately 24 hours following collection from the male, whereas in the present study the first separation analysis was not performed until day 1 (approximately 30 hours after collection). As a result, the sperm in the present study may have been either less motile or of lower buoyant density, or a combination of the two compared to sperm separated immediately after collection. Therefore, after approximately 30 hours of storage the sperm cells in the present study were unable to separate in a fashion seen in previous studies. Although not expected, it could be possible that only 30 hours of storage may affect separation patterns of sperm using density gradient centrifugation. This may be true, as research using 3 boars shows that motility and progressive motility of sperm cells

was higher ($P < 0.05$) in the 90% layer compared to the 45% layer on day 0 (day of collection). Sperm motility on day 0 was 76 ± 9% in the 90% layer and 36 ± 2% in the 45% layer. However, on day 1, there was no difference ($P > 0.05$) in motility between the 45% and 90% layers (71 ± 8% vs. 46 ± 4%). The motility of the whole sample (sperm not separated by density gradient centrifugation) was not different ($P = 0.9$) between days 0 (87 ± 2%) and 1 (83 ± 3%) (Appendix D).

The disagreement between this study and other studies for the lack of differences in motility between the density layers may also be due to processing of the sperm cells following density gradient centrifugation. In the previous reports, the sperm cells were removed from the percoll gradient and re-suspended in a buffer solution to remove the cells from the potentially toxic effects of the percoll prior to motility analysis (Avery and Greve, 1995). However, the manufacturer product information sheet states that percoll is non-toxic (P1644, Sigma, St. Louis, MO). In the present study, the sperm cells were not removed from the percoll prior to motility analysis in an attempt to prevent further centrifugal stress on the sperm cells. In the present study, the longest time period sperm cells might have been in the percoll prior to analysis was 30-45 minutes. Research demonstrates that percoll does not appear to have a toxic effect on sperm cells. Sperm suspended in either the 45% or 90% density layers had similar ($P > 0.05$) motility to sperm cells suspended in Androhep extender over a 45 minute analysis period. Sperm samples were first pelleted by centrifugation (800 x g for 5 minutes at room temperature) and then re-suspended in the appropriate treatment. Although treatment did not have an effect, there was an effect of time ($P < 0.0001$) in this analysis (Appendix E).

Similar to motility, there were no differences in sperm morphology between the density layers following density gradient centrifugation. These results do not agree with previous research demonstrating a higher percentage of normal sperm in the high density gradient and higher percentage of abnormal sperm in the low density gradient. Ball et al. (2001) used a discontinuous 40% and 80% percoll gradient to separate stallion sperm and found approximately 60% normal sperm in the high density layer compared to 40% normal sperm in the low density layer. It is unknown at this time why similar results were not detected in the present study. In the previous study, the sperm analyzed for morphological analysis in the high density layer were reportedly removed from the sperm pellet located at the base of the layer and the sperm analyzed from the low density layer came from the low/high density layer interface. In the present study, the gradients were fractionated into layers based on volume, and not visual appraisal of the gradient interface and sperm pellet in the high density gradient. The use of qualitative fractionation used in the previously mentioned study may have led to the differences seen in the sperm morphological characteristics that may not have been present if quantitative fractionation (i.e. fractionation into layers by volume) was used. Since all the sperm present in the 2 mLs of the 90% high density layer were analyzed instead of just the sperm in the pellet at the base of the layer, it may be possible that more morphologically abnormal sperm were present in the 2 mLs of the high density layer, and therefore the percentage of morphologically normal sperm in the high density layer was not different from sperm in the low density layer.

Specific modifications to the structure and integrity of the sperm plasma membrane may be responsible for the differences in sperm motility and morphology between the high

density and low density layers as seen in previous studies (Aitken et al., 1993b; Ball et al., 2001). Lipid peroxidation of polyunsaturated fatty acids (PUFAs) in the sperm plasma membrane could lead to a decrease in the percentage of PUFAs found in the sperm cell (Cerolini et al., 2000). The loss of PUFAs may disrupt the membrane and increase membrane permeability, leading to a decrease in sperm progressive motility and buoyant density. Research evaluating the effects of hydrogen peroxide on pulmonary artery endothelial cells found that damage to the structure and function of the plasma membrane manifested as a loss of membrane fluidity and increase in leakiness of the plasma membrane. It was thought that lipid peroxidation was associated with this decrease in membrane quality (Block, 1991). An increase in the leakiness of the plasma membrane of sperm cells due to lipid peroxidation may also decrease the sperm cell progressive motility and buoyant density.

Data support the hypothesis that sperm progressive motility and buoyant density of the sperm cell may decrease in response to lipid peroxidation. Ejaculates from three mature boars were used in a study evaluating the effects of a 600 μM hydrogen peroxide challenge for 24 hours in hypothermic liquid storage (17-18ºC). Following the hydrogen peroxide challenge and density gradient centrifugation, a lower ($P = 0.005$) percentage of hydrogen peroxide challenged boar sperm were recovered in the 90% density layer (27 ± 5%) compared to the percentage of sperm recovered in the 90% density layer of the control (66 ± 7%). Although the buoyant density of the sperm cells may have been altered by lipid peroxidation in response to the hydrogen peroxide challenge, the motility of the sperm cell also likely influenced the ability of the sperm cell to penetrate the 90% density layer. The motility and progressive motility of the hydrogen peroxide treated sperm cells was lower ($P <$

0.02) compared to the control sperm cells immediately before centrifugation (71 ± 6% vs 90 ± 1% and 29 ± 4% vs 46 ± 2%, respectively). As are result, the motility and progressive motility were higher ($P < 0.05$) in the 90% density layer of the control (87 ± 3%, 62 ± 5%) compared to the 90% density layer of the hydrogen peroxide challenged sperm (14 ± 3%, 7 ± 2%). These results suggest that hydrogen peroxide is able to affect the separation characteristics of sperm cells likely through alterations of the motility, progressive motility and buoyant density of the cell (Appendix C).

As previously discussed, disruption of the sperm plasma membrane may lead to a decrease in the buoyant density through an increase in permeability of the sperm plasma membrane (Block, 1991). If such events occur to sperm during storage post-collection, then the percentage of sperm cells that separate to the 90% density layer would decrease over the storage period. Similarly, the percentage of sperm cells that seperate to the 45% density layer would increase over the storage period. In the present study, a lower percentage of sperm cells were found in the 90% density layer on day 7 of storage post-collection compared to day 1 of storage post-collection for sperm cells collected during the 1 time per week period. This indicates that the sperm cell buoyant density may be altered during the storage period. Motility and progressive motility of the sperm cell may play a role in whether the sperm cell can migrate to the 90% layer; however, motility and progressive motility were similar between days 1 and 7 of storage post-collection for the 1 time per week period. Since the sperm motility and progressive motility were not different between day 1 and 7, this would suggest that buoyant density plays an important role in determining the ability of sperm cells to separate to the high density layer. A similar separation response was seen for sperm cells

collected during the multiple collections period as the percentage of sperm found in the 45% layer was higher on days 4 and 7 compared to day 1. Unlike the 1 time per week period, however, there was a decrease in motility and progressive motility from day 1 to 7. Therefore, it appears that both sperm cell motility, progressive motility and buoyant density work together to determine how sperm cells separate through the density gradients.

The cause for this loss of motility and buoyant density may be from lipid peroxidation of the sperm cell plasma membrane. This may explain why previous research has demonstrated that sperm cells removed from the low density layer produce higher levels of reactive oxygen species and are more susceptible to plasma membrane lipid peroxidation (Aitken et al., 1993b; Ball et al., 2001). Lipid peroxidation during storage may be the cause for the decrease in the percentage of PUFAs seen in boar sperm over a 5 day hypothermic liquid storage period post-collection (Cerolini et al., 2000). Therefore, one could speculate that a lower percentage of PUFAs would be present in sperm cells recovered from the low density layer compared to sperm cells from the high density layer.

In the present study, however, differences in sperm lipid peroxidation were detected between the high and low density layers in sperm collected during the 3 day rest period only. No differences were found for sperm lipid peroxidation between the high and low density layers for the 1 time per week period and multiple collections period. The reasons for the inconsitent results between the collection periods for sperm lipid peroxidation are not known. In addition, there also were no differences in the sperm cell PUFA content between the high and low density layers. The inability to find consistent differences for sperm cell lipid peroxidation and fatty acid composition between the high and low density layers may be due

to the fact that there were also no differences for sperm motility and morphology between the high and low density layers. Since there were no differences for lipid peroxidation or changes in the fatty acid composition of the sperm cell, it is uncertain what molecular factor(s) altered the sperm motility and buoyant density over the 7 day storage period. Further research is needed to determine factors that affect sperm cell separation using density gradient centrifugation.

Conclusions

The results of this study suggest that boar sperm cells decrease in their ability to penetrate through a two-step discontinuous density gradient of 45% and 90% isotonic percoll over a 7 day storage period post-collection as measured by the percent of total sperm recovered from each layer. The changes in sperm cell penetration appear to be affected by collection period and day of storage post-collection through the sperm properties of motility, progressive motility and buoyant density. The percent of sperm cells recovered in the high density 90% layer decreased from day 1 to 7 for the 1 time per week period, while the percent of sperm cells recovered in the low density 45% layer increased from day 1 to 7 for the multiple collections period. In addition, sperm motility may also play a role in sperm separation as there was decrease in sperm motility and progressive motility from day 1 to 7 of storage seen in sperm from the multiple collections period. However there were no differences in sperm motility between the density layers at any day of storage. The changes observed in sperm separation do not appear to be related to sperm cell morphology, lipid peroxidation of the plasma membrane or fatty acid composition of the sperm cell. Collectively, these data suggest that collection period and the day of storage post-collection

affect sperm motility, progressive motility and buoyant density, however, the molecular factors driving these changes are not known.

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Table 3.1. Effect of collection period on the ejaculate characteristics volume, concentration and total sperm in the ejaculate.

Ejaculate parameter	Collection period			SEM
	1 time/week	Multiple collections	3 day rest	
Volume, mLs	208	242	215	32
Concentration, x 10^6/mLs	341[a]	139[b]	211[b]	30
Total sperm, x 10^9	66[a]	32[b]	41[b]	5

[a,b]Means in a row without a common superscript are different ($P<0.05$)

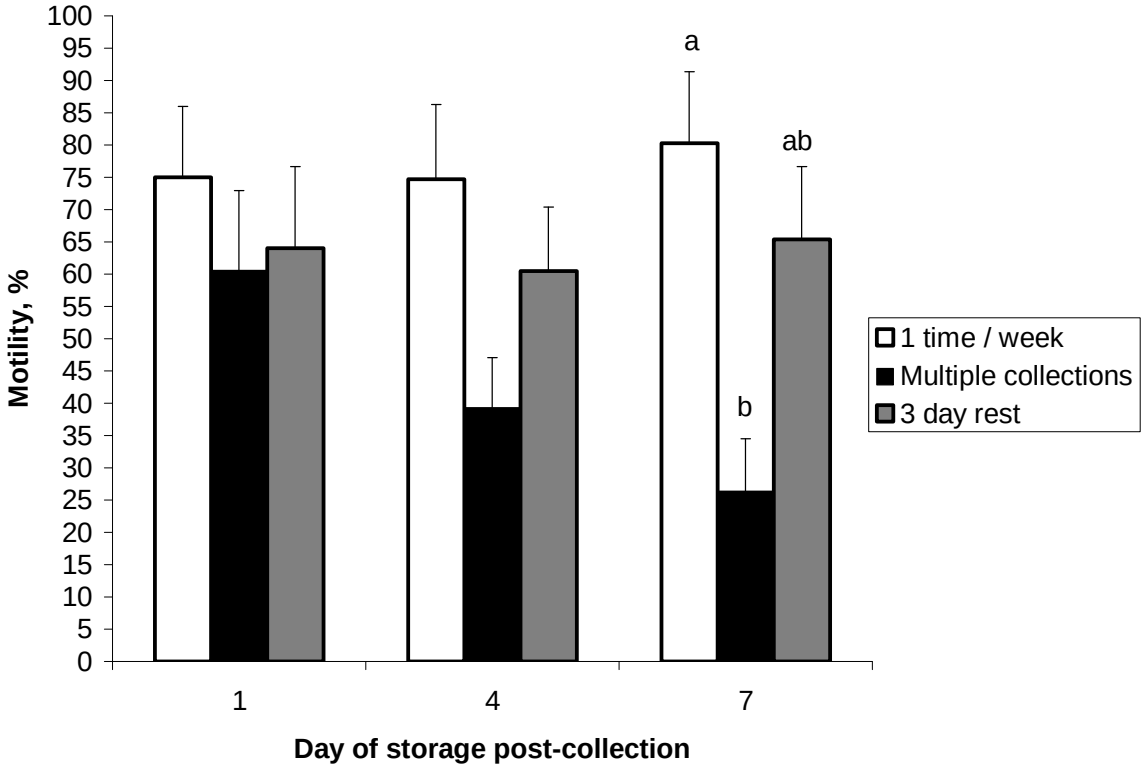


Figure 3.1. Effect of collection period and day of storage post-collection on the percentage of motile sperm in the extended ejaculate, sorted by day of storage. The data were arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.

[ab]Means are different within day of storage ($P < 0.05$)

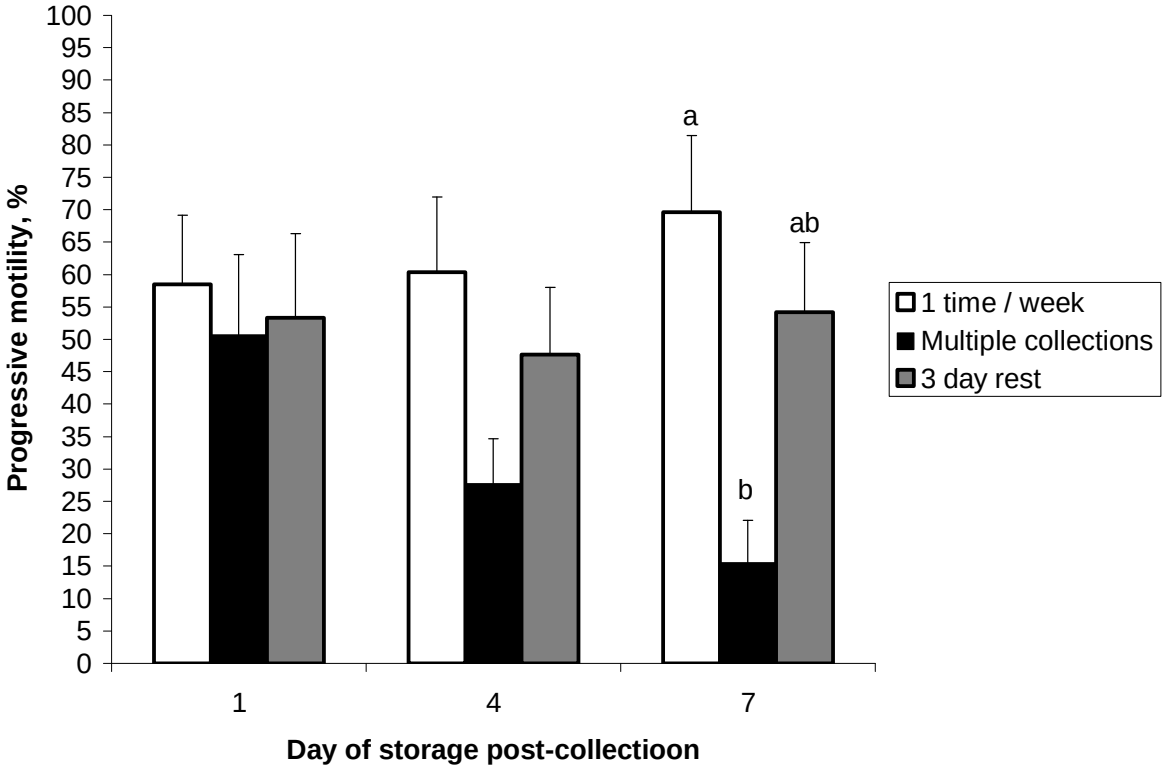


Figure 3.2. Effect of collection period and day of storage post-collection on the percentage of progressively motile sperm in the extended ejaculate, sorted by day of storage. The data were arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.

[ab]Means are different within day of storage ($P < 0.05$)

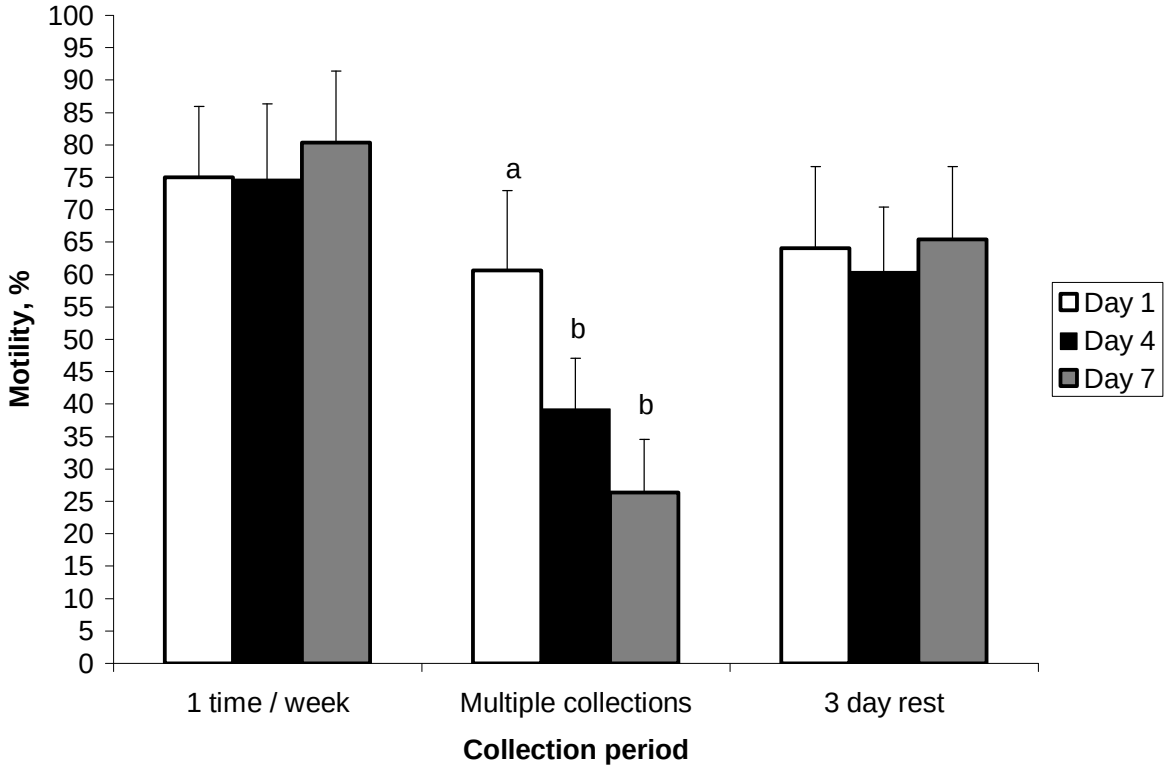


Figure 3.3. Effect of collection period and day of storage post-collection on the percentage of motile sperm in the extended ejaculate, sorted by collection period. The data were arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.

[ab]Means are different within day of storage ($P < 0.05$)

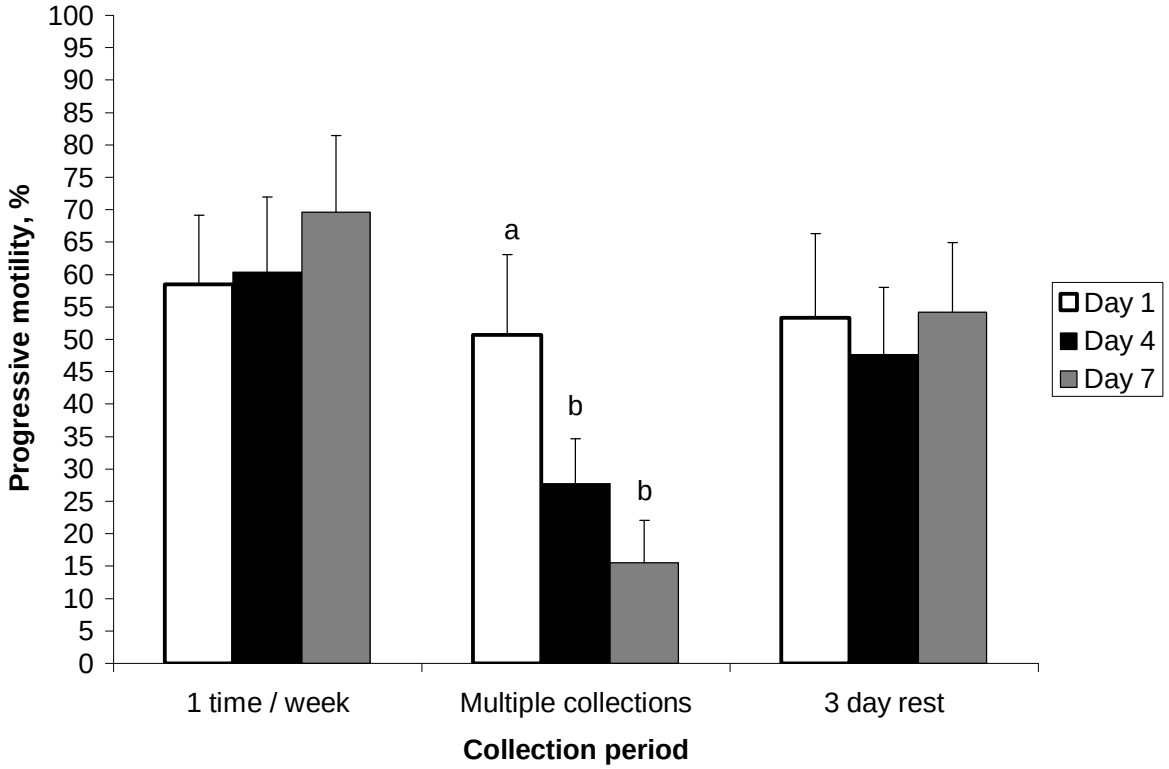


Figure 3.4. Effect of collection period and day of storage post-collection on the percentage of progressively motile sperm in the extended ejaculate, sorted by collection period. The data were arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.
[ab]Means are different within day of storage ($P < 0.05$)

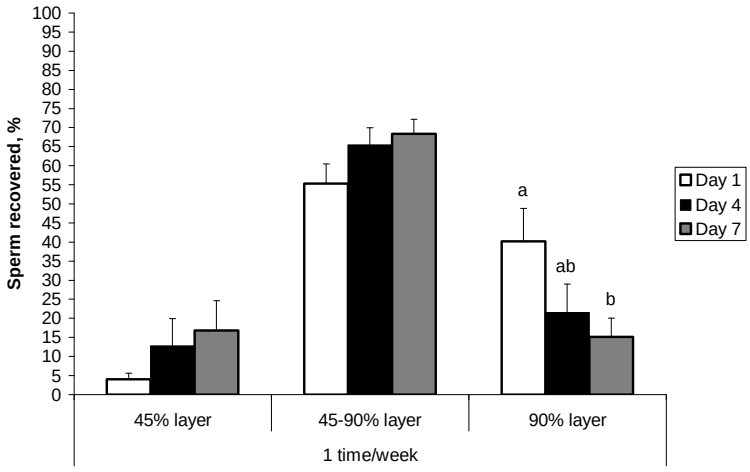
Table 3.2. Effect of collection period and day of storage on the percentage of normal and total abnormal sperm.

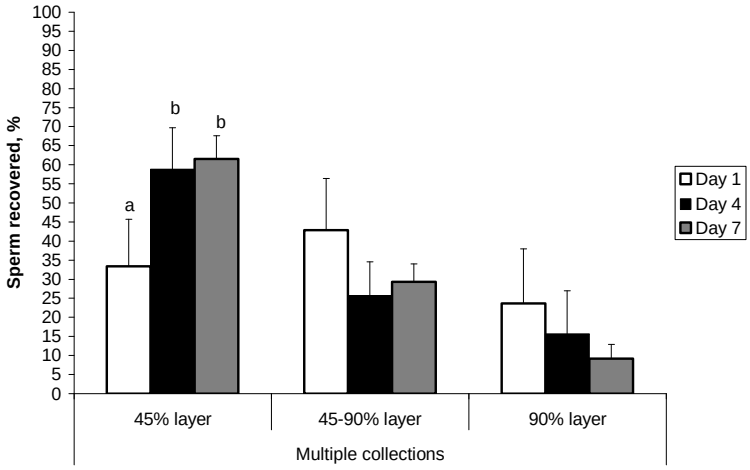
	Day of storage post-collection								
	1			4			7		
Sperm morphological parameter[1], %	1 time/week	Multiple collections	3 day rest	1 time/week	Multiple collections	3 day rest	1 time/week	Multiple collections	3 day rest
Normal	81.2 ± 11.4	75.7 ± 7.4	67.2 ± 10.0	80.5 ± 10.7	77.5 ± 8.5	76.7 ± 10.7	83.2 ± 11.7	81.8 ± 6.8	75.5 ± 11.9
Distal cytoplasmic droplet	7.0 ± 4.3	4.8 ± 2.3	3.3 ± 1.8	6.8 ± 4.1	5.0 ± 1.5	4.3 ± 1.7	4.7 ± 3.7	3.8 ± 2.1	4.2 ± 1.9
Total abnormal	18.8 ± 11.4	24.3 ± 7.4	33.0 ± 9.9	19.5 ± 10.1	22.7 ± 8.7	23.3 ± 10.7	16.8 ± 11.7	18.3 ± 6.8	24.5 ± 11.9

The data was arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.

[1]Definitions: Total abnormal (Total attached cytoplasmic droplets + abnormal tails + abnormal heads)

Figure 3.5. Effect of collection period and day of storage post-collection on the percentage of sperm recovered from each density layer after density gradient centrifugation. The data were arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.
[ab]Means are different within layer within collection period ($P < 0.05$)


Sperm recovered, %
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
a
ab
b
Day 1
Day 4
Day 7
45% layer
45-90% layer
90% layer
1 time/week


Sperm recovered, %
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
a
b
b
Day 1
Day 4
Day 7
45% layer
45-90% layer
90% layer
Multiple collections

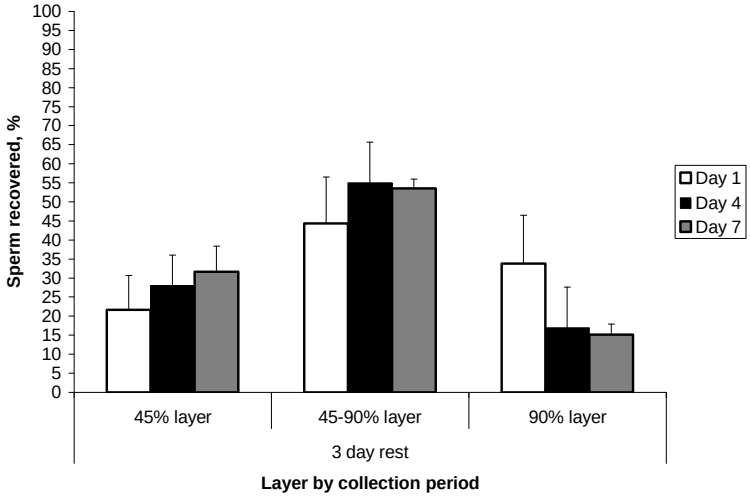

Sperm recovered, %
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
Day 1
Day 4
Day 7
45% layer
45-90% layer
90% layer
3 day rest
Layer by collection period

Table 3.3. Effect of density gradient centrifugation and day of storage post-collection on percentage of normal, distal cytoplasmic droplet, total attached cytoplasmic droplets and total abnormal sperm.

	Day of storage post-collection								
	1			4			7		
Sperm morphological parameter[1], %	45%	45/90%	90%	45%	45/90%	90%	45%	45/90%	90%
Normal	74.6 ± 5.1	79.1 ± 5.8	80.9 ± 5.7	78.8 ± 5.5	81.5 ± 5.7	81.4 ± 5.9	78.7 ± 5.2	82.5 ± 6.1	78.8 ± 5.7
Distal cytoplasmic droplet	10.5 ± 1.8[a]	5.6 ± 1.8[ab]	3.3 ± 1.5[b]	6.3 ± 1.5	4.7 ± 1.5	3.6 ± 1.3	6.6 ± 2.0	4.4 ± 1.8	4.1 ± 1.8
Total attached cytoplasmic droplets	20.1 ± 4.5	16.7 ± 5.2	11.6 ± 4.8	16.7 ± 4.7	15.6 ± 5.3	13.9 ± 5.3	17.5 ± 4.5	14.8 ± 5.7	12.4 ± 5.1
Total abnormal	25.4 ± 5.1	20.9 ± 5.8	19.1 ± 5.7	21.3 ± 5.5	18.5 ± 5.7	18.6 ± 5.9	21.3 ± 5.2	17.5 ± 6.1	21.2 ± 5.7

The data was arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.

[1]Definitions: Total abnormal (Total attached cytoplasmic droplets + abnormal tail + abnormal head)

[ab]Means within day of storage and row without similar superscripts are different ($P < 0.05$)

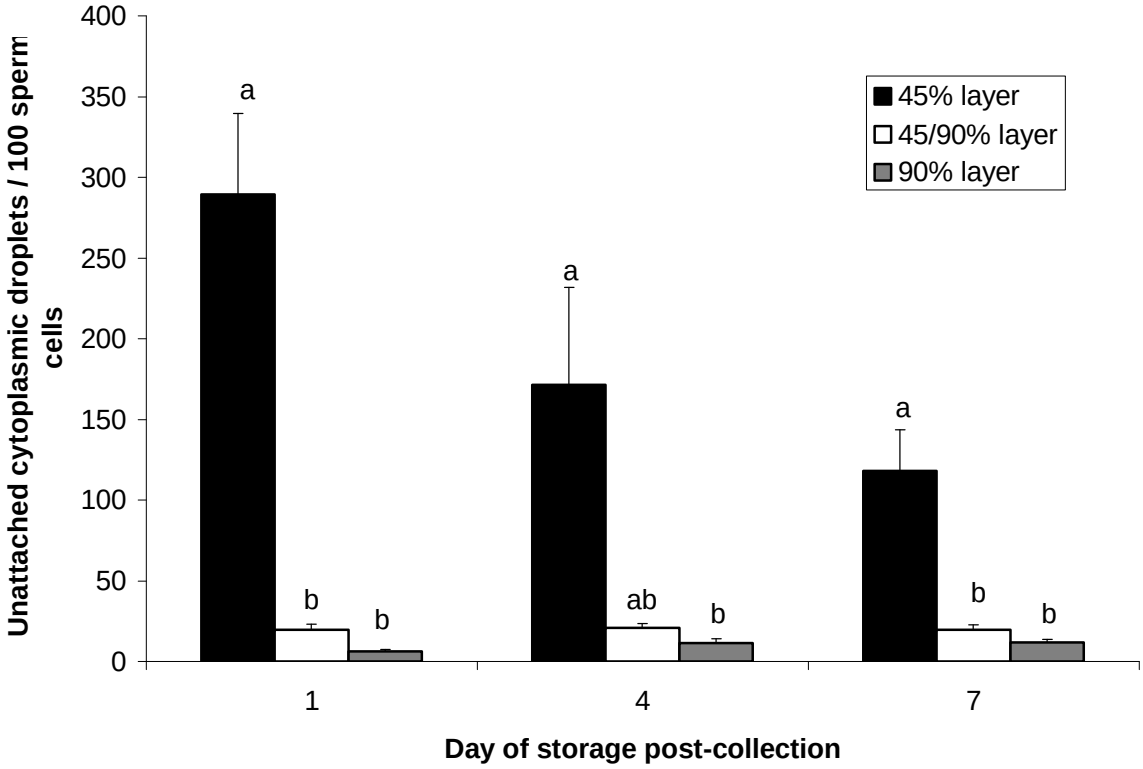


Figure 3.6. Effect of day of storage post-collection on the concentration of unattached cytoplasmic droplets in the density layers after density gradient centrifugation.
[ab]Means are different within day of storage post-collection ($P < 0.05$)

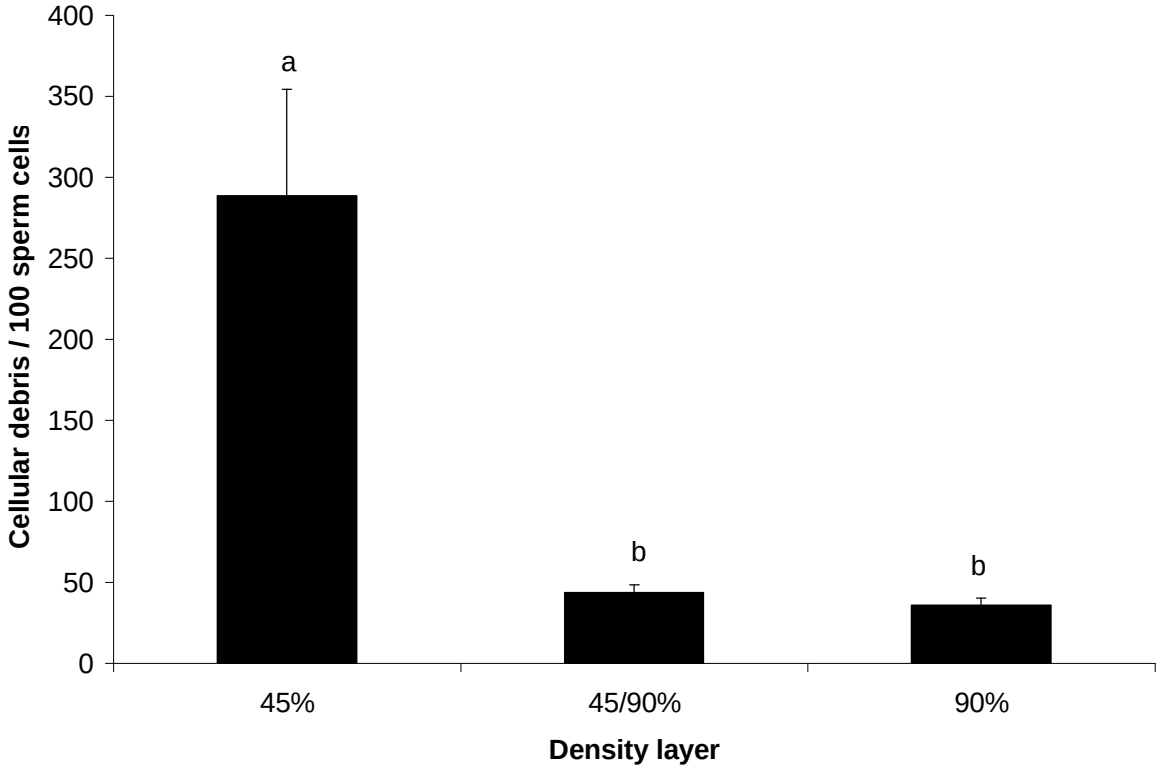
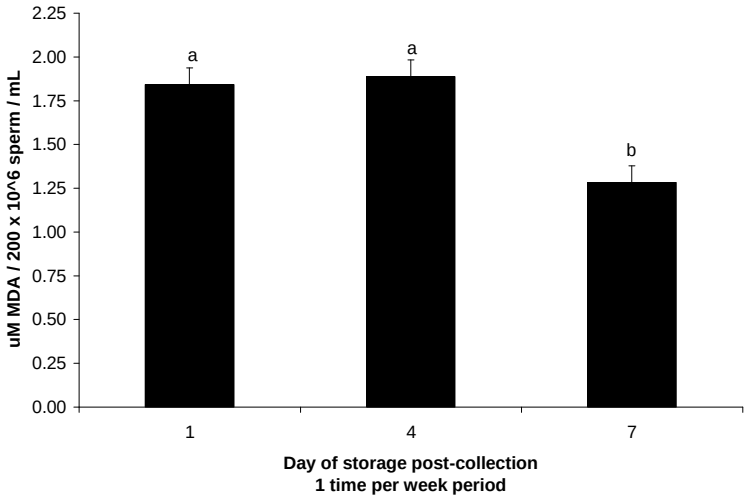
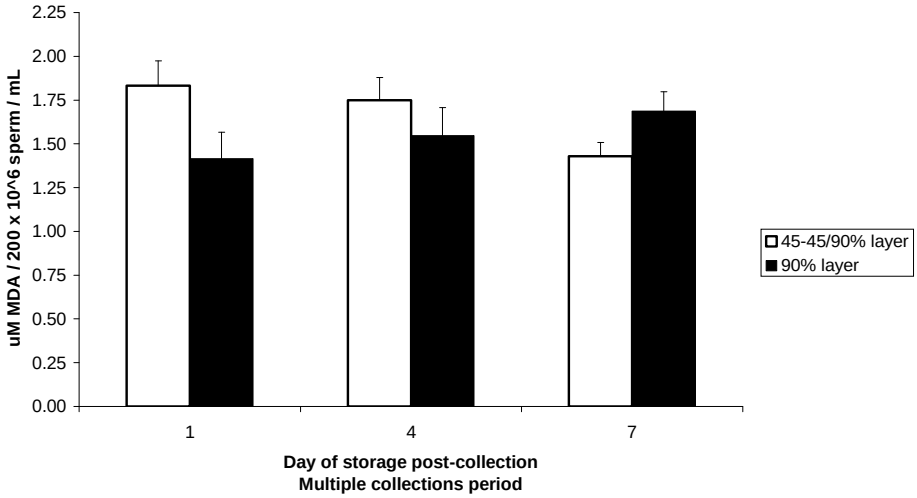


Figure 3.7. Concentration of cellular debris in the density layers after density gradient centrifugation.
[ab]Means without a common superscript are different ($P < 0.05$)

Figure 3.8. Effect of collection period, density layer and day of storage on sperm lipid peroxidation (as measured by the concentration of MDA).
[ab]Means without a common superscript within collection period are different ($P < 0.05$)


2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
uM MDA / 200 x 10^6 sperm / mL
a
a
b
1
4
7
Day of storage post-collection
1 time per week period


2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
uM MDA / 200 x 10^6 sperm / mL
1
4
7
Day of storage post-collection
Multiple collections period
45-45/90% layer
90% layer

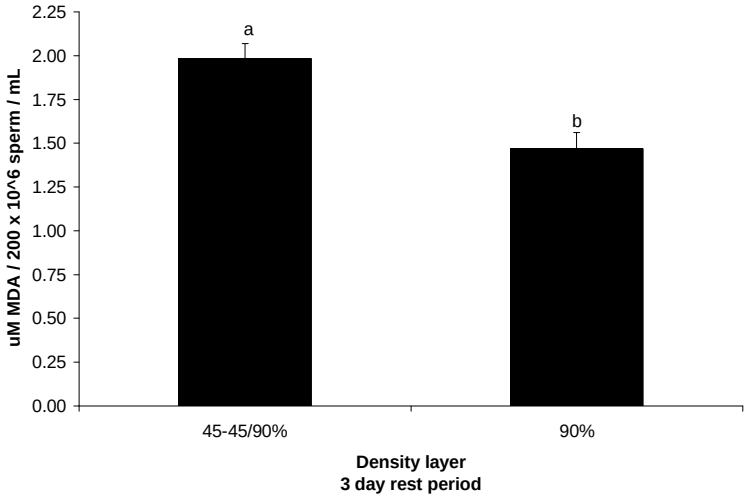
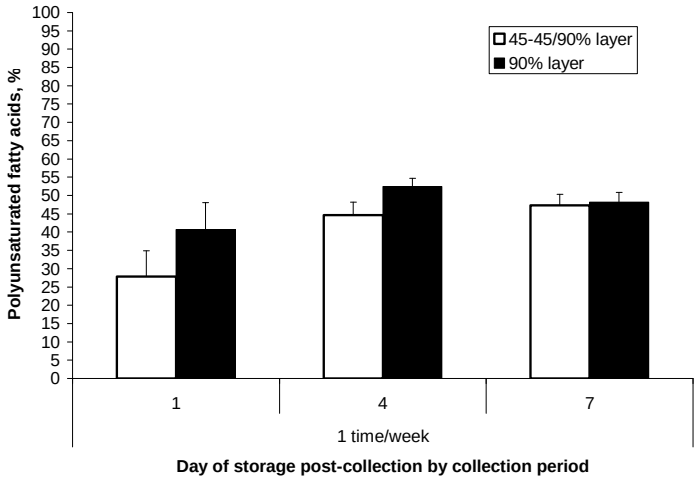
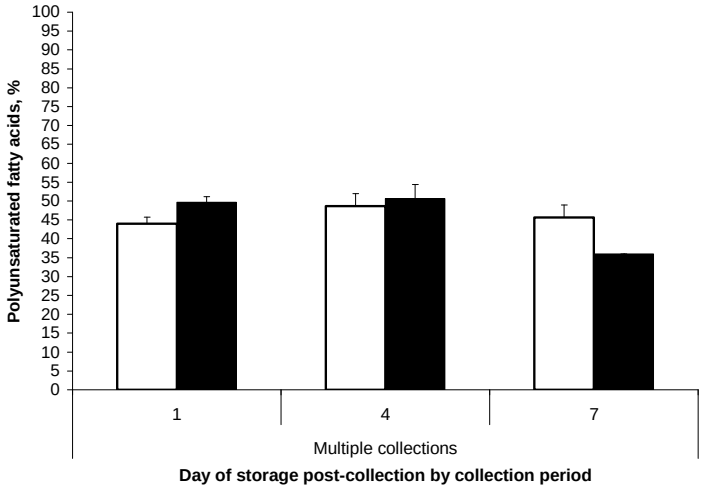
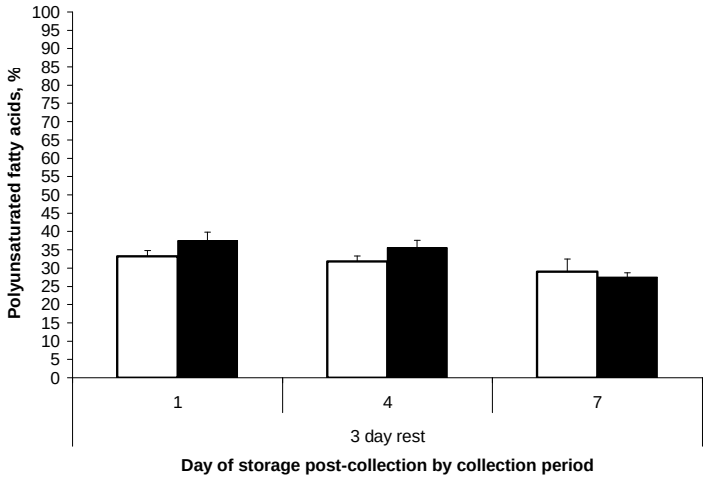

2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
uM MDA / 200 x 10^6 sperm / mL
a
b
45-45/90%
90%
Density layer
3 day rest period

Figure 3.9. Percentage of polyunsaturated fatty acids (PUFAs) measured from the density layers after density gradient centrifugation sorted by day of storage post-collection and collection period.


45-45/90% layer
90% layer
Polyunsaturated fatty acids, %
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
1
4
7
1 time/week
Day of storage post-collection by collection period


Polyunsaturated fatty acids, %
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
1
4
7
Multiple collections
Day of storage post-collection by collection period


Polyunsaturated fatty acids, %
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
1
4
7
3 day rest
Day of storage post-collection by collection period

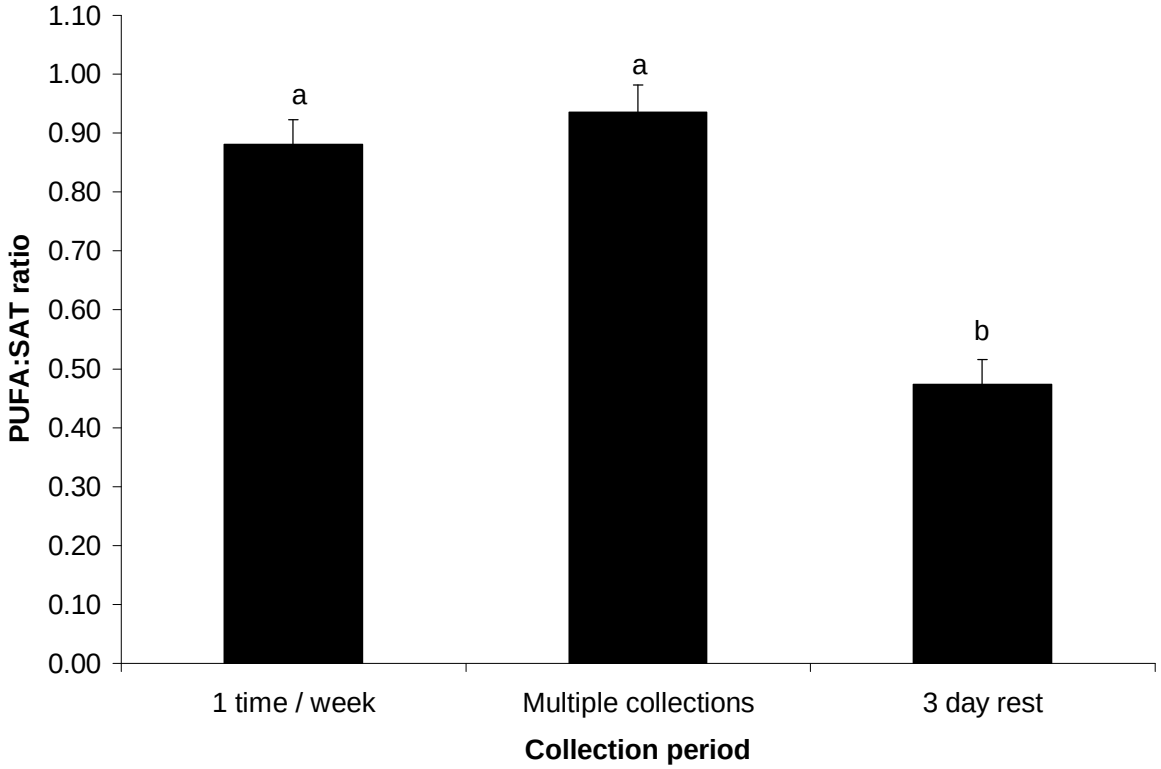


Figure 3.10. Polyunsaturated fatty acids (PUFAS) to saturated fatty acids (SAT) ratio measured from sperm collected from boars maintained on the 1 time per week, multiple collections and 3 day rest periods.
[ab]Means without a common superscript are different ($P < 0.0001$)

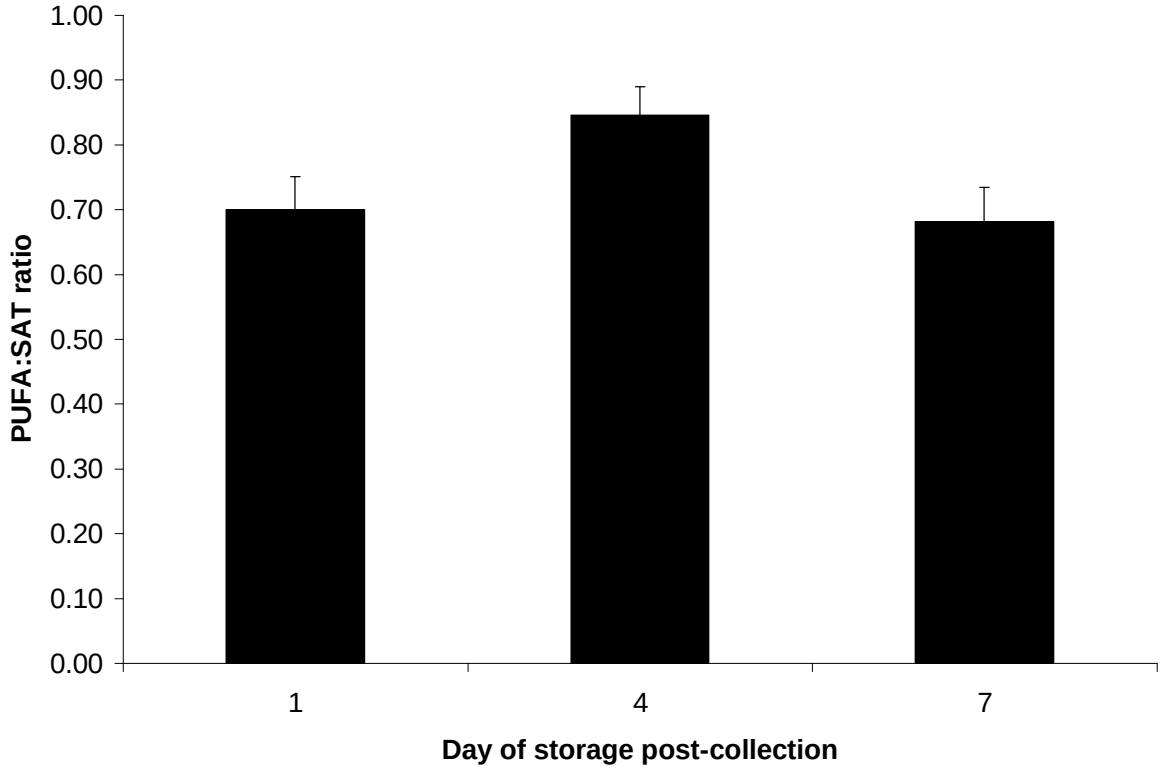


Figure 3.11. Polyunsaturated fatty acids (PUFAS) to saturated fatty acids (SAT) ratio by day of storage post collection.

CHAPTER 4

EFFECT OF DIETARY SELENIUM ON BOAR SPERM QUALITY

Abstract

The primary objective of this research was to evaluate the effect of dietary selenium on sperm production and sperm quality. The dietary treatments were a non-supplemented negative control basal diet or the basal diet supplemented at 0.3 ppm with either organic selenium or inorganic selenium. A secondary objective was to examine changes in sperm quality over a six day storage period post-collection. Boars were fed the dietary treatments from the time of weaning (20.97 ± 0.18 d of age) until the end of the study (382.97 ± 0.18 d of age). Following training to a collection dummy, six boars from each dietary treatment were randomly selected for semen evaluation over a six month period (January, March, May and June). From January through May, boars were maintained on a 1 time per week collection frequency. In June boars were subjected to a high collection frequency of six collections in four days with the sixth collection used for ejaculate and semen analysis. Ejaculates were extended in a 5-day commercially available semen extender and evaluated on day 1 and day 6 of storage post-collection (day 0 = day of collection). Boars fed the organic selenium diet had higher ($P < 0.01$) levels of selenium in the blood compared to the negative control diet boars, but no differences ($P = 0.18$) were found between the dietary treatments for selenium in the semen. Dietary treatment did not affect growth parameters during the nursery and finishing phases ($P > 0.05$) or the semen collection phase ($P > 0.3$). Dietary treatment did not affect volume, concentration or total sperm in the ejaculate ($P > 0.5$). Dietary treatment did not affect ($P > 0.2$) sperm motility, progressive motility, morphology, membrane lipid peroxidation and glutathione peroxidase activity. Following density gradient centrifugation, sperm motility, progressive motility, the percentage of

morphologically normal sperm and the percentage of sperm recovered were higher ($P < 0.01$) in the 90% density layer compared to the 45% density layer on day 1 but not day 6 ($P > 0.1$) of storage post-collection. Lipid peroxidation and glutathione peroxidase activity was higher ($P < 0.01$) in March and May compared to January. After a high collection frequency in June, the level of MDA was lower ($P < 0.01$) compared to January, March and May while the glutathione peroxidase activity was higher ($P < 0.05$) compared to January and March. These results indicate that dietary treatment affected selenium levels in the blood, but did not affect sperm quality. In addition, boar sperm cells decrease in progressive motility and buoyant density over a six day storage period which appears to affect sperm motility, morphology and percent of sperm recovered in the high and low density layers following density gradient centrifugation.

Introduction

Previous reports have demonstrated that males consuming diets low in selenium produce sperm with low motility and increased structural abnormalities related to the midpiece (Hansen and Deguchi, 1996; Maiorino et al., 1999). The underlying cause of the reduced sperm quality from a selenium deficiency is not known, but may be a result of abnormal development of the sperm cell during spermatogenesis (Behne et al., 1996). Selenium may affect sperm quality through its relationship with the glutathione peroxidase (GPX) enzyme. Research suggests that the selenium containing GPX enzyme is important for preserving the structural integrity of the sperm plasma membrane (Wu et al., 1979). In addition, the GPX enzyme is an essential antioxidant responsible for neutralizing reactive

oxygen species and thereby protecting the sperm plasma membrane from lipid peroxidation (Drevet, 2006; Storey, 2008).

Studies evaluating the effect of dietary selenium have shown beneficial effects for boar sperm quality. Marin-Guzman et al. (1997) found that boars supplemented with 0.5 ppm of selenium produced sperm with better motility and morphology and had improved fertility compared to boars fed the selenium deficient diet. Other research has suggested the source of selenium may be important. Jacyno and Kawecka (2002) found that boars supplemented with an organic source of selenium had higher sperm concentration and total sperm numbers in the ejaculate as well as a lower percentage of abnormal sperm compared to boars supplemented with an inorganic source of selenium.

The objectives of this study were to determine the effects of dietary selenium on boar sperm production and quality, and to examine changes in sperm quality over a six day storage period post-collection.

Materials and Methods

Animal Care and Use

All procedures that involved the use of animals were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC ID # 07-063-A).

Growing phase in nursery and finishing. A total of 30 crossbred boars of mixed genotypic frequencies (Yorkshire x Landrace x Large White sows bred to Hampshire x Duroc x Pietran boars) were used in the study. The boars were weaned at approximately 21 d of age (20.97 ± 0.18 d of age) and moved into a side-wall baffle ventilated nursery room with 24 pens (1.82m x 0.91m). During the five week long nursery portion, boars were housed two

to a pen and provided *ad libitum* access to feed (0.17m x 0.3m feeder space) and water from two nipple waterers. The boars were then moved into a curtain-sided underslat ventilated finishing facility. The finishing phase lasted fourteen weeks. The boars were penned (1.84m x 2.84m) in their original group of two. Access to feed (0.91m feeder space) and water (two nipple waterers) was provided *ad libitum*.

Semen collection phase. Following the finishing phase, boars (n=18) were moved to individual stalls (2m x 0.7m) located in an environmentally controlled gestation building employing a curtain-sided, under slat ventilation system. Supplemental ventilation fans and a drip cooling system were set to activate when ambient temperatures reached 23ºC and 26ºC, respectively. The boars were provided *ad libitum* access to water from a single nipple waterer. The boars were hand-fed daily into a concrete trough that spanned the width of the stall. The boars were trained to mount a collection dummy sow between 160 and 190 d of age. Eighteen boars were trained to collect from the dummy sow over a three week training period.

Experimental design

Dietary treatments. Boars were fed one of three corn-soybean meal based dietary treatments: 1) a negative control basal diet containing no supplemental selenium; 2) an inorganic selenium diet supplemented with 0.3 parts per million (ppm) of inorganic selenium from sodium selenite and 3) an organic selenium diet supplemented with 0.3 ppm of organic selenium from selenium enriched yeast (Sel-Plex 2000, Alltech Biotechnology Center, Nicholasville, KY). The dietary treatments were formulated to meet or exceed NRC recommendations with the exception of selenium supplementation for the negative control

diet for growing swine and boars for each phase of the study. Dietary treatments were administered to the boars beginning in the nursery phase immediately after weaning and continued until the end of the study.

During the growth phase in the nursery and finisher boars were fed the dietary treatments as a two-phase nursery program. The phase one nursery diet was fed for two weeks and phase two nursery diet was fed for three weeks. The finishing phase lasted fourteen weeks. Four diets were fed during the finishing phase, a two-phase grower diet and a two-phase finisher diet. The grower diets and the first finisher diet were fed for four weeks each, the second finisher diet was fed for two weeks. During the semen collection phase, the boars were restricted fed 2 kg from approximately 154 d of age to 277 d of age and then 2.5 kg from approximately 278 d of age to 382 d of age.

The compositions of all experimental diets by phase used in the study are presented in Table 4.1. Calculated intakes of minerals and vitamins during the semen collection phase when diets were restricted fed are presented in Table 4.2.

Growing phase in nursery and finishing. A total of 30 crossbred boars were used in the growth phase of the study. At weaning, boars were weighed, blocked by weight and randomly allocated to pen and dietary treatment within block. A total of 15 pens containing two pigs per pen were used, with 5 replicates per treatment. Boars were retained with the same pen mate during the finisher phase and kept on the same diets.

Semen collection phase. At approximately 160 d of age, 15 boars were randomly selected (n = 5 per treatment) to be trained to collect from a dummy sow. Ten of the 15 boars were trained to the dummy after three weeks (n = 3 for negative control; n = 3 for inorganic

selenium and n = 4 for organic selenium). At approximately 190 d of age, another 8 boars (n = 3 for negative control and inorganic selenium treatments and n = 2 for organic selenium treatment) were trained to collect. The total number of boars used in the semen collection phase was 18 (n = 6 boars per treatment). Following training to a dummy, the boars were randomly assigned to be collected either Tuesday or Thursday on a 1 time per week collection frequency.

Semen analysis was performed every other month beginning in January of 2008 until May. Semen analyses were performed during the second week of the month. In June boars were subjected to a multiple collection frequency of six collections in four days. The final ejaculate during the multiple collection frequency was extended for laboratory analysis. Ejaculates not used for semen analysis during the 1 time per week collection frequency or the multiple collections period were measured for volume and concentration and then discarded. Following completion of the multiple collection frequency, the study was concluded.

Ejaculates were collected using the gloved-hand technique (Almond et al., 1998) into a pre-weighted collection thermos containing a plastic bag (IMV International Corp., Maple Grove, MN) and warmed to 37ºC. The collection thermos was covered with a filter (IMV International, Eden Prairie, MN) to separate the fluid fraction (semen) of the ejaculate from the gel fraction. The volume of semen was estimated by weighing each ejaculate with a gravimetric electronic balance (V-300, Acculab, Edgewood, NY) and using the conversion of 1 g of semen equals 1 mL. The concentration of sperm cells in the semen was evaluated using a photometer (SpermaCue™, Minitube of America, Verona, WI). The total number of sperm cells in the semen was calculated by multiplying the volume by the concentration.

Ejaculate volume, concentration and total sperm cell numbers were recorded following each collection. Following collection, ejaculates were stored in a water bath pre-warmed to 37ºC (Minitube of America, Verona, WI). Ejaculates were transferred to the lab approximately 1.5 hours after the first ejaculate had been collected.

Upon arrival at the laboratory, semen was placed into a water bath pre-warmed to 37ºC. The ejaculates were extended to a concentration of 75 x 10^6 sperm/mL. All ejaculates were extended using a commercially available 5-day extender (Androhep; Minitube of America, Verona, WI) and stored in a semen cooling unit (Minitube of America, Verona, WI, USA) at 17-18°C until analysis. A total of 300 mLs of extended semen were made for each boar and stored in 50 ml tubes. There was a total of 3.7 x 10^9 sperm in the 50 mL tube. During storage the 50 ml tubes were laid flat in the semen cooler and were gently inverted once per day to re-suspend the sperm cells into solution.

Laboratory analyses for semen quality estimates were performed on days 1 and 6 post-collection, except the GPX assay, which was performed on day 1 only. Day 0 was considered the day of collection. The following semen quality estimates were performed for each sample: the percentage of motile and progressively motile sperm, the percentage sperm classified with normal morphology or abnormal morphology. Abnormal morphology included the following: abnormal tail, abnormal head, and sperm possessing a cytoplasmic droplet in the proximal, distal or as part of the distal midpiece reflex. In addition, the concentration of malondialdehyde (MDA) and sperm cell fatty acid composition was measured for each sample. Sperm were also subjected to density gradient centrifugation and

sperm motility, progressive motility, morphology, cellular particles and the percent of sperm recovered from each density layer were evaluated.

Body weight, blood collection and testicular measurements

Boars were weighed and testicular measurements determined during the growth phase at weaning (20.97 ± 0.18 d of age) and at the end of each dietary phase (nursery: approximately 35 and 56 d of age; finishing: approximately 84, 112, 140 and 154 d of age). In the nursery phase, body weights in kilograms were taken by placing the boar on a movable electronic scale (Mosdal Seed Carts; Broad View, MT). Weights in the finishing phase and semen collection phase were recorded in pounds using a stationary scale (Ezi Weigh 2; Tru Test; Mineral Well, TX). Weights recorded in pounds were later converted to kilograms.

Testicular measurements in centimeters were taken by the same technician throughout the study to determine testicular size. The height and width of the testicles was taken using digital calipers (Fisher Scientific; Atlanta, GA) during the nursery phase. Height was determined using the right testicle by measuring from the top to the bottom. Width was determined by measuring the width of both testicles. During the finishing phase and semen collection phase testicular measurements were taken as described above, except that a flexible tape measure was used. Testicle area was calculated by taking the right testicle height multiplied by the width of both testicles.

Blood was collected at the end of each dietary phase in the nursery and one time in the finishing phase at approximately 112 d of age in order to avoid creating fear of human contact prior to training for semen collection. Blood was collected in the nursery phase by placing the boars on their backs in a V-shaped wooden trough and restraining their front and

back legs while a technician collected the blood via venipuncture of the jugular vein through the jugular furrow using a 1 or 1.5 in, 20 gauge needle depending on the size of the piglet (Becton Dickinson; Franklin Lakes, NJ). A volume of approximately 3 mLs was collected and stored in a cooler lined with ice packs at about 4ºC. Blood samples were transferred back to the lab and kept at 4ºC. Samples were processed by centrifugation at 2500 rpm for 20 minutes at room temperature. The plasma was decanted just above the buffy coat and placed into a plastic tube, capped and stored at -20ºC until subsequent analyses. In the finisher phase, blood was collected from the boar by manual restraint using a snare. Similar to the nursery phase, blood was collected from the jugular vein via venipuncture of the jugular vein through the jugular furrow using a 3.5 inch, 18 gauge, thin-walled needle attached to a 5 cc syringe.

During the semen collection phase, blood samples were taken as the boar was being collected while mounted on the dummy sow. Blood was collected via venipuncture of a marginal ear vein with a 21 gauge, 0.75 in butterfly needle (Vaccutainer Blood Collection Set; Becton Dickinson; Franklin Lakes, NJ) connected to a 3 mL syringe. The blood was processed as previously described.

Sperm quality analyses

Motility analysis. Motility analysis was performed using the Sperm Vision™ digital semen evaluation software (Minitube of America, Verona, WI) using images obtained by a digital camera attached to phase contrast microscope (Model BX41; Olympus Optical; Tokyo, Japan) with a 20x phase objective. Prior to analysis, a 500 µL aliquot from each sample was diluted with 500 µLs of 17ºC Androhep extender and incubated at 37°C for 30

minutes. Four-chamber Leja slides (IMV International Corp., Maple Grove, MN) were pre-warmed to 37°C on a slide warmer (Minitube of America, Verona, WI) before motility analysis. Five microscopic fields were analyzed for each sample, in duplicate, for a total of ten fields analyzed per sample. Approximately 250-400 total cells were analyzed for the percentage of sperm exhibiting motility and progressive forward motility.

Sperm morphological and cellular particles analysis. Evaluation of sperm morphology and cellular particles was performed using a phase contrast microscope (Zeiss; West Germany) using a 45x objective. Prior to analysis, a 50 µL aliquot from each gradient for each boar was fixed with 5 µLs of 10% buffered formalin. For sperm morphology analysis, the sample was diluted for evaluation and 10 µL of the diluted sample was placed on a hemocytometer, covered with an 18mm x 18mm coverslip and then given at least 5 minutes for the sperm and cellular particles to settle before analysis. The analysis consisted of classifying all intact sperm and cellular particles within the triple lines of the hemocytometer. The morphology and concentration of sperm in each layer (45%, 45/90% and 90%) was determined from four hemocytomer counts. The average number of sperm evaluated per sample was 199.01 ± 4.16 (mean ± standard error).

For sperm morphology, sperm were classified according to Almond et. al. (1998) and Lovercamp et al. (2007). Intact sperm were classified into one of the following six morphological groups: normal, abnormal head, abnormal tail, proximal cytoplasmic droplet, distal cytoplasmic droplet and the distal midpiece reflex with a cytoplasmic droplet. Spermatozoa classified as a proximal cytoplasmic droplet possessed a cytoplasmic droplet located on the anterior half of the sperm tail midpiece proximal to the sperm head.

Spermatozoa classified as distal cytoplasmic droplet possessed a cytoplasmic droplet located on the posterior half of the sperm midpiece. All spermatozoa classified as distal midpiece reflex possessed a cytoplasmic droplet in the 180° bend of the tail. Spermatozoa with tail defects observed without a distal cytoplasmic droplet in the bend of the tail were classified as an abnormal tail.

Cellular particles were evaluated using a slightly modified version of the analysis described in Lessley and Garner (1983). Briefly, the cellular particles evaluated were unattached cytoplasmic droplets and cellular debris. Cellular debris included sloughed epithelial cells from the male reproductive tract, leukocytes, unidentifiable debris and broken heads and tails (Glover et al., 1990; Brunzel, 1994; Strasinger, 2001). The number of unattached cytoplasmic droplets and cellular debris were counted and the data are reported as the number of unattached cytoplasmic droplets or cellular debris quantified per 100 sperm cells.

Density gradient centrifugation. Sperm cells were separated into layers for evaluation of sperm cell motility, progressive motility, morphology and the percent of sperm recovered from each layer. Separation of sperm cells was accomplished by centrifugation of extended sperm on each day of analysis using a percoll (P-1644, Aldrich, Milwaukee, WI) 2-step discontinuous gradient centrifugation procedure with 45% and 90% isotonic percoll solutions. The 45% and 90% percoll solutions were made from an iso-osmotic 100% percoll-saline solution prepared from a stock percoll suspension (1.130 g/mL; P-1644, Aldrich, Milwaukee, WI) and a 10-fold saline stock solution, according to Vincent and Nadeau (1984)

as described in Harrison et al. (1992). The individual 45% (~1.055 g/mL) and 90% (~1.110 g/mL) percoll solutions were prepared the night before analysis and kept separate.

On each day of analysis, the discontinuous percoll gradient was prepared by sequential layering of 3mls of the 90% percoll solution and 3 mls of the 45% percoll solution into a 15ml conical tube. One percoll gradient tube was prepared for each sample on the day of analysis. For each sample, four mls of extended sperm were gently added on top of the 45% layer. After the addition of sperm, the percoll gradient tubes were subjected to centrifugation (Model Marathon 3200; Fisher Scientific, Pittsburgh, PA) for 5 minutes at 200 x g followed by 15 minutes at 1000 x g. Following centrifugation, the original 45% (3mLs) and 90% (3 mLs) layers were removed in the following manner to produce three individual layers containing sperm cells: 1) a 45% layer (2 mLs), 2) a 45/90% interface layer (2 mLs) and 3) a 90% layer (2 mLs). These three layers were formed by first removing the 4 mLs of extender. Next, the first two mLs of the percoll gradient were removed and designated as the 45% layer. The following two mLs were then removed and designated as the 45/90% interface layer. The remaining two mLs were then removed and designated as the 90% layer. A 100 µL sample from each layer for each sample was removed and placed into a separate bullet tube for motility and morphological analysis.

Sperm lipid peroxidation. Spontaneous sperm lipid peroxidation activity was evaluated using the MDA-586 kit (OxisResearch, Portland, OR, USA). The assay detects the presence of a carbonyl compound, malondiadehyde (MDA), which is an end-product of the lipid peroxidation cascade resulting from the breakdown of lipid peroxides. For each sample, 5 mLs of extended semen containing 75 x 10^6/mL sperm were washed twice in PBS at 3900

rpm for 5 minutes in a hinged rotor centrifuge (Model Marathon 3200; Fisher Scientific, Pittsburgh, PA) at room temperature to form a sperm pellet. After each wash the pellet was then gently re-suspended in 1 mL of PBS. The pellet was finally re-suspended in 1 mL of PBS to a final concentration of 375 x 10^6/mL for quantification of MDA. The MDA assay was performed to measure free MDA by incubating the samples at 45°C for 60 minutes. After the incubation, the samples were centrifuged at 10,000 x g for 10 minutes to obtain a clear supernatant, as directed by the protocol. Samples were immediately plated and absorbances measured in a 96-well plate at a wavelength of 586 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT). Intra- and inter-assay coefficients of variation for sixteen assay batches were 3.51 and 18.34%, respectively based on a known 2.5 μM amount of the MDA standard that was pre-made, stored frozen and measured in duplicate on each assay day. The MDA-586 assay kit was validated for boar sperm using a spike and recovery technique of known concentrations of the MDA standard into three samples of boar sperm stored in the 5-day extender for 2 days. The recoveries were (mean ± standard error) 77.0 ± 2.5%, 83.0 ± 3.0% and 89.5 ± 1.5% (Appendix A).

Glutathione peroxidase assay. Sperm cell glutathione peroxidase (GPX) activity was evaluated using the Glutathione Peroxidase assay kit (Cayman Chemical Company, Ann Arbor, MI). The GPX enzyme was removed from sperm cells by homogenization (1 time for 30 seconds) and sonication (3 times at 10 seconds each) of the sperm pellet in a cold buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA and 1 mM DTT). Samples remained on ice during the extraction procedures. The samples were concentrated using centrifugal filter devices (Amicon Ultra-4, Millipore Corporation, Billerica, MA). Following concentration of the

samples, the total protein content was determined using a variation of the Lowry assay (DC protein assay kit, Bio-Rad Laboratories, Hercules, CA).

The GPX assay determines activity indirectly by evaluating the oxidation of NADPH to $NADP^+$ at an absorbance of 340 nm. The activity of GPX in the sample is determined using the NADPH extinction coefficient that will cause the oxidation of 1 nmol of NADPH to $NADP^+$ per minute at 25ºC. The results for the assay were standardized by reporting sperm GPX activity (nmol/min/ml) per 10 mg/ml of sperm protein. Intra- and inter-assay coefficients of variation for four assay batches were 2.20 and 2.10%, respectively based on a provided positive control sample of bovine erythrocyte GPX that was stored frozen and measured in duplicate on each assay day. The GPX assay kit was validated for boar sperm using a spike and recovery technique of known concentrations of the positive control bovine erythrocyte GPX into three samples of boar sperm GPX. Processing of the sperm prior to analysis was performed as previously described. The recoveries were (mean ± standard error) 128.1 ± 14.8%, 104.9 ± 9.4% and 102.4 ± 7.8% (Appendix B).

Selenium analysis by the Nuclear Reactor Program at North Carolina State Univeristy

Selenium was evaluated in the three experimental diets, blood plasma and semen from samples collected during the May analysis period of the study. The mash feed that was analyzed for selenium in each diet was from the batch fed for 1.5 months prior to the May blood plasma and semen analyses. Feed samples were measured in duplicate for each diet, with the arithmetic means reported ± standard error of the mean.

The presence of selenium for the study was determined using Instrumental Neutron Activation Analysis (INAA; McKown and Morris, 1978) procedures at the nuclear reactor on

the campus of North Carolina State University (Nuclear Reactor Program, Department of Nuclear Engineering, Raleigh, NC). Briefly, the samples were activated for 12 hours in the reactor. After 2-3 weeks the short lived interfering isotopes decayed away. The activated trace radioisotopes (selenium-75) in the samples were analyzed and quantified by gamma spectrometry systems to measure gamma ray decay signatures. Certified elemental standards, traceable standard reference material controls, method blanks, and sample duplicates were processed along with samples for quality assurance.

Statistical analysis

All statistical analyses were performed using the mixed procedure of SAS (SAS Ins., Cary, NC). The variance/covariance structure used for the repeated measures analyses was determined by finding the appropriate structure with the lowest fit statistics. Prior to analysis, all percentage data were normalized with an arcsine transformation (Snedecor and Cochran, 1989). Results for percentage data are reported as non-transformed arithmetic means ± standard error of the mean. All other data are reported as LS means ± standard error of the mean. For all models, the Tukey-Kramer adjustment was used to determine differences among means of independent variables when significant effects were observed.

Three boars were removed from the semen analysis portion of the study. One boar was removed from the negative control diet treatment due to death, one boar was removed from the inorganic selenium diet treatment due to testicular injury and one boar was removed from the organic selenium diet treatment for medication due to lameness.

Two additional cumulative variables were derived from the morphological analysis for each ejaculate and are included in the statistical analysis for sperm morphology: 1) a total

attached cytoplasmic droplet group (proximal cytoplasmic droplet + distal cytoplasmic droplet + distal midpiece reflex); and 2) a total abnormality group (abnormal tail + abnormal head + total attached cytoplasmic droplets).

The percentage of sperm recovered from each density layer was calculated by first determining the total number of sperm recovered from each layer. Next, the total number of sperm was calculated by summing the sperm from each layer. Finally, the percentage of sperm from each layer was calculated by dividing the total sperm from each layer by the total number of sperm recovered from all three layers, multiplied by 100.

Selenium analysis. Data for selenium analysis in the blood plasma and semen of boars was performed on blood and semen collected during the May analysis period. The data were analyzed as a complete randomized design. Boar was considered the experimental unit. The model included diet (negative control, inorganic selenium, organic selenium).

Body weight and testicular measurements – growth phase. Data for body weight, average daily feed intake, gain to feed, testicular area and testicle area to body weight ratio of boars during the growth phase in the nursery and finisher phases were analyzed as a complete randomized block design using analysis of variance procedures for repeated measures (Gill and Hafs, 1971). Pen was considered the experimental unit. The model included block, diet (negative control, inorganic selenium, organic selenium), time (weaning, nursery 1-2; finisher 1-4), and the diet x time interaction. The variance/covariance structure used was compound symmetry heterogeneous. The error term used to test treatment effects was pen nested within diet. Age at dietary phase was considered the repeated measure.

Body weight, testicular measurements, ejaculate characteristics and glutathione peroxidase activity – semen collection phase. Data for body weight, testicular size, ejaculate characteristics and glutathione peroxidase activity of boars during the semen collection phase were analyzed as a complete randomized design using analysis of variance procedures for repeated measures. Boar was considered the experimental unit. The model included diet (negative control, inorganic selenium, organic selenium), month (January, March, May, June) and the diet x month interaction. The variance/covariance structure used was unstructured. The error term used to test treatment effects was boar nested within diet. Month was considered the repeated measure.

Sperm quality analysis. Data for sperm motility, progressive motility, morphology, lipid peroxidation and fatty acid composition were analyzed as a complete randomized design using analysis of variance procedures for repeated measures. Ejaculate was considered the experimental unit. The model included diet (negative control, inorganic selenium, organic selenium), month (January, March, May, June), day of storage (day 1, 6) and all appropriate interactions. The variance/covariance structure used was unstructured. The error term used to test for treatment effects was boar nested within the diet x month interaction. Day of storage was considered the repeated measure. A month x day of storage interaction was present for motility ($P = 0.03$). Therefore additional analyses were conducted to evaluate differences among day of storage within month. Furthermore, analyses were conducted to evaluate differences among month within day of storage.

Sperm quality analysis for the density gradient centrifugation. Data for the density gradient analysis of the 45%, 45/90% and 90% layers evaluated sperm motility, progressive

motility, sperm morphology, cellular particles and percent of recovered sperm from each layer and were analyzed as a complete randomized design using analysis of variance procedures for repeated measures. Ejaculate was considered the experimental unit. The model included diet (negative control, inorganic selenium, organic selenium), month (January, March, May, June), layer (45%, 45/90%, 90%), day of storage (day 1, 6) and all appropriate interactions. The concentration of sperm measured by Sperm Vision™ was used as a covariate in the model for the motility analysis to account for differences in sperm concentrations in each layer. Similarly, the number of total sperm determined from the four hemocytometer counts was used as a covariate in the model for the morphology analysis to account for differences in sperm concentrations in each layer. The variance/covariance structure used was compound symmetry. The error term used to test for treatment effects was boar nested within the diet x month x density layer interaction. Day of storage was considered the repeated measure. A density layer x day of storage interaction was present for sperm motility ($P = 0.0004$), progressive motility ($P < 0.0001$) and morphology ($P < 0.05$). Therefore additional analyses were conducted to evaluate differences among layers within day of storage. A month x layer x day of storage interaction was present for the percentage of sperm recovered from the layers ($P < 0.0001$). Therefore additional analyses were conducted to evaluate differences among layers within month and day of storage.

Results

Selenium analysis

The levels of selenium in the three experimental diets can be found in Table 4.3. No detectable levels of selenium were found in the negative control diet. The inorganic selenium

and organic selenium diets contained 0.31 and 0.37 ppm of selenium, respectively. Table 4.3. also contains the levels of selenium in blood plasma and semen for the three diets. There was an effect of diet ($P = 0.003$) on the level of selenium in the blood plasma. Higher ($P = 0.003$) levels of selenium were found in the organic selenium diet compared to the negative control diet, while the inorganic selenium diet was not different ($P > 0.05$) from the organic selenium and negative control diets. There was no effect ($P = 0.18$) of diet on the level of selenium in the semen.

One boar from the organic selenium diet was removed from the statistical analysis due to medication received for lameness. The medication for the lameness was administered prior to the blood collection used for determining the levels of selenium in the blood. Since the medication was not administered to all boars, and the medication may have affected feed intake, it was decided to drop the boar from the analysis for selenium levels in the blood plasma. If the boar were included in the analysis for selenium levels in the blood plasma, the organic selenium diet would have been higher ($P < 0.03$) compared to the inorganic selenium and negative control diets, however, no effect ($P = 0.59$) would have been found for selenium levels in the semen.

Body weight and testicular measurements

Growing phase in nursery and finishing. There was an effect of time ($P < 0.0001$) on pen weight, average daily feed intake, gain to finish ratio, testicle area and testicle area to body weight ratio during the nursery and finishing phases (Table 4.4.). Generally speaking, there was an increase ($P < 0.05$) in pen weight, average daily feed intake and testicle area and a decrease in gain to feed ratio ($P < 0.05$) as the boars aged. The testicle to body weight ratio

decreased ($P < 0.05$) initially, but then increased ($P < 0.05$) as boars matured during puberty. Diet did not affect pen weight ($P = 0.6$), average daily feed intake ($P = 0.6$), gain to feed ratio ($P = 0.3$), testicle area ($P = 0.9$) and testicle to body weight ratio ($P = 0.9$) during the nursery and finishing phases.

Semen collection phase. There was an effect of month ($P < 0.0001$) on boar weight, testicle area and testicle area to body weight ratio during the semen collection phase (Table 4.5.). Boar weight and testicle area increased ($P < 0.05$) during each month of semen analysis. However, the testicle area to body weight ratios for January, March and May were lower ($P < 0.003$) compared to June. Diet did not affect boar weight ($P = 0.8$), testicle area ($P = 0.9$) and testicle to body weight ratio ($P = 0.3$) during the semen collection phase.

Ejaculate analyses

The ejaculate characteristics for semen volume, concentration and total sperm in the ejaculate during the semen collection phase are presented by month in Table 4.6. The diet x month interaction for the ejaculate characteristics was not significant ($P > 0.1$). The boars in the study were collected on a 1 time per week collection frequency in January, March and May and a high collection frequency of six collections in four days in June.

There was an effect of month on semen volume ($P = 0.02$), concentration ($P < 0.0001$) and total sperm ($P < 0.0001$). For semen volume, May was higher ($P = 0.01$) compared to January and June. Both semen concentration and numbers of total sperm in the ejaculate were higher ($P < 0.01$) in January, March and May compared to June. Dietary treatment did not affect semen volume ($P = 0.9$), concentration ($P = 0.5$) and total sperm ($P = 0.8$) (Table 4.7.).

Sperm quality analyses

Motility analysis. The boars in the study were collected on a 1 time per week collection frequency in January, March and May and a high collection frequency of six collections in four days in June. A month x day interaction ($P = 0.03$) was present for the sperm motility, therefore differences were evaluated among day of storage within month (Figure 4.1.). In January, sperm motility decreased ($P < 0.001$) from day 1 to day 6. There were no differences ($P > 0.05$) in motility between days 1 and 6 for sperm samples collected in March, May and June. Additional analyses were conducted to evaluate differences among month within days of storage (Figure 4.2). Both days 1 and 6 in June were lower ($P < 0.05$) for sperm motility compared to days 1 and 6 in January, March and May. Dietary treatment did not affect ($P = 0.8$) sperm motility (Table 4.8.).

A month effect ($P < 0.0001$; Figure 4.3.) and day effect ($P = 0.0009$; Figure 4.4.) were present for the sperm progressive motility, however the month x day interaction was not significant ($P = 0.5$). Sperm progressive motility was lower ($P < 0.001$) in June compared to January, March and May. Sperm progressive motility was lower ($P = 0.0009$) on day 6 compared to day 1 of storage post-collection. Dietary treatment did not affect ($P = 0.5$) sperm progressive motility (Table 4.8.).

Morphology analysis. There was no effect of diet ($P > 0.2$) on the sperm morphological parameters, except for the proximal cytoplasmic droplet ($P = 0.03$). Boars fed the negative control diet produced a higher ($P = 0.02$) percentage of sperm with a proximal cytoplasmic droplet compared to boars fed the inorganic selenium diet. Table 4.8. contains

the morphological parameters evaluated by dietary treatment. There was no effect of month ($P > 0.2$) or day of storage post-collection ($P > 0.3$) on sperm morphology.

Density gradient centrifugation analyses

Motility analysis. A density layer x day of storage interaction ($P = 0.0004$) was present for sperm motility (Figure 4.5.). On day 1, sperm motility was lower ($P = 0.0009$) in the 45% layer compared to the 90% layer, however, no differences in sperm motility were detected between the 45% and 90% layers on day 6 ($P = 0.7$). There was no effect of dietary treatment or month on motility for the density layers ($P > 0.4$).

There was a significant density layer x day of storage interaction ($P < 0.0001$) for sperm progressive motility (Figure 4.6.). On day 1, sperm progressive motility was lower ($P < 0.01$) in the 45% layer compared to the 90% layer, however, no differences in sperm motility were detected between the 45% and 90% layers on day 6 ($P = 1.0$). There was no effect of dietary treatment or month on progressive motility for the density layers ($P > 0.3$).

Morphology analysis. A density layer x day of storage interaction ($P < 0.05$) was present for all the sperm morphological parameters except abnormal heads (Table 4.9.). There was a higher ($P < 0.0001$) percentage of normal sperm in the 90% layer compared to the 45% gradient on day 1, but not on day 6 ($P = 0.14$). The reciprocal was true for the percentage of abnormal sperm in the 45% and 90% layers. The percentage of total attached cytoplasmic droplets sperm was higher in the 45% layer compared to the 90% layer on day 1 ($P< 0.0001$) but not day 6 ($P = 0.5$).

Percent of sperm recovered. The percentage of sperm recovered from each layer is presented by month, day and density layer due to a significant three way interaction ($P <$

0.0001; Figure 4.7.). A greater ($P < 0.001$) percentage of sperm were recovered from the 90% gradient compared to the 45% gradient on day 1, but not day 6 ($P > 0.05$) in January, March and May samples. No differences ($P > 0.05$) were found for the percentage of sperm recovered from the low and high density layers for the June analysis.

Cellular particles. Estimates for unattached cytoplasmic droplets and cellular debris are presented by density layer ($P < 0.0001$; Table 4.10.). The concentration of unattached cytoplasmic droplets and cellular debris was higher ($P < 0.0001$) in the 45% layer compared to the 90% layer.

Lipid peroxidation. A month effect ($P < 0.0001$) was present for the amount of malondialdehyde (MDA) produced per 375 x 10^6 sperm / mL (Figure 4.8.). The boars in the study were collected on a 1 time per week collection frequency in January, March and May and a high collection frequency of six collections in four days in June. There was a higher ($P = 0.01$) concentration of MDA in March and May compared to January. The concentration of MDA was lower ($P < 0.0001$) in June compared to January, March and May. The concentration of MDA was not affected by dietary treatment ($P = 0.4$) or day of storage post-collection ($P = 0.3$).

Sperm cell glutathione peroxidase activity. The effect of month ($P < 0.0001$) on sperm glutathione peroxidase (GPX) activity is presented in Figure 4.9. The sperm GPX activity was lowest ($P < 0.01$) in January, while sperm GPX activity in June was higher ($P < 0.05$) compared to January and March, but not May ($P = 0.06$). The sperm GPX activity was not affected by dietary treatment ($P = 0.8$).

Discussion

Previous research has shown that boars consuming a diet supplemented with selenium have better sperm quality compared to boars consuming a diet deficient for selenium (Marin-Guzman et al., 1997). Other research with selenium has shown that boars fed a diet containing organic selenium had better sperm production and sperm morphology compared to boars consuming a diet supplemented with inorganic selenium (Jacyno and Kawecka, 2002). It is thought selenium improves sperm quality through the GPX enzyme, which is a selenium containing enzyme that is associated with the male reproductive tract and sperm cells (Drevet, 2006). The GPX enzyme is an antioxidant and helps protect the sperm cell plasma membrane from damage due to lipid peroxidation via reactive oxygen species. Therefore, in theory, boars consuming a diet supplemented with an organic source of selenium should have improved sperm motility and morphology, and should demonstrate lower levels of lipid peroxidation compared to boars consuming diets not supplemented with selenium or supplemented with an inorganic source of selenium. It might also be expected that boars fed an organic selenium diet would also have better sperm quality when stored at hypothermic conditions in a liquid extender for a given period of time post-collection. Therefore, this study was conducted to evaluate the effect of dietary selenium on sperm production and sperm quality and to examine changes in sperm quality over a six day storage period post-collection.

In the present study, boars fed the organic diet had a higher level of selenium in the blood plasma compared to the boars fed the negative control diet. However, the inorganic diet was not different compared to the organic and negative control diets. Marin-Guzman et

al. (1997) fed boars a basal diet supplemented with 0.5 ppm of inorganic selenium from sodium selenite from the time of weaning until approximately 18 months of age. The basal diet had a level of 0.063 ppm selenium and was considered deficient for selenium. At 145 kg, boars on the supplemented selenium diet contained 0.225 ppm of selenium in the blood whereas the boars on the non-supplemented basal diet contained 0.033 ppm of selenium. In the present study, at a similar weight (182 kg) to the boars in Marin-Guzman et al. (1997), the level of selenium in the blood of the boars fed the negative control diet, inorganic selenium diet or organic selenium diet was 0.16 ppm, 0.18 ppm, and 0.21 ppm, respectively. The ability of Marin-Guzman et al. (1997) to achieve higher levels of selenium in the blood may be due to either the higher level of selenium supplementation or the deficient level of selenium in the non-supplemented basal diet. In the present study, the non-supplemented basal diet did not have detectable levels of selenium in the mash feed. However, detectable levels of selenium were found in the blood plasma of boars consuming the non-supplmented basal diet. From this observation, one might conclude that the non-supplemented basal diet was not deficient for selenium and was able to provide adequate selenium to the boars through the corn and soybean meal in the diet.

In agreement with the present study, Jacyno and Kawecka (2002) did not find differences of selenium levels in the blood of boars fed either an organic (blood selenium level = 0.359 ppm) or inorganic diet (blood selenium level = 0.345 ppm) diet supplemented at 0.2 ppm of selenium. The results of the present study also agree with Mahan and Parrett (1996), who found no differences in blood selenium levels for growing and finishing swine fed either an organic selenium diet or an inorganic selenium diet supplemented at 0.3 ppm.

In the present study, there also were no differences in selenium levels of the semen from boars on the different diets. These results disagree with Marin-Guzman et al. (1997), who found higher levels of selenium in the semen of boars fed the supplemented selenium diet (0.134 ppm selenium) compared to boars fed the non-supplemented basal diet (0.031 ppm selenium). A possible reason for this discrepancy is that the boars in the present study were supplemented at 0.3 ppm selenium, whereas the boars in Marin-Guzman et al. (1997) were supplemented at 0.5 ppm selenium. The results of the present study, however, are similar to Jacyno and Kawecka (2002) who also found no differences in selenium levels when measuring seminal plasma from boars fed either the organic selenium or inorganic selenium diet. Previous research had indicated that an organic source of selenium from selenium-enriched yeast (Mahan and Parrett, 1996) was more bio-available than an inorganic source of selenium due to results showing a higher level of selenium in loins of growing and finishing pigs. However, the results of this study and previous studies indicate the improved bio-availability of organic selenium over inorganic selenium demonstrated for muscle tissue may not be applicable to the boar reproductive tract. It may be that the reproductive tract strictly regulates the level of selenium in the testicle and the secondary sex glands. As a result, no differences in selenium levels would be detected in the semen.

There were no differences in ejaculate volume, sperm concentration and total sperm numbers for the boars on the dietary treatments. These results agree with previous research that also did not find differences for sperm production measures when supplemented with selenium (Segerson et al., 1981; Henson et al., 1983; Marin-Guzman et al., 1997). Results from Jacyno and Kawecka (2002), however, indicated that boars fed a diet containing

organic selenium had higher sperm concentration and total sperm numbers in the ejaculate compared to boars fed a diet containing inorganic selenium. A reason for this difference in results may be due to the reproductive ability for sperm production of the boars used in the trials. The boars in the present study were 64% higher for volume, 39% higher for sperm concentration and 149% higher for total sperm numbers compared to the boars used in Jacyno and Kawecka (2002). Perhaps the lower productivity of the boars used in Jacyno and Kawecka (2002) made them more responsive to the organic selenium diet compared to inorganic selenium diet. The boars used in both studies were evaluated for sperm production and quality at approximately the same age (approximately 190-300 d of age).

The dietary treatments did not affect sperm motility and sperm morphology at collection and after a 6 day storage period post-collection. Additionally, there was no dietary treatment x month interaction for the sperm quality measures, indicating the dietary treatments responded equally to the high collection frequency used in June. All three dietary treatments produced ejaculates with acceptable levels of sperm motility (> 80% motility and > 65% progressive motility). Likewise, all three dietary treatments produced a high percentage of morphologically normal sperm (> 80%) and a low percentage of sperm with attached cytoplasmic droplets (< 15%), tail abnormalities (< 2%) and head abnormalities (< 1%). These levels for sperm quality are considered acceptable in the swine industry for use in insemination programs (Flowers, 1997). It is surprising that the negative control diet did not have a lower sperm quality compared to the selenium supplemented diets. It appears that enough selenium was present in the corn and soybean meal of the basal diet to ensure adequate selenium to produce sperm of acceptable quality.

Sperm cell motility decreased from day 1 to day 6 of storage post-collection for the January analysis, however, a decrease in motility was not observed from day 1 to 6 for samples collected in March, May and June. Prior to storage, the sperm in the present study were diluted in a commercially available 5-day extender (Androhep). Other studies using 5-day and long-term (greater than 5-7 days) extenders have also reported that sperm cells are able to maintain motility over a six day storage period (Kuster and Althouse, 1999). The boars in the study were collected on a 1 time per week collection frequency in January, March and May and a high collection frequency of six collections in four days in June. The motility of sperm collected following the high collection frequency in June were lower on days 1 and 6 compared to the 1 time per week collection frequency used in January, March and May. This agrees with previous reports demonstrating a reduction in motility of sperm samples following a high collection frequency (Schilling and Vengust, 1987; Bonet et al., 1991; Frangež et al., 2004). It is interesting, however, that there was not a decline in motility from day 1 to 6 of storage for the samples collected following the high collection frequency in June. The lack of a decline in motility over the storage period is surprising since one would speculate that the high collection frequency would alter the sperm maturation in the epididymis and would intensify the stress on the sperm cell, leading to an inability to maintain similar motility on day 6 of storage compared to day 1 (Franca et al., 2005). Progressive sperm motility was affected by month and day of storage, but not a month x day of storage interaction. Similar to motility, progressive motility was not different for the analyses in January, March and May, but was lower in June, likely due to the high collection frequency. Progressive motility also declined from day 1 to day 6 of storage post-collection.

Density gradient centrifugation separates the sperm cells into sub-populations allowing investigation of the sperm quality within these unique sub-populations. Previous research investigating sperm from humans (Aitken et al., 1993), equids (Ball et al., 2001) and swine (Popwell and Flowers, 2004) indicated that sperm recovered from the high density layer are viable, highly motile and have a higher percentage of sperm with normal morphology compared to sperm recovered in the low density layer. The results of the present study agree with these previous reports. In the present study, sperm motility, progressive motility and morphology were higher in the 90% density layer compared to the 45% density layer on day 1 of storage post-collection. Interestingly, the sperm quality differences between the high and low density layers seen on day 1 of storage were not observed on day 6 of storage post-collection, as there were no differences between the 45% and 90% layers for sperm motility, progressive motility and morphology. Day of storage also affected the buoyant density of sperm cells over the storage period post-collection. In January, March and May, the percentage of sperm recovered from the 90% density layer was higher compared to the percentage of sperm recovered from the 45% density layer on day 1. In January and March, there were no differences in the percentage of sperm recovered from the 45% and 90% density layers on day 6. Interestingly, there were no differences in the percentage of sperm recovered between the 45% and 90% density layers for the June analysis. The reason for this observation is not known, but is likely due to the altered rate at which the sperm cells passed through the epididymis, which may affect their ability to mature prior to ejaculation.

As discussed previously, there was not a reduction in sperm motility over the 6 day storage period for 3 out of the 4 months evaluated. However, there was a decline in sperm

progressive motility over the 6 day storage period. This information suggests that sperm progressive motility is more important for determine if a sperm cell is able to penetrate the high density layer compared to motility since a reduction of progressive motility, but not motility, was observed over the storage period post-collection. These results indicate that evaluation of sperm for progressive motility may be of greater importance than evaluation of overall motility (Fraser, 1984). This data agrees with previous reports that suggest sperm cells separate through density gradients based on the velocity of their forward motility (Grant et al., 1994; Popwell and Flowers, 2004). However, sperm cell progressive motility is likely not the only factor affecting how sperm cells separate on density gradients. The sperm cell buoyant density also plays a role in determining whether a sperm cell can penetrate the high density layer. The failure of sperm to sediment through a 100% Percoll layer led Lessley and Garner (1983) to speculate that the buoyant density of sperm cells was less than 1.13 gm/ml (1.13 gm/ml being the calculated density of the 100% gradient). Therefore the observations in the present study suggest that on day 6 of storage, the buoyant density has been altered to prevent a greater percentage of sperm from penetrating the high density layer. Most likely the progressive motility and buoyant density properties of the sperm cell work synergistically to propel the sperm cell through the density gradients.

The factors affecting sperm cell progressive motility and buoyant density during storage post-collection are not known. Reactive oxygen species may affect the sperm cell through lipid peroxidation of the polyunsaturated fatty acids (PUFAs) in the sperm plasma membrane. Research has shown that boar sperm decreased in the percentage of PUFAs over a storage period of five days (Cerolini et al., 2000). Disruption of the plasma membrane from

lipid peroxidation could increase membrane permeability and thereby decrease the compactness of the sperm cell, leading to a decrease in buoyant density of the sperm. Research evaluating the effects of hydrogen peroxide on pulmonary artery endothelial cells found that damage to the structure and function of the plasma membrane manifested as a loss of membrane fluidity and increase in the leakiness of the plasma membrane. It was thought that lipid peroxidation was associated with this decreases in membrane quality (Block, 1991). However, in the present study there were no differences in the level of lipid peroxidation between days 1 and 6 of storage post-collection. Therefore, lipid peroxidation does not appear to play a role in altering the sperm cell during storage post-collection, and changes seen in progressive motility and buoyant density are probably caused by some other factor.

The GPX enzymes contain a post-transcriptionally incorporated selenocysteine residue (Chambers et al., 1986) and are important for combating the effects of reactive oxygen species and preventing the lipid peroxidation cascade in the sperm plasma membrane (Storey, 2008). Therefore, it was hypothesized that the boars receiving the negative control diet would have higher sperm lipid peroxidation levels and lower sperm GPX activity compared to the organic selenium and inorganic selenium diet boars since the negative control boars would have less selenium available for incorporating into the GPX enzyme. However, in the present study there was no effect of dietary treatment on sperm lipid peroxidation levels and sperm GPX activity. Nevertheless, a difference in the levels of lipid peroxidation and GPX activity due to month was detected. During the 1 time per week collection frequency, the levels of lipid peroxidation were higher in March and May compared to January, and then decreased significantly in June during the high collection

frequency. Interestingly, sperm GPX activity also increased in March and May compared to January, and then increased significantly during June. A reason for these responses is not known. The lower levels of lipid peroxidation and GPX activity in January might be indicative of an immature boar reproductive tract that is producing immature sperm with low levels of GPX activity and possibly low susceptibility to lipid peroxidation or low levels of reactive oxygen species activity. The increases in lipid peroxidation levels and GPX activity seen in March and May could signify the maturation of the boar reproductive tract.

The decrease in lipid peroxidation levels and corresponding increase in sperm GPX activity observed for the June analysis presents an interesting scenario. These results would seem to suggest that the increased activity of GPX prevented lipid peroxidation in the sperm cell plasma membrane, thereby leading to a reduced level of lipid peroxidation. However, this explanation is questionable, since previous research has shown an increase in lipid peroxidation levels following high collection frequencies in boars (Strezezek et al., 2000). Lower fertility has been observed for boars subjected to a high collection frequency (Bonet et al., 1991; Frangež et al., 2004). The decreased fertility is thought to be caused by reduction in sperm motility and increase in sperm abnormalities (Bonet et al., 1991). However, recent research has shown that low levels of reactive oxygen species may be important for proper sperm maturational events necessary for fertilization, such as capacitation, zona binding and acrosomal exocytosis for penetration of the oocyte zona pellucida (Asquith et al., 2005; O'Flaherty et al., 2006). Therefore, one might speculate that another cause for the reduced fertility seen in boars on a high collection frequency may be an increase in antioxidant activity that suppresses reactive oxygen species activity, and in doing so, also prevents the

low-level reactive oxygen species critical for sperm maturational events needed in successful fertilization. The increased levels of GPX activity in June may be responsible for the decreased levels of lipid peroxidation observed, but also may be responsible for the reduced fertility that would be expected had these boars been used for breeding purposes.

Conclusions

Feeding diets supplemented with 0.3 ppm of either organic selenium or inorganic selenium to boars from the time of weaning through semen collection resulted in a higher level of selenium in the blood for the boars consuming the organic selenium diet compared to the boars consuming the non-supplemented negative control diet. However, there were no differences between the dietary treatments for selenium levels in the semen, nor were there any differences in sperm production, motility, progressive motility, morphology, lipid peroxidation or GPX activity. Following density gradient centrifugation, sperm motility, progressive motility, morphology and the percentage of sperm recovered were higher in the 90% density layer compared to the 45% density layer on day 1, but not day 6 of storage post-collection. These data indicate that changes occur to boar sperm cells over a 6 day storage period post-collection that affect their ability to separate on a density gradient. The alterations in these sperm cells are likely due to changes in progressive motility and buoyant density, however, lipid peroxidation of the sperm cell plasma membrane does not appear to play a role. Further investigations into the mechanisms affecting sperm cell progressive motility and buoyant density over the storage period post-collection may help explain the decrease in boar sperm fertility seen over time post-collection.

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Table 4.1. Composition of experimental diets fed throughout the trial (as fed basis)

	Dietary phase[1]						
	Nursery 1	Nursery 2	Grower 1	Grower 2	Finisher 1	Finisher 2	Maintenance
Age of pig (days)	21-35	36-56	57-84	85-112	113-140	141-168	169-382
Ingredient, %							
Corn	47.780	54.752	69.960	74.830	77.910	77.910	84.300
Soybean meal, without hulls, solvent extract	16.520	32.950	23.260	19.450	16.500	16.500	12.250
Dried whey	20.000	5.000	-	-	-	-	-
Fish meal	5.000	-	-	-	-	-	-
Blood meal	1.500	-	-	-	-	-	-
Spray dried animal plasma	3.000	-	-	-	-	-	-
Fat	4.000	4.000	4.000	3.000	3.000	3.000	-
Monocalcium phosphate	0.763	1.472	0.900	0.900	0.850	0.850	1.550
Calcium carbonate	0.572	1.051	1.000	1.000	0.950	0.950	1.110
Zinc oxide	0.340	-	-	-	-	-	-
L-lysine-HCl	0.160	0.150	0.280	0.220	0.190	0.190	0.190
Salt	0.100	0.400	0.500	0.500	0.500	0.500	0.500
Copper sulfate	0.080	0.084	-	-	-	-	-
Starter vitamin[2]	0.020	0.020	-	-	-	-	-
Grower vitamin[3]	0.050	0.050	0.050	0.050	0.050	0.050	0.050
DL-methionine	0.060	0.021	-	-	-	-	-
Threonine	0.005	-	-	-	-	-	-
Trace mineral[4]	0.050	0.050	0.050	0.050	0.050	0.050	0.050
Total, %	100.000	100.000	100.000	100.000	100.000	100.000	100.000
Calculated analysis							
ME, kcal/kg	3450.00	3472.00	3509.00	3465.00	3469.00	3469.00	3306.00
Crude protein, %	21.06	20.96	17.12	15.66	14.48	14.48	13.00
Calcium, %	0.85	0.83	0.65	0.63	0.60	0.60	0.77
Phosphorus, %	0.76	0.73	0.55	0.53	0.51	0.51	0.65
Lysine, %	1.48	1.31	1.11	0.96	0.85	0.85	0.74
Methionine, %	0.41	0.34	0.27	0.26	0.24	0.24	0.23
Threonine, %	0.93	0.80	0.63	0.58	0.53	0.53	0.47
Tryptophan, %	0.26	0.26	0.19	0.17	0.15	0.15	0.13

[1]Inorganic Se (IS diet; Sodium selenite) or organic Se (OS diet; Se-enriched yeast - Sel-Plex 2000) was added to the non-supplemented negative control (CON diet) to achieve a level of 0.3 ppm Se in the final diet

[2]Starter vitamin premix provided 6.8 IU/kg Vit E, 4.4 ppm menadione, 0.1 ppm pyridoxine in the final diet

[3]Grower vitamin premix provided 1360.85 IU/kg Vit A, 136.09 IU/kg Vit D3, 6.8 IU/kg Vit E, 14.99 ppm Niacin, 12.49 ppm Pantothenic acid, 0.02 ppm Vit B12, 3.50 ppm Riboflavin in the final diet

[4]Provided 20 ppm Cu, 0.75 ppm I, 70 ppm Fe, 50 ppm Mn and 90 ppm Zn in the final diet

Table 4.2. Calculated consumption of minerals and vitamins for the boars during the semen collection phase.

Minerals	Calculated amount in diet	Calculated amount consumed per day for 2.0 kg[1]	Calculated amount consumed per day for 2.5 kg[2]
Copper, ppm	20.00	40.00	50.00
Iodine, ppm	0.75	1.50	1.88
Iron, ppm	70.00	140.00	175.00
Manganese, ppm	50.00	100.00	125.00
Zinc, ppm	90.00	180.00	225.00
Selenium, ppm[3]	0.30	0.60	0.75
Vitamins			
A, IU	1360.85	2721.70	3402.13
D3, IU	136.09	272.18	340.23
E, IU	6.80	13.60	17.00
Niacin, ppm	14.99	29.98	37.48
Pantothenic acid, ppm	12.49	24.98	31.23
B12, ppm	0.02	0.04	0.05
Riboflavin, ppm	3.50	7.00	8.75

[1]Boars were fed 2.0 kg per day from 154 to 277 d of age

[2]Boars were fed 2.5 kg per day from 278 to 382 d of age

[2]The basal diet did not contain any supplemental selenium

Table 4.3. Level of selenium measured in the dietary treatments, blood plasma and semen..

	Biological matrix containing selenium		
Dietary treatment[1]	Feed, ppm[2]	Blood plasma, ppm[3]	Semen, ppm[3]
Negative control	non-detectable	0.16 ± 0.008^{a}	0.12 ± 0.01
Inorganic selenium	0.31 ± 0.04	0.18 ± 0.008^{ab}	0.11 ± 0.01
Organic selenium	0.37 ± 0.06	0.21 ± 0.008^{b}	0.15 ± 0.01

[1]The negative control basal diet contained no supplemental selenium. The inorganic selenium and organic selenium diets were formulated to contain 0.3 ppm selenium

[2]The mash feed that was analyzed for selenium in each diet was from the batch fed for 1.5 months prior to the May blood plasma and semen analyses.

[3]The blood plasma and semen samples analyzed were collected during the May analysis period

[ab]Means within a column without a common superscript are different ($P < 0.05$)

Table 4.4. Effect of time on growth parameters during the nursery and finishing phases.

	Growth parameter[1]				
Dietary phase[2]	Weight, kg	Average daily feed intake	Gain:Feed	Testicle area, cm^2	Testicle area to body weight ratio
Weaning (21d)	7.03 ± 0.15^{a}	-	-	15.15 ± 0.50^{a}	2.16 ± 0.12^{a}
Nursery 1 (35d)	11.24 ± 0.29^{b}	0.36 ± 0.02^{a}	0.55 ± 0.02^{a}	18.60 ± 0.46^{b}	1.66 ± 0.05^{b}
Nursery 2 (56d)	23.07 ± 0.53^{c}	0.84 ± 0.03^{b}	0.64 ± 0.01^{b}	25.63 ± 0.74^{c}	1.12 ± 0.03^{c}
Grower 1 (84d)	45.25 ± 1.18^{d}	1.55 ± 0.05^{c}	0.51 ± 0.01^{a}	54.39 ± 1.69^{d}	1.20 ± 0.03^{c}
Grower 2 (112d)	72.71 ± 1.70^{e}	2.19 ± 0.05^{d}	0.45 ± 0.01^{d}	193.08 ± 9.02^{e}	2.66 ± 0.12^{d}
Finisher 1 (140d)	98.40 ± 2.31^{f}	2.56 ± 0.07^{e}	0.36 ± 0.01^{e}	419.45 ± 18.41^{f}	4.28 ± 0.18^{e}
Finisher 2 (154d)	108.45 ± 2.14^{g}	1.72 ± 0.01^{f}	0.42 ± 0.01^{d}	387.64 ± 12.22^{f}	3.59 ± 0.11^{f}

[1]Testicle area was calculated by taking the right testicle height multiplied by the width of both testicles

[2]Approximate age of boars at the end of each phase in parentheses

[a-g]Means within a column without a common superscript are different ($P < 0.05$)

Table 4.5. Effect of month on growth parameters during semen collection phase.

Growth parameter[1]	Month of semen collection[2,3]			
	January (228d)	March (277d)	May (347d)	June (382d)
Weight, kg	137.34 ± 2.93[a]	146.44 ± 2.94[b]	182.85 ± 2.87[c]	199.68 ± 3.17[d]
Testicle area, cm^2	536.39 ± 18.33[a]	612.61 ± 23.39[b]	766.70 ± 45.38[c]	1031.75 ± 65.27[d]
Testicle area to body weight ratio	3.91 ± 0.11[a]	4.19 ± 0.16[a]	4.19 ± 0.25[a]	5.15 ± 0.30[b]

[1]Testicle area was calculated by taking the right testicle height multiplied by the width of both testicles

[2]Approximate age of boars in parentheses

[3]Collection frequencies: January, March and May - 1 time per week; June - 6 collections in 4 days

[a-d]Means within a row without a common superscript are different ($P < 0.05$)

Table 4.6. Effect of month on ejaculate parameters.

Semen parameter[1]	Month of semen collection[2,3]			
	January (228d) 1 time per week	March (277d) 1 time per week	May (347d) 1 time per week	June (382d) 6 coll. in 4 days
Volume, mLs	160.47 ± 10.06[a]	189.33 ± 13.37[ab]	197.00 ± 11.86[b]	161.27 ± 11.46[a]
Concentration, x 10^6 sperm / mL	349.07 ± 18.67[a]	326.53 ± 18.06[a]	351.33 ± 19.36[a]	111.27 ± 8.57[b]
Total sperm, x 10^9 sperm	54.34 ± 2.80[a]	60.94 ± 5.14[a]	68.85 ± 5.21[a]	18.08 ± 1.81[b]

[1]Total sperm calculated by taking volume multiplied by concentration

[2]Approximate age of boars in parentheses

[3]Collection frequencies: January, March and May - 1 time/week; June - 6 collections in 4 days

[ab]Means within a row without a common superscript are different ($P < 0.05$)

Table 4.7. Effect of diet on ejaculate parameters.

	Dietary treatment		
Semen parameter[1]	Negative control	Inorganic selenium	Organic selenium
Volume, mLs	173.63 ± 13.38	175.89 ± 13.38	181.53 ± 13.38
Concentration, x 10^6 sperm / mL	286.40 ± 21.12	299.10 ± 21.12	268.15 ± 21.12
Total sperm, x 10^9 sperm	50.37 ± 5.37	52.54 ± 5.37	48.74 ± 5.37

[1]Total sperm calculated by multiplying volume by concentration

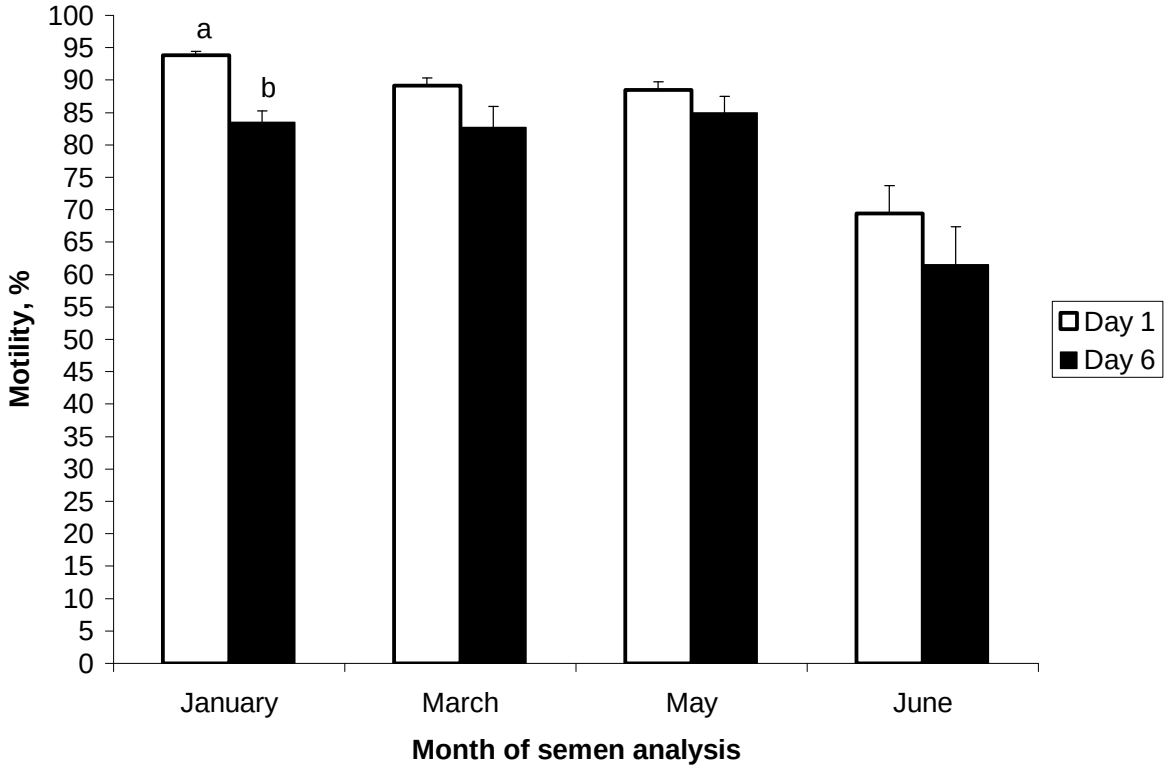


Figure 4.1. Effect of the day of storage for the percentage of motile sperm. The data were arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.
[ab]Means are different within month ($P < 0.05$)

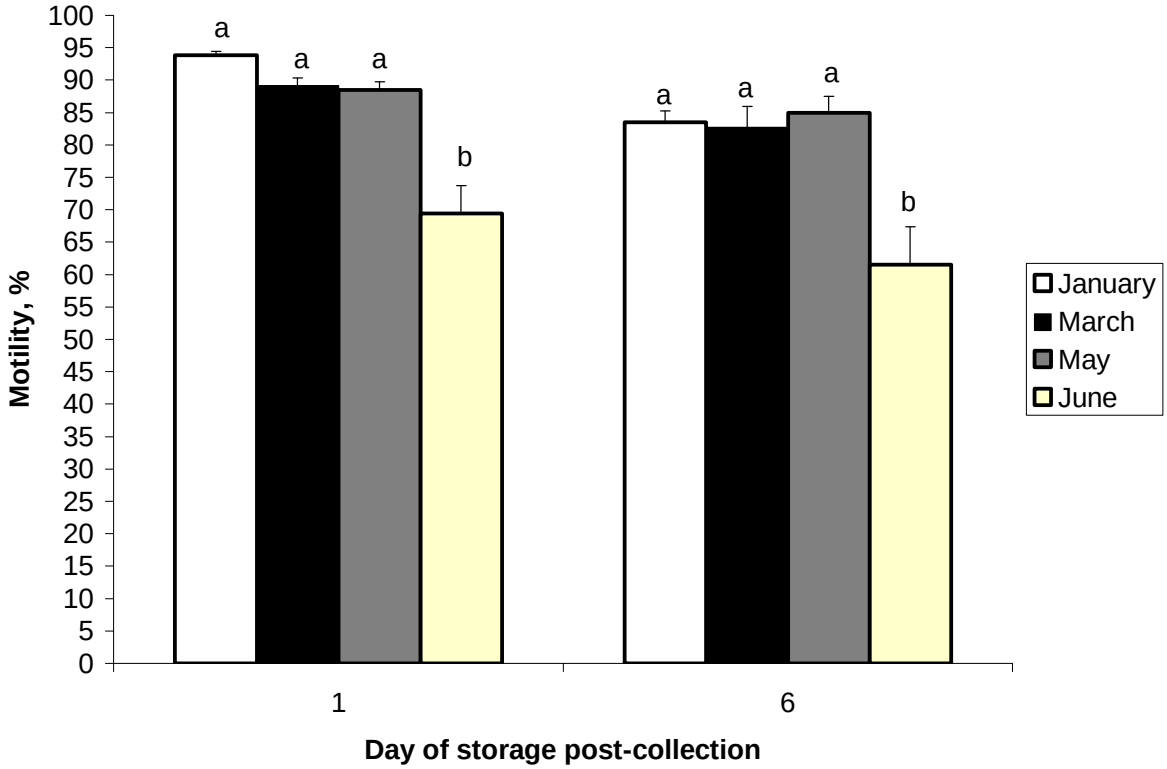


Figure 4.2. Effect of month for the percentage of motile sperm. The data were arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.
[ab]Means are different within day of storage ($P < 0.05$)

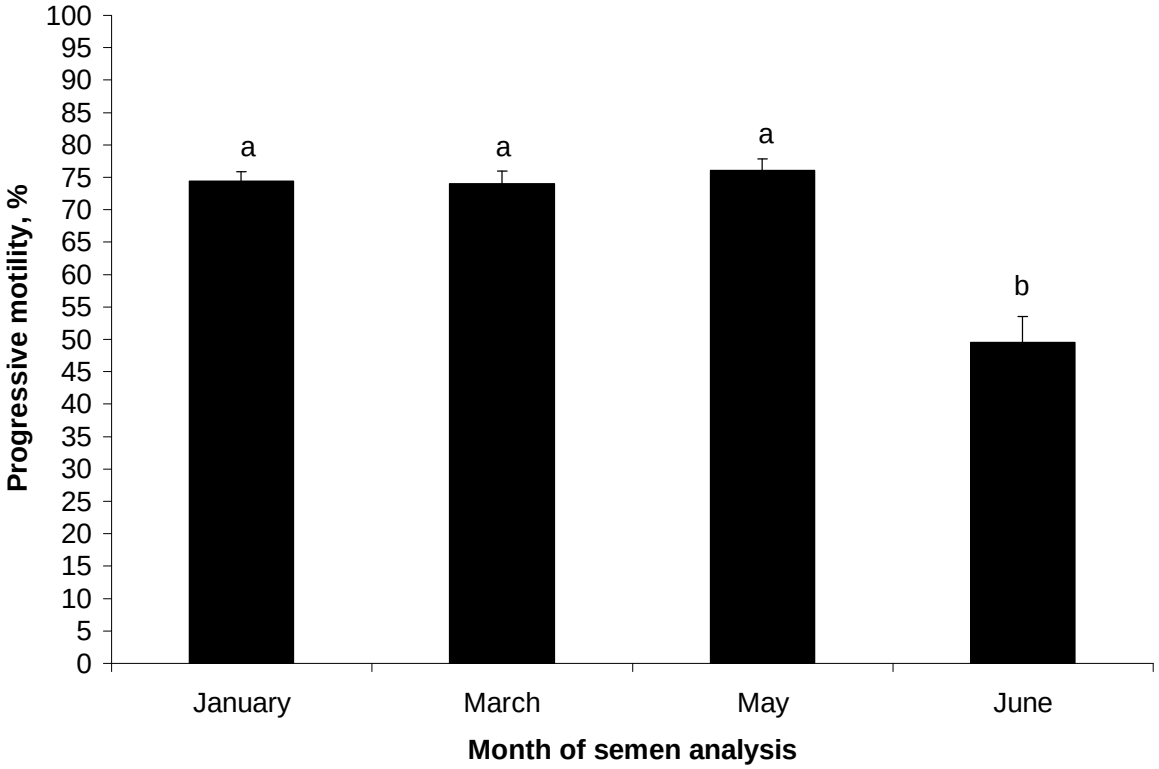


Figure 4.3. Effect of month for the percentage of progressively motile sperm. The data were arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.
[ab]Means without a common superscript are different ($P < 0.05$)

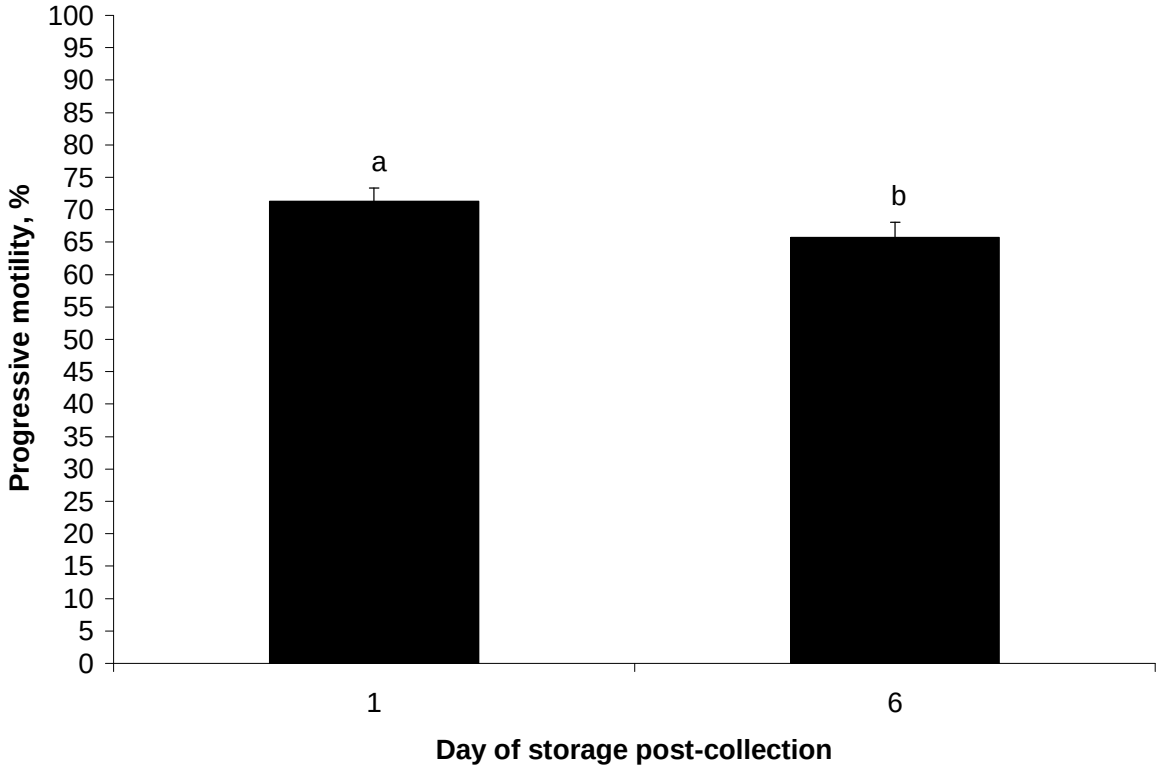


Figure 4.4. Effect of day of storage post-collection for the percentage of progressively motile sperm. The data were arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.
[ab]Means without a common superscript are different ($P < 0.05$)

Table 4.8. Effect of dietary treatment on sperm motility, progressive motility and morphological parameters.

	Dietary treatment		
Motility parameter, %	Negative control	Inorganic selenium	Organic selenium
Motility	81.15 ± 2.30	80.10 ± 2.57	83.92 ± 2.43
Progressive motility	67.60 ± 2.56	66.15 ± 2.90	71.79 ± 2.79
Morphological parameter[1], %			
Normal	83.34 ± 2.66	86.55 ± 2.52	90.50 ± 0.96
Proximal cytoplasmic droplet	8.70 ± 2.10[a]	1.93 ± 0.33[b]	3.55 ± 0.61[ab]
Distal cytoplasmic droplet	5.58 ± 0.83	4.94 ± 1.24	2.71 ± 0.32
Distal midpiece reflex[2]	0.49 ± 0.10	4.20 ± 1.14	1.32 ± 0.36
Total attached cytoplasmic droplets	14.76 ± 2.55	11.07 ± 2.26	7.58 ± 0.86
Abnormal tail	1.31 ± 0.22	1.84 ± 0.34	1.28 ± 0.17
Abnormal head	0.58 ± 0.11	0.54 ± 0.11	0.65 ± 0.14
Total abnormal	16.66 ± 2.66	13.45 ± 2.53	9.50 ± 0.95

The data was arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.

[1]Definitions: Total attached cytoplasmic droplets (proximal + distal + distal midpiece reflex); Total abnormal (total attached cytoplasmic droplets + abnormal tail + abnormal head)

[2]The distal midpiece reflex sperm contained a cytoplasmic droplet in the midpiece reflex

[ab]Means within a row without a common superscript are different ($P < 0.05$)

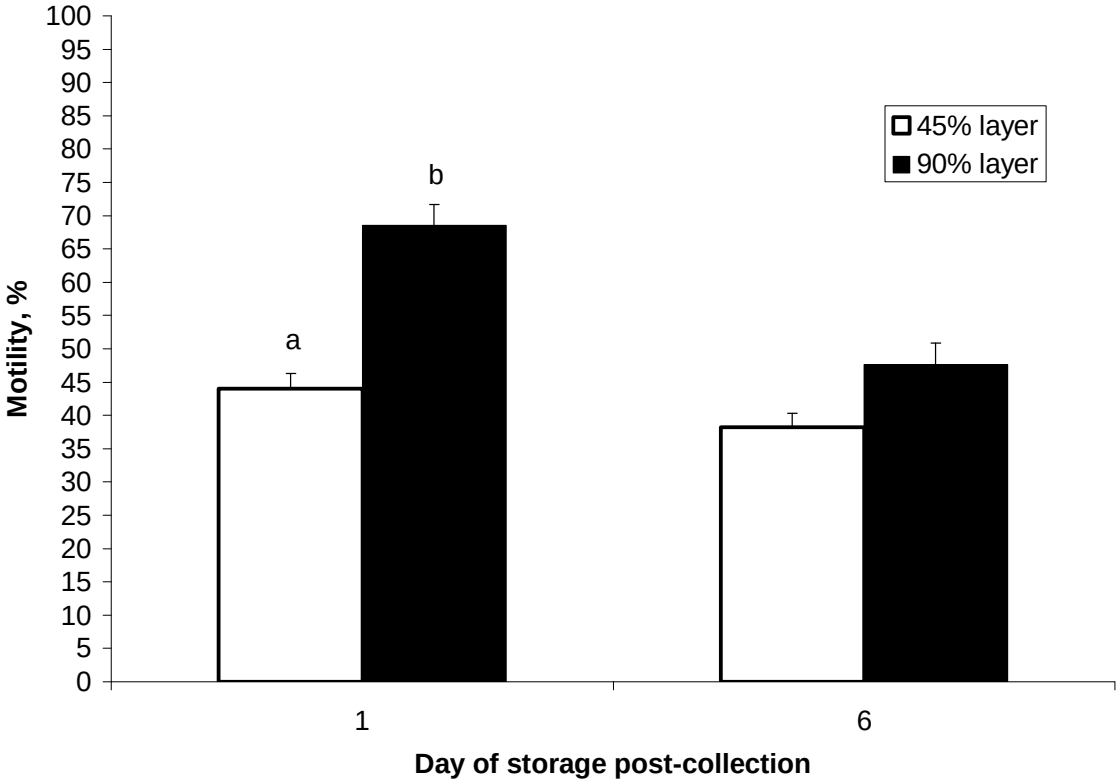


Figure 4.5. Effect of density layer for the percentage of motile sperm. The data were arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.

[ab]Means are different within day of storage ($P < 0.05$)

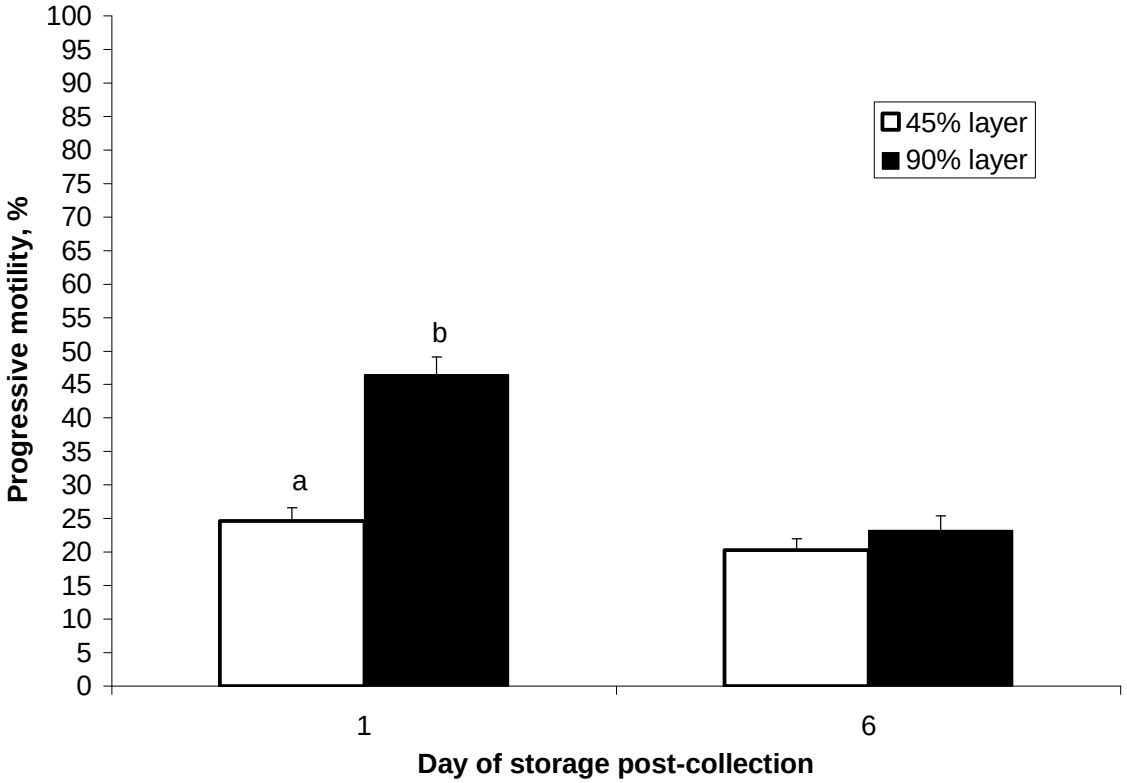
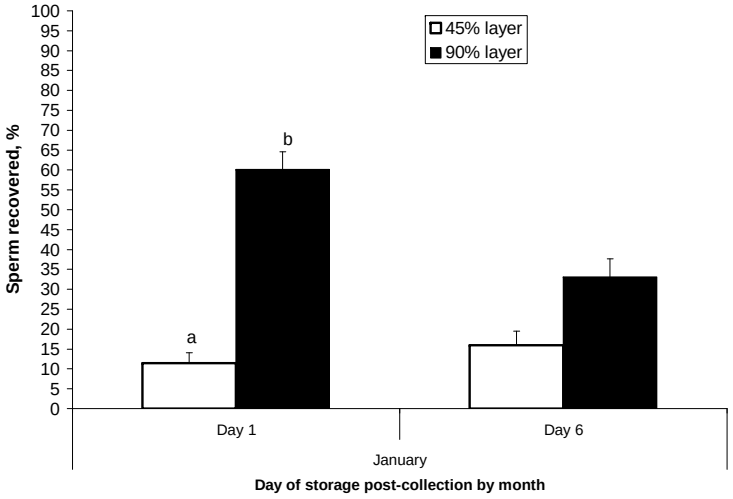
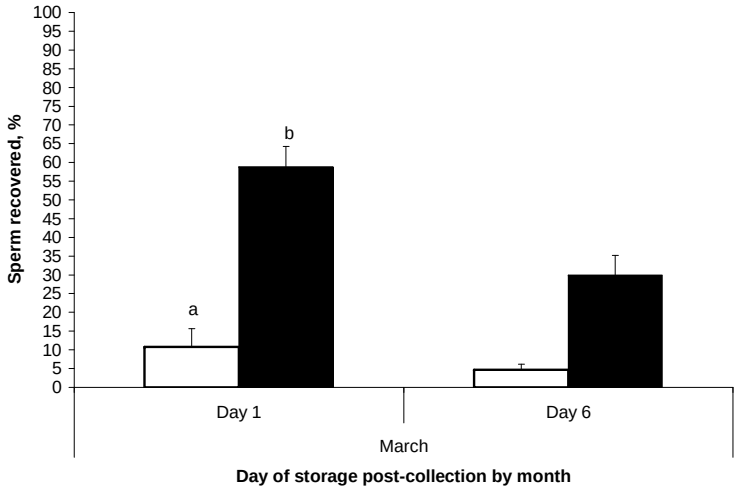
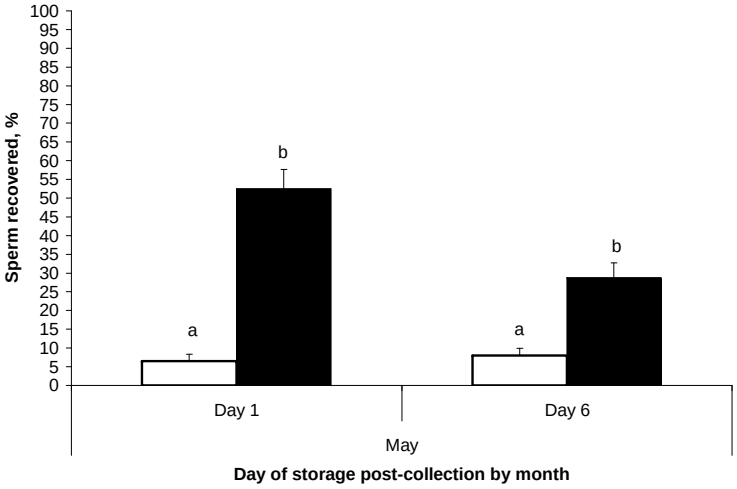


Figure 4.6. Effect of density layer for the percentage of progressively motile sperm. The data were arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.
[ab]Means are different within day of storage ($P < 0.05$)

Figure 4.7. Effect of density layer for the percentage of sperm recovered from the low and high density layers. The data were arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.
[ab]Means are different within day of storage and month ($P < 0.05$)


45% layer
90% layer
Sperm recovered, %
a
b
Day 1
Day 6
January
Day of storage post-collection by month


Sperm recovered, %
a
b
Day 1
Day 6
March
Day of storage post-collection by month


Sperm recovered, %
a
b
a
b
Day 1
Day 6
May
Day of storage post-collection by month

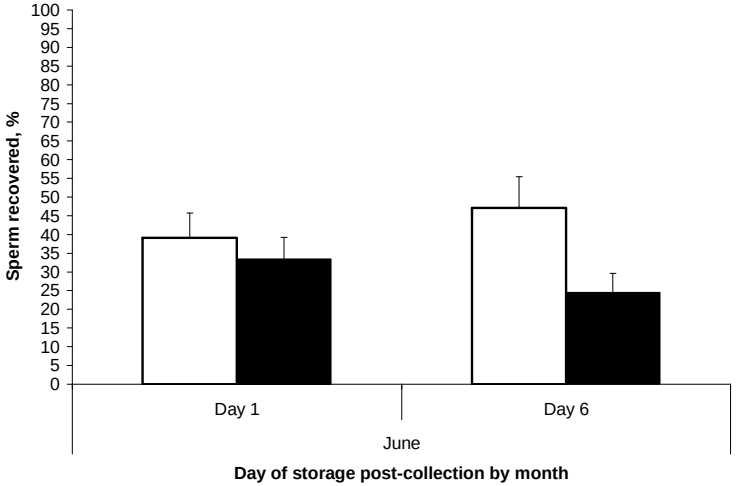

Sperm recovered, %
Day 1
Day 6
June
Day of storage post-collection by month

Table 4.9. Effect of density layer on the sperm morphological parameters.

	Day of storage post-collection			
	Day 1		Day 6	
Sperm morphological parameter[1], %	45% layer	90% layer	45% layer	90% layer
Normal	74.54 ± 2.12[a]	94.23 ± 1.48[c]	84.31 ± 1.65[b]	89.60 ± 1.81[b]
Proximal cytoplasmic droplet	5.98 ± 1.04[a]	2.23 ± 0.67[c]	4.57 ± 0.96[b]	3.75 ± 1.19[b]
Distal cytoplasmic droplet	12.24 ± 1.29[a]	1.84 ± 0.64[c]	5.88 ± 0.80[b]	3.70 ± 0.67[b]
Distal midpiece reflex[2]	2.64 ± 0.59[a]	0.73 ± 0.45[c]	1.80 ± 0.56[b]	1.66 ± 0.61[b]
Total attached cytoplasmic droplets	20.85 ± 1.95[a]	4.79 ± 1.36[c]	12.25 ± 1.56[b]	9.11 ± 1.74[b]
Abnormal tail	3.38 ± 0.45[a]	0.49 ± 0.10[b]	2.46 ± 0.41[a]	0.85 ± 0.12[b]
Abnormal head	1.24 ± 0.17	0.47 ± 0.07	0.98 ± 0.21	0.43 ± 0.07
Total abnormal	25.47 ± 2.13[a]	5.76 ± 1.48[c]	15.69 ± 1.65[b]	10.39 ± 1.81[b]

The data was arcsine transformed for statistical analysis. The data reported are arithmetic means and standard errors.

[1]Definitions: Total attached cytoplasmic droplets (proximal + distal + distal midpiece reflex); Total abnormal (Tot. att. cyto. drop. + abnormal tail + abnormal head)

[2]The distal midpiece reflex sperm contained a cytoplasmic droplet in the midpiece reflex

[abc]Means within a row without common superscripts are different ($P < 0.05$)

Table 4.10. Effect of density layer on the cellular particles.

	Density layer	
Cellular particle	45%	90%
Unattached cytoplasmic droplets / 100 sperm	92.21 ± 6.42[a]	1.81 ± 6.47[b]
Cellular debris[1] / 100 sperm	175.97 ± 15.29[a]	16.25 ± 15.35[b]

[1]Cellular debris included sloughed epithelial cells from the male reproductive tract, leukocytes, unidentifiable debris and broken heads and tails

[ab]Within row, means without a common superscript are different ($P < 0.05$)

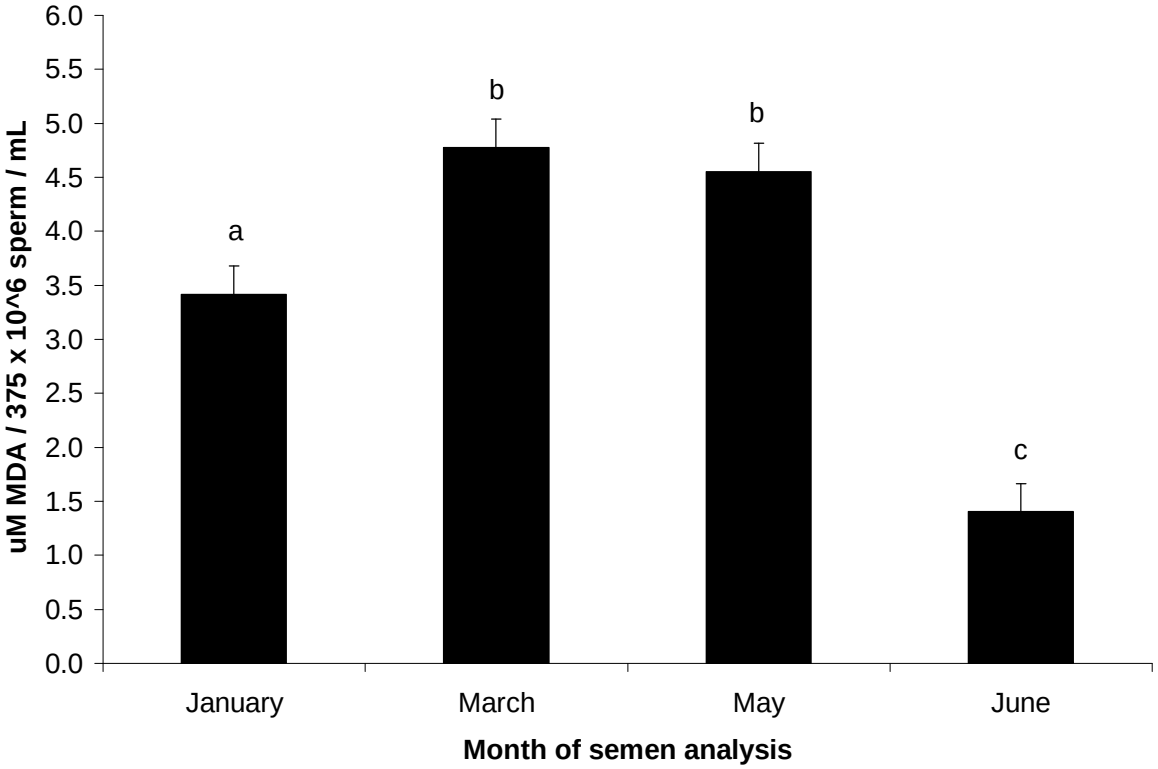


Figure 4.8. Concentration of MDA measured from sperm collected during the semen collection phase.
[abc]Means without a common superscript are different ($P < 0.05$)

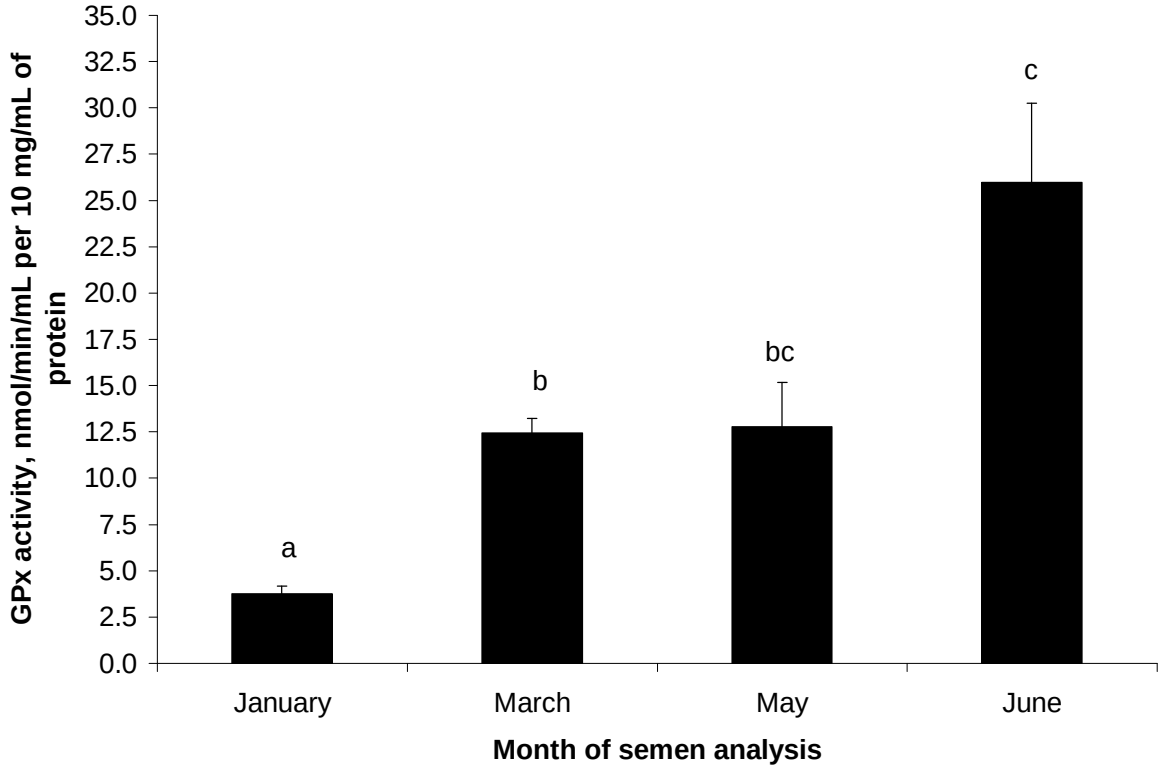


Figure 4.9. Sperm cell glutathione peroxidase (GPX) activity measured from sperm collected during the semen collection phase.
[abc]Means without a common superscript are different ($P < 0.05$)

CHAPTER 5

GENERAL CONCLUSIONS

Achieving a high level of reproductive efficiency is critical for success in the swine industry. The adoption of artificial insemination (AI) on sow farms in the United States has continued to increase due to its ability to maximize the number of piglets produced per boar ejaculate. Therefore, it is essential to the reproductive efficiency of modern swine production that research is performed to investigate factors that influence the fertility of the male gamete during the storage period post-collection. Logically, those factors that affect sperm quality between the time the semen is collected, diluted in a semen extender and inseminated are likely responsible for the decline in sperm quality and fertility over the storage period post-collection. The presence and activity of reactive oxygen species may be responsible for this decline. Therefore, the purpose of this research was to investigate factors that affect the presence and activity of reactive oxygen species in the porcine ejaculate and changes in sperm quality over storage time post-collection. Insight into the relationship of reactive oxygen species and mammalian sperm cells will help researchers better understand sperm cell physiology and livestock producers improve the reproductive efficiency of their operation by managing males for production of optimum sperm quality and/or use of extending media for storage of sperm cells prior to insemination.

In the first experiment, (chapter two), collection frequency, extender and storage time were examined to determine their effects on sperm quality and reactive oxygen species activity as measured by lipid peroxidation. The results of this study suggests that a high collection frequency reduces sperm motility, but does not affect sperm morphology or sperm

plasma membrane lipid peroxidation compared to a normal collection frequency. In addition, sperm membrane lipid peroxidation increases over a 7 day storage period in a short-term extender (BTS), but not a long-term extender (Androhep). Therefore, to ensure high quality AI doses, producers should use a normal collection frequency and use a long-term semen extender.

The results of the study reported in chapter three suggest that changes in sperm cell separation patterns using density gradient centrifugation over a 7 day storage period are affected by collection frequency and day of storage post-collection, most likely through alterations of the sperm properties progressive motility and buoyant density, but not through sperm membrane lipid peroxidation. These results indicate the changes that occur to sperm quality could be responsible for the decline in fertility observed during hypothermic liquid storage of boar sperm cells.

In chapter four, the effects of feeding a non-supplemented diet or diets supplemented with 0.3 ppm of either organic selenium or inorganic selenium to boars from the time of weaning through semen collection were examined to determine their effect on sperm quality. Although a higher level of selenium in the blood was found for boars consuming the organic selenium diet compared to boars consuming the non-supplemented negative control diet, no differences were found between the dietary treatments for selenium levels in the semen or sperm quality. Modifications in sperm separation patterns using density gradient centrifugation indicate that the change in separation patterns seen over storage time and are likely due to declines in sperm cell progressive motility and buoyant density. The factors

affecting these sperm property changes are not known, although lipid peroxidation does not seem to play a role.

Overall, reactive oxygen species activity as measured by lipid peroxidation appears to be most affected by the interaction of extender type used and the storage time post-collection, while collection frequency does not appear to have an effect. Changes seen in sperm cell separation patterns using density gradient centrifugation are likely affected by declines in sperm cell progressive motility and buoyant density over storage time, but do not appear to be a result of sperm membrane lipid peroxidation. These results indicate maintaining boars on a once a week collection frequency and using a long-term extender will prevent an increase in lipid peroxidation of sperm cells, however, changes in sperm cell separation patterns will still occur over the storage period post-collection. These changes in sperm cell separation patterns warrant further investigation to determine their effect on the decline in sperm cell fertility over the storage period post-collection.

APPENDICES

Appendix A

Validation of the MDA-586 assay kit

Spike and recovery experiments are used to determine whether the sample matrix (sample matrix is defined as either an undiluted biological sample or a mixture of the biological sample with a sample diluent) potentially interferes with the detection of the analyte. Spike and recovery is based on the difference between the diluent used to prepare the standard curve and the biological sample matrix.

The MDA-586 assay (OxisResearch, Portland, OR) detects the presence of a carbonyl compound, malondiadehyde (MDA), which is an end-product of the lipid peroxidation cascade. The MDA-586 assay kit was validated for boar sperm using a spike and recovery technique of known concentrations of the MDA standard into three samples of boar sperm stored in the long-term extender for 2 days. Processing of the sperm prior to analysis was performed as previously described. The recoveries (mean ± standard error) were 77.0 ± 2.5% for the low spike, 83.0 ± 3.0% and 89.5 ± 1.5%.

Table A.1. Spike and recovery of malondialdehyde (MDA) standard in three boar sperm samples. Samples were assayed by adding 75 µL of sample and 25 µL of spike stock solution calculated to yield the intended 0, 2.5 (low spike), 7.5 (medium spike) or 15 (high spike) µM of MDA. Recoveries for spiked test samples were calculated by comparison to the measured recovery of spiked diluent control. For example, the percent of MDA recovered for the low spike of sample B1 was calculated by taking the total MDA minus the no spike MDA and dividing that number by the low spike diluent control (3.30 – 1.63 = 1.67, then 1.67 ÷ 2.22 = 75.2%). Diluent for the diluent control and preparation of spike stock solutions was the same as the standard diluent. All samples were measured in duplicate. The recoveries were (mean ± standard error) 77.0 ± 2.5% for the low spike, 83.0 ± 3.0% and 89.5 ± 1.5%.

		Low spike - 2.5 uM			Medium spike - 7.5 uM			High spike - 15 uM		
			minus			minus			minus	
Sample	No spike - 0 uM	Total	no spike	% recovered	Total	no spike	% recovered	Total	no spike	% recovered
Diluent control	0.01	2.22	2.22	100.0%	6.41	6.41	100.0%	12.48	12.48	100.0%
B1	1.63	3.30	1.67	75.2%	7.33	5.70	88.9%	13.02	11.39	91.3%
B2	2.81	4.63	1.82	82.0%	7.89	5.08	79.3%	13.60	10.79	86.5%
B3	3.14	4.78	1.64	73.9%	8.32	5.18	80.8%	14.45	11.31	90.6%
	Mean recovery			77.0%			83.0%			89.4%
	Standard deviation			4.3%			5.2%			2.6%
	Standard error			2.5%			3.0%			1.5%

Appendix B

Validation of the Glutathione Peroxidase assay kit

Spike and recovery experiments are used to determine whether the sample matrix (sample matrix is defined as either an undiluted biological sample or a mixture of the biological sample with a sample diluent) potentially interferes with the detection of the analyte. Spike and recovery is based on the difference between the diluent used to prepare the standard curve and the biological sample matrix.

The GPX assay (Cayman Chemical Company, Ann Arbor, MI) determines activity indirectly by evaluating the oxidation of NADPH to $NADP^+$ at an absorbance of 340 nm. The activity of GPX in the sample is determined using the NADPH extinction coefficient that will cause the oxidation of 1 nmol of NADPH to $NADP^+$ per minute at 25ºC. The results for the assay were standardized by reporting sperm GPX activity (nmol/min/ml) per 10 mg/ml of sperm protein.

Table B.1. Spike and recovery of bovine erythrocyte GPX standard in three boar sperm samples. The GPX assay kit was validated for boar sperm using a spike and recovery technique of known concentrations of the positive control bovine erythrocyte GPX into three samples of boar sperm GPX. Samples were assayed by adding 15 µL of sample and 5 µL of spike stock solution calculated to yield the intended 0, 25% (low spike), 50% (medium spike) or 75% (high spike) of positive control. Recoveries for spiked test samples were calculated by comparison to the measured recovery of spiked diluent control. For example, the percent of GPX activity recovered for the low spike of sample B1 was calculated by taking the total GPX activity of the positive control minus the no spike GPX activity of the positive control and dividing that number by the low spike diluent control (29.93 – 12.23 = 17.70, then 17.70 ÷ 13.88 = 127.5%). Diluent for the diluent control and preparation of spike stock solutions was the same as the standard diluent. All samples were measured in duplicate. Processing of the sperm prior to analysis was performed as previously described above in the dissertation. The recoveries were (mean ± standard error) 128.1 ± 14.8% for the low spike, 104.9 ± 9.4% and 102.4 ± 7.8%.

		Low spike - 25% of positive control			Medium spike - 50% of positive control			High spike - 75% of positive control		
Sample	No spike - 0% of positive control	Total	minus no spike	% recovered	Total	minus no spike	% recovered	Total	minus no spike	% recovered
Diluent control	N.D.	13.88	13.88	100.0%	38.46	38.46	100.0%	48.52	48.52	100.0%
B1	12.23	29.93	17.70	127.5%	46.35	34.13	88.7%	57.94	45.72	94.2%
B2	12.48	33.87	21.39	154.1%	59.09	46.61	121.2%	69.79	57.31	118.1%
B3	13.88	28.14	14.26	102.8%	54.12	40.24	104.6%	59.98	46.10	95.0%
	Mean recovery			128.1%			104.9%			102.4%
	Standard deviation			25.7%			16.2%			13.6%
	Standard error			14.8%			9.4%			7.8%

Appendix C

Effect of hydrogen peroxide challenge on boar sperm separation using density gradient centrifugation

Objective: Evaluate the effect of hydrogen peroxide treatment on separation patterns of boar sperm cells using a two-step discontinuous density gradient of 45% and 90% iso-tonic percoll for density gradient centrifugation.

Materials and methods: Boar sperm samples were collected from four healthy mature boars housed at the Swine Education Unit (Raleigh, NC). Sperm samples were diluted to a concentration of 150 x 10^6 sperm / ml in a commercially available long-term extender (Androhep). The diluted sperm for each boar was split into two equal parts. One part was the untreated control, while the other was treated with hydrogen peroxide for a final hydrogen peroxide concentration of 600 μM in the sample. The samples were then laid flat in a semen cooler for 24 hrs. The following day the sperm samples were measured for motility prior to loading on the percoll gradients for density gradient centrifugation. The percoll gradients were prepared before centrifugation and evaluated after centrifugation as described previously in the dissertation. Motility was measured before centrifugation and in the gradients after centrifugation. Sperm concentration was determined in the gradients after centrifugation.

Results and discussion: Following a 600 μM hydrogen peroxide challenge for 24 hours in liquid storage (17-18ºC), a lower ($P = 0.005$) percentage of hydrogen peroxide challenged sperm were recovered in the 90% gradient (27%) compared to the percentage of sperm recovered in the 90% gradient of the control (67%). Although the buoyant density of the sperm cells may have been altered by lipid peroxidation in response to the hydrogen peroxide challenge, motility of the sperm cell also likely influenced the ability of the sperm cell to penetrate the 90% gradient. The motility and progressive motility of the hydrogen peroxide treated sperm cells before centrifugation was lower ($P < 0.02$) compared to the control sperm cells before centrifugation (71% vs 90% and 30% vs 46%, respectively).

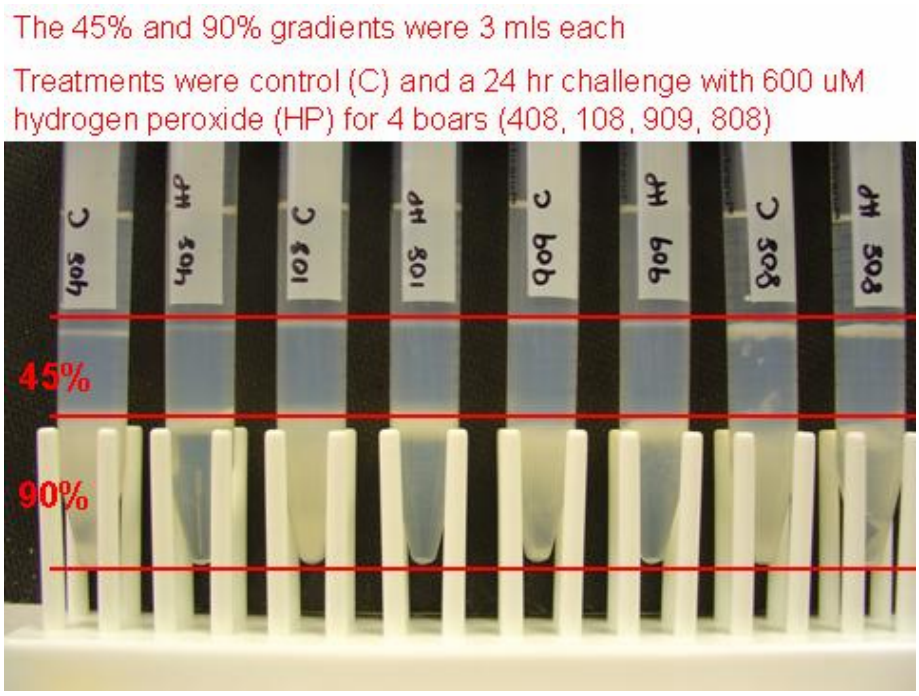


Figure C.1. Visual evaluation of the samples following 24 hour hydrogen peroxide challenge and density gradient centrifugation.

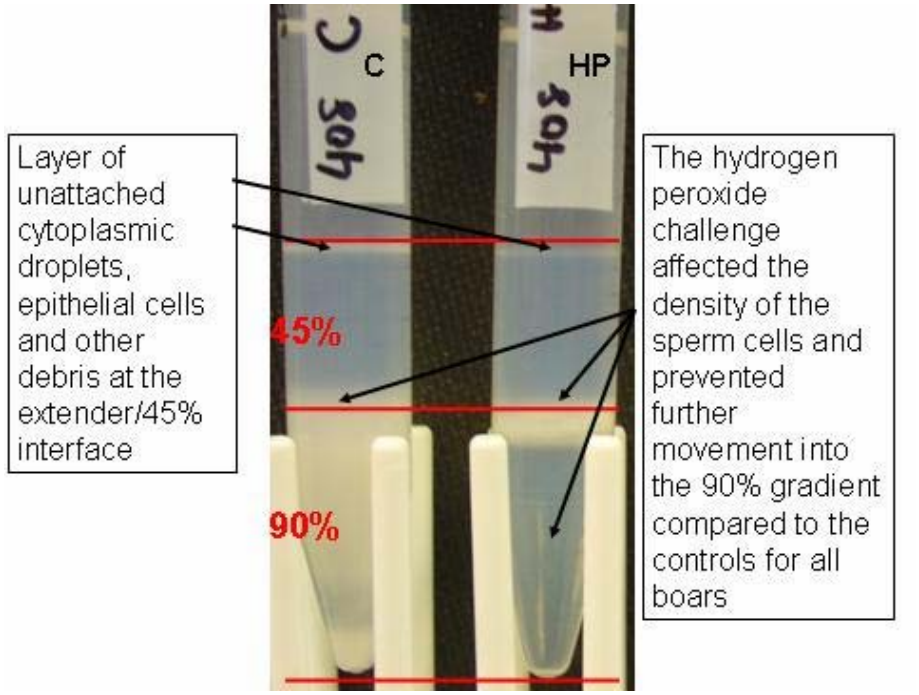


Figure C.2. Visual evaluation and description of separation characteristics of the samples following 24 hour hydrogen peroxide challenge and density gradient centrifugation.

Table C.1. Effect of 24 hour hydrogen peroxide challenge on sperm motility and progressive motility.

	Sperm motility parameter, %	
Treatment	Motility	Progressive motility
Control	90.10 ± 0.84[a]	45.63 ± 2.28[a]
Hydrogen peroxide	71.40 ± 5.71[b]	29.45 ± 4.27[b]

The data were arcsine tranformed for statistical analysis

The data reported are arithmetic means and standard errors

[ab]Within column, means without similar superscripts are different (P < 0.05)

Table C.2. Effect of 24 hour hydrogen peroxide challenge on percentage of sperm recovered from the 90% layer after density gradient centrifugation.

	Sperm recovered in 90% density layer		
Treatment	Sperm recovered, %	Motility, %	Progressive motility, %
Control	66.58 ±7.12[a]	86.90 ± 3.82[a]	62.03 ± 5.02[a]
Hydrogen peroxide	26.85 ± 4.96[b]	13.83 ± 2.86[b]	6.83 ± 2.03[b]

The data were arcsine tranformed for statistical analysis

The data reported are arithmetic means and standard errors

[ab]Within column, means without similar superscripts are different (P < 0.05)

Appendix D

Effect of day of storage on separation of boar sperm cells using density gradient centrifugation

Objective: Evaluate the effect of day of storage on separation patterns of boar sperm cells using a two-step discontinuous density gradient of 45% and 90% iso-tonic percoll for density gradient centrifugation.

Materials and methods: Boar sperm samples were collected from three healthy mature boars housed at the Swine Education Unit (Raleigh, NC). Sperm samples were diluted to a concentration of 75 x 10^6 sperm / ml in a commercially available long-term extender (Androhep). The diluted sperm for each boar was split into three equal parts for evaluation on day 0, 1 and 5 post-collection (day 0 = day of collection). The samples were then laid flat in a semen cooler for storage. The following day the sperm samples were measured for motility prior to loading on the percoll gradients for density gradient centrifugation. The percoll gradients were prepared before centrifugation and evaluated after centrifugation as described previously in the dissertation. Motility was measured before centrifugation and in the gradients after centrifugation.

Results and discussion: Although not expected, it could be possible that only 30 hours of storage may affect the separation patterns of sperm using density gradient centrifugation. The motility of sperm cells was higher ($P = 0.004$) in the 90% gradient compared to the 45% gradient on day 0 (day of collection) but not day 1 ($P = 0.056$), but not day five ($P = 1.0$). Motility of the whole sample (sperm not separated by density gradient centrifugation) was not different ($P > 0.6$) between days 0, 1 and 5 of storage. Progressive motility was higher in the 90% gradient compared to the 45% gradient on days 0 ($P < 0.0001$) and 1 ($P = 0.014$), but not day 5 ($P = 1.0$). Progressive motility of the whole sample (sperm not separated by density gradient centrifugation) was higher ($P = 0.004$) on day 0 compared to day 5.

Table D.1. Effect of day of storage on separation of boar sperm cells using density gradient centrifugation.

		Sperm motility parameter, %	
Day of storage	Sperm sample	Motility	Progressive motility
Day 0	Whole sample	87.43 ± 2.28	72.98 ± 3.29[y]
	45% gradient	36.25 ± 2.18[a]	14.22 ± 2.63[a]
	90% gradient	76.58 ± 9.04[b]	59.62 ± 11.90[b]
Day 1	Whole sample	83.67 ± 2.54	67.48 ± 3.65[yz]
	45% gradient	46.20 ± 4.34	20.27 ± 4.94[a]
	90% gradient	71.40 ± 8.09	51.72 ± 10.22[b]
Day 5	Whole sample	76.40 ± 4.91	47.77 ± 7.58[z]
	45% gradient	40.95 ± 5.75	18.73 ± 3.45
	90% gradient	43.23 ± 9.82	17.03 ± 6.60

The data was arcsine transformed for statistical analysis.

The data reported are arithmetic means and standard errors.

[ab]Within column, means are different within day between 45% and 90% gradient ($P < 0.05$)

[yz]Within column, means are different for whole sample ($P < 0.05$)

Appendix E

Effect of media on boar sperm motility over an incubation of 45 minutes

Objective: Evaluate the effect of media on boar sperm motility over an incubation of 45 minutes.

Materials and methods: Boar sperm samples were collected from three healthy mature boars housed at the Swine Education Unit (Raleigh, NC). Sperm samples were diluted to a concentration of 75 x 10^6 sperm / ml in a commercially available long-term extender (Androhep). The samples were then laid flat in a semen cooler for storage. The percoll gradients (45% and 90%) were prepared before centrifugation and partitioned after centrifugation as described previously in the dissertation. On day five of storage, the sperm samples were pelleted by centrifugation (800 x g for 5 minutes at room temperature) and then re-suspended in the appropriate treatment; either the 45% gradient, the 90% gradient or in Androhep (control). All media was at room temperature when the sperm were re-suspended and stayed at room temperature during the 45 minute analysis.

Results and discussion: There does not appear to be a toxic effect of percoll, as sperm cells suspended in either 45% or 90% density layers had similar ($P > 0.05$) motility and progressive motility to sperm cells suspended in Androhep extender over a 45 minute analysis period. Although treatment did not have an effect, there was an effect of time ($P < 0.0001$) in this analysis for both motility and progressive motility.

Table E.1. Effect of media on boar sperm motility over an incubation of 45 minutes.

		Sperm motility parameter, %	
Time	Treatment	Motility	Progressive motility
0 minutes	Androhep control	77.67 ± 7.36	53.20 ± 9.37
	45% gradient	65.43 ± 11.88	30.43 ± 10.26
	90% gradient	73.97 ± 9.30	34.93 ± 11.75
15 minutes	Androhep control	66.30 ± 8.11	43.67 ± 10.56
	45% gradient	64.17 ± 13.42	28.00 ± 11.03
	90% gradient	60.27 ± 12.53	29.17 ± 12.26
30 minutes	Androhep control	51.77 ± 10.88	22.57 ± 9.17
	45% gradient	47.50 ± 11.37	34.77 ± 12.80
	90% gradient	49.30 ± 16.08	23.43 ± 11.39
45 minutes	Androhep control	64.37 ± 10.36	37.57 ± 8.68
	45% gradient	49.70 ± 17.14	18.33 ± 9.84
	90% gradient	48.23 ± 18.32	21.67 ± 11.65

The data was arcsine transformed for statistical analysis.

The data reported are arithmetic means and standard errors.

Time effect for both motility and progressive motility ($P < 0.0001$)

Appendix F

Training boars to collect from a dummy sow

Boars trained to collect from dummy sow (chapter 4) were trained according to the follow experimental design. Eighteen boars were trained to collect from the dummy sow over a three week training period. The boars were allowed five minutes to interact with the dummy, mount, and collect during each training session. Training periods occurred every day (except during the weekend) until the boar was trained or the three week period ended. Boars were considered trained when semen was collected for three consecutive days. The training collection pen (2.43 x 3.65 m) held a dummy sow (0.3 m wide and 1.21 m long; Minitube of America, Inc., Verona, WI) bolted to a 1 inch plywood board with slats nailed to the plywood behind the dummy sow to provide traction to the boar while mounting. During the training period boars could move freely on either side and behind the dummy, but not in front. This was done to encourage the boar to mount the dummy when attempting to escape from beside the dummy as the technician could gently force the boar onto the dummy.

At 160 d of age, 15 boars were randomly selected (n = 5 per treatment) to be trained to collect from a dummy sow. Nine of the 15 boars were trained to the dummy after three weeks (n = 3 for negative control; n = 3 for inorganic selenium and n = 4 for organic selenium). At 190 day of age, another 8 boars (3 from negative control and inorganic selenium treatments and 2 from organic selenium treatment) were trained to collect. All eight boars were successfully trained. Following training to a dummy, the boars were randomly placed on a 1x per week collection frequency to be collected either Tuesday or Thursday.

During the training period for the eighteen boars, a group of eight boars were randomly selected to participate in a study to assess the effect of age of boar and dummy height on ability to train for semen collection to a dummy sow. This study was performed as part of the requirements for a undergraduate research honors project (see abstract below). The boars were trained to mount a collection dummy sow between 160 and 190 days of age at two different dummy heights. A 2 x 2 factorial design was used to assess the effects of low (47.5cm) versus high (53.5cm) dummy height on boars at 160 and 190 days of age. Boars were randomly assigned to treatments (n=4). The following six dependent variables were measured: 1) percentage of boars trained; 2) days needed to train; 3) libido score; 4) semen concentration; 5) semen volume; 6) total sperm. Boar libido was tested on a scale of 0 to 5 depending on amount of interest in the dummy: 0 – No interest or contact with the dummy during the training session 1 – Sniffs and rubs against the dummy but does not chew on, bite or charge it 2 – Aggressively interacts with the dummy – bites, chews on and charges it 3 – Mounts the dummy, but does not extend penis 4 – Mounts the dummy, extends penis, but does not collect 5 – Mounts the dummy, extends penis and is collected.

Abstract submitted for undergraduate honors research project

The objective of this study was to determine the effect of age and dummy height on training boars for semen collection and semen production. A 2 x 2 factorial design was used to assess the effects of low (47.5cm) versus high (53.5cm) dummy height on boars at 160 and 190 days of age. Boars were randomly assigned to treatments (n=4). The following dependent variables were measured: 1) percentage of boars trained; 2) days needed to train; 3) libido score; 4) semen concentration; 5) semen volume; 6) total sperm. Libido was tested on a scale of 0 to 5 depending on amount of interest in the dummy (0 - no interest, 5 - semen collection). Boars were considered trained when semen was collected for three consecutive days. There was a trend (P = 0.08) for dummy height in which boars were trained 75% of the time at the high setting compared to those that trained 100% of the time at the low setting. There was also a trend (P = 0.17) for age to affect the number of days needed to train; boars at 190d required 4.1d compared to 7.2d for the 160d boars. There were no differences (P = 0.4) for libido scores. As semen concentration was measured, a trend (P = 0.07) was found among age where boars at 190d had a higher concentration consisting of 196 x 10^6 sperm/mL vs 123 x 10^6 sperm/ mL for boars at 160d. Although there was no significance between amount of volume produced, results for total sperm in the ejaculate (concentration x volume) were significant (P = 0.04) for age where boars at 190d produced about twice as many sperm per ejaculate (30 billion) as those at 160d (16 billion). These data suggest that a low dummy setting should be used and that older boars will likely train faster and produce more semen.

Appendix G

Hydrogen peroxide assay

Objective: Evaluation of the Hydrogen Peroxide assay (Northwest Life Science Specialties, LLC, Vancouver, WA) for boar sperm.

Materials and methods: The ejaculate from a healthy, mature boar was collected and divided into three parts for evaluation. One part was kept as the neat ejaculate (355 x 10^6 sperm /ml), the other two parts were extended to a concentration of 37 x 10^6 sperm /ml in either BTS or Androhep. Analysis for the hydrogen peroxide assay was performed on 375 x 10^6 sperm /ml. Centrifugation (3600 rpm for 10 minutes at room temperature) was used to concentrate the sperm to the 375 x 10^6 sperm /ml concentration. On day 0 (day of collection), the hydrogen peroxide assay was performed for the neat ejaculate and the sperm extended in BTS and Androhep (sperm were measured approximately 45 minutes after extending). On day 7, the assay was performed on the extended sperm samples only. Samples were plated and absorbances were measured in a 96-well plate at a wavelength of 590 nm using a microplate reader.

The hydrogen peroxide assay is based on the oxidation of ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) by peroxides. The (Fe^{3+}) then forms a complex with an indicator dye an indicator dye xylenol orange which causes an increase in absorbance at 560-590 nm measurable as a purple colored complex.

Results and discussion: Figure G.1. contains the level of hydrogen peroxide in the neat ejaculate, and the BTS and Androhep extenders as measured on day 0 and day 7. The neat ejaculate was measured on day 0 only. The sperm extended in BTS had the highest levels of hydrogen peroxide on day 0. The hydrogen peroxide levels for the sperm extended in Androhep were non-detectable. The levels of hydrogen peroxide were non-detectable on day 7 for both BTS and Androhep. A spike and recovery assay was used to spike 50 uM of hydrogen peroxide into three sample matices, seminal plasma, BTS extender (no sperm) and the neat ejaculate (day 0 only). No hydrogen peroxide was recovered from the BTS extender or the neat ejaculate. On day 1, 62% of the spiked hydrogen peroxide was recovered, whereas 74% was recovered from the seminal plasma on day 7 (Figure G.2).

Conclusions: Due to the inability to detect hydrogen peroxide on day 7 of storage, poor recovery of hydrogen peroxide in different sample matrices and cost, it was decided to not use this assay in the studies.

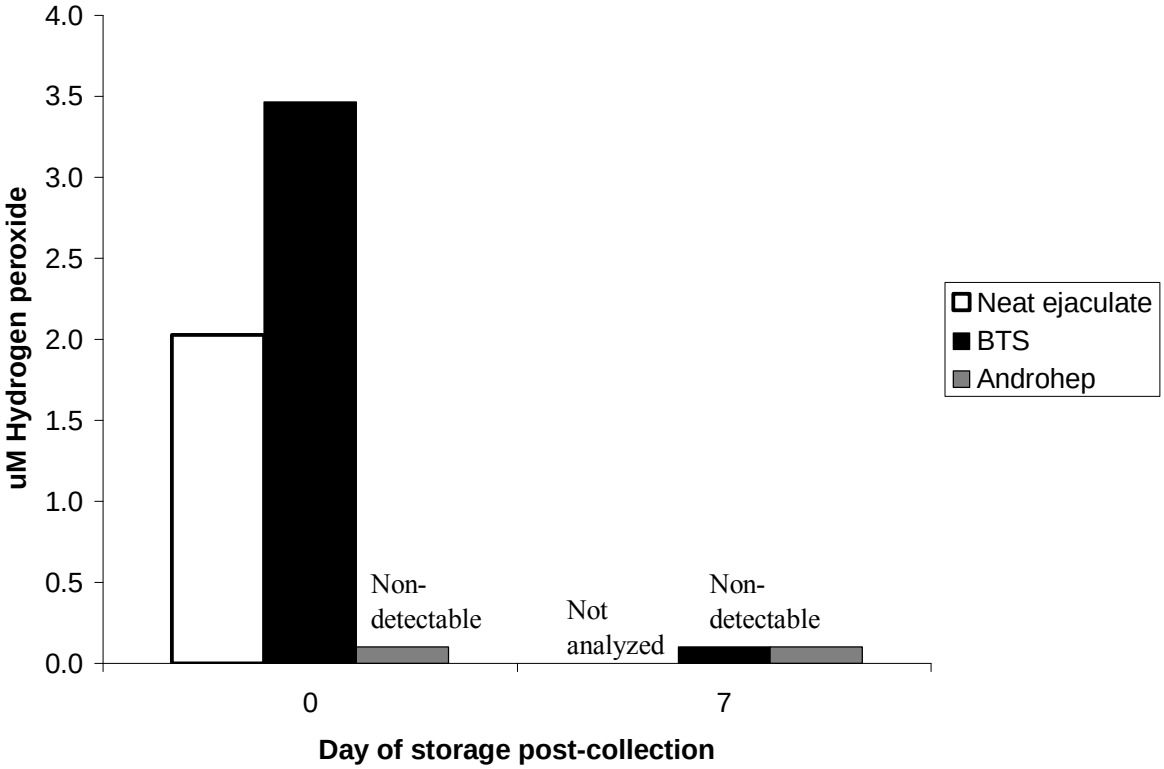


Figure G.1. Levels of hydrogen peroxide for sperm stored neat (no extender), in BTS and in Androhep extenders over 7 days of storage post-collection.

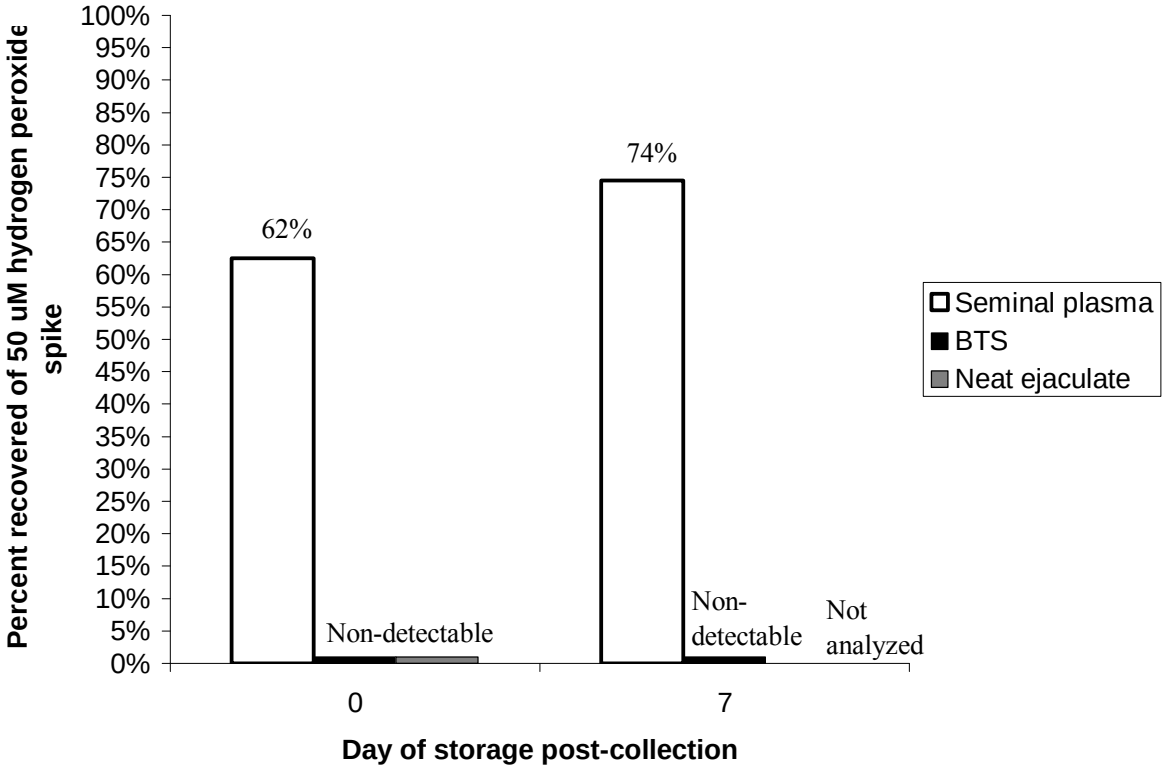


Figure G.2. Percent recovered of 50 uM hydrogen peroxide spiked into seminal plasma, BTS extender and neat ejaculate (i.e. ejaculate not extended).

Appendix H

Superoxide anion assay

Objective: Evaluation of the superoxide anion assay (LumiMax Superoxide anion detection kit, Stratagene, La Jolla, CA) for boar sperm.

Materials and methods: The ejaculate from a healthy, mature boar was collected and extended to a concentration of 37.5 x 10^6 sperm /ml in Androhep extender. On the day of analysis, the extended sperm diluted into concentrations for evaluation, 5, 10 and 15 x 10^6 sperm /ml as recommended by the assay instruction manual. For each concentration, the sperm was analyzed in three ways: 1) sperm in assay buffer containing luminol solution, enhancer solution and phorbol 12-myristate 13-acetate (PMA), 2) sperm and assay buffer minus the enhancer solution, and 3) sperm and assay buffer minus the PMA. Samples were plated and luminescence was measured in a 96-well plate at a wavelength of 590 nm using a microplate reader equipped with a 460/40 emission filter (the excitation wheel contained a plug) to read the sample at 416 nm at 30 second intervals for 2 minutes.

The superoxide anion assay is based on the principle that superoxide anion oxidizes luminol in a reaction that produces photons of light that are readily measured with a luminometer. The assay can be used in any preparation that contains a suspected generator of superoxide anions.

Results and discussion: No luminescence was detected using the superoxide anion assay. The reasons for the inability to detect results are unknown. The likely cause is the inability of the assay to identify superoxide anion due to the short half-life of this molecule.

Conclusions: Due to the inability to detect superoxide anion, and the fact that this assay determines superoxide anion qualitatively and not quantitatively, it was decided to not use this assay in the studies.