DISSERTATION

IMPROVING LONG-TERM STORAGE AND CRYOPRESERVATION OF BOAR SPERM USING ANTIOXIDANTS (GAMETEGUARD®) WITH NOVEL EXPERIMENTAL APPROACHES

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Myles Jon Shepherd

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Doctoral Committee:

Advisor: Jennifer Barfield
Co-Advisor: Gerrit Bouma

Clint Schwab
Lisa Herickhoff
Zach Weller

ABSTRACT

IMPROVING LONG-TERM STORAGE AND CRYOPRESERVATION OF BOAR SPERM USING ANTIOXIDANTS (GAMETEGUARD®) WITH NOVEL EXPERIMENTAL APPROACHES

In an industry where about 80% of the pork produced is the result of artificial insemination, the use of technologies to improve boar sperm preservation becomes of interest. Typically, cooled semen stored at 15°C to 17°C for up to seven days is used, although this leaves sperm vulnerable to the natural aging process and damage from reactive oxygen species (ROS). The high polyunsaturated fatty acid (PUFA) content of boar sperm is highly susceptible to lipid peroxidation and free radical formation which presents a challenge when processing sperm for cryopreservation [1, 2].

The first chapter of this dissertation will discuss the challenges of using semen technologies in the global pork industry as it relates to managing ROS generation and oxidative stress in sperm, as well as the role of antioxidants in mitigating oxidative damage. This lays the foundation for the research presented here which focuses on the impact of supplementing cooled aged boar semen with novel antioxidants and evaluating the impacts on sperm quality, conception rate, farrowing rate and litter size. The first chapter also provides background to the assessment of potential sublethal sperm modifications using mean fluorescent intensity (MFI). Moreover, the variability of boar sperm cryopreservation and tools used to screen the factors that impact the success of cryopreservation using the novel approach of the Plackett-Burman experimental design will be discussed.

To determine the impacts new technologies have on sperm fertilizing potential, a multi-assay approach is necessary to generate a full picture of sperm quality. Flow cytometry is a powerful tool for assessing quality of cooled stored boar sperm but is currently underutilized in routine assessment of insemination doses. Boar genetic centers that produce semen doses for artificial insemination largely rely on motility and morphology to determine potential fertilizing ability of sperm doses, yet motility may not detect subfertility. To fully utilize flow cytometers capabilities, multi-assays may be developed into one co-staining protocol coupled with evaluation of MFI. In addition, emerging capabilities of flow cytometers such as temperature-controlled stages equipped with plate readers enables rapid evaluation of many samples. The study performed in chapter two aimed to validate a 4-panel co-staining protocol utilizing the commonly employed 3-panel (SYBR-14, propidium iodide, PNA conjugated Alexa Fluor® 647) with and without MitoTracker® Orange. Through this investigation it was determined that no difference was detected in membrane permeability and acrosome integrity between the two panels described above, yet mitochondrial membrane potential evaluation was enabled. The staining protocols and evaluation temperatures were carried out at 17°C, 22°C, and 37°C and also assessed at 10, 20, 40, and 60 minutes. Findings in sperm quality assessment of membrane quality, acrosome intactness, and mitochondrial membrane potential demonstrated that cooled stored boar sperm were ideally stained and evaluated at 22°C for up to 60 minutes meaning there was no difference detected in sperm quality over time using this temperature ($P<0.05$). Finally, MFI of SYBR-14, PNA conjugated Alexa Fluor® 647, MitoTracker orange, and merocyanine 540 were assessed as potential subfertility markers by computing correlations between boar housing temperatures 55, 15, 10, 5, and 1 day prior to collection. MitoTracker® orange and merocyanine 540 MFI were both found to be potential indicators of subfertility. Taken together,

larger flow cytometry panels, provide researchers with more tools in one assay for determining potential subfertility and the panel studied in this chapter was subsequently utilized for the research performed in chapter three.

The use of artificial insemination with cooled stored boar semen accounts for 99% of porcine inseminations worldwide. The semen used to inseminate sows is typically stored between 15°C to 17°C with a shelf life of around 5 days leaving sperm exposed to oxidative damage. The research summarized in chapter three examines the use of GameteGuard®-CP (Membrane Protective Technologies, Inc. Fort Collins, CO), a natural plant derived blend of antioxidants, supplemented in one commercially available semen extender on sperm quality, conception rate, farrowing rate, and litter size. Ejaculates from 16 commercial Duroc boars were used in a split-ejaculate single-sire mating study executed over 10 weeks. Cooled stored sperm was evaluated for motility, viability, acrosome integrity, membrane stability, mitochondrial membrane potential, DNA intactness, and total antioxidant reactivity on both day 1 and 4 post collection. Sows (n=1476) were inseminated after synchronized ovulation using day 4 post-collected sperm extended in AndroStar Plus® or GameteGuard®-CP supplemented extender. On day 4 post-collection, treated sperm maintained viability, membrane stability, acrosome intactness, mitochondrial membrane potential, and DNA intactness ($P<0.01$) with GameteGuard®-CP treatments. Control doses demonstrated a decline from day 1 to 4 for all sperm parameters evaluated above ($P<0.01$). Conception rate and farrowing rate were significantly improved by 7.4% and 9.7%, respectively ($P<0.05$), but there was no difference detected between mean litter outcomes. The finding of the chapter three study demonstrates that antioxidant supplementation using GameteGuard®-CP maintains sperm quality over time,

thereby improving the shelf life of sperm which translates to improved conception and farrowing rates when using aged semen.

Cryopreservation of sperm is a routine technology in many livestock species, but not in swine. Frozen sperm must result in acceptable conception rates and produce 11-12 piglets/litter to be competitive with traditional cooled semen. The development of an extender that results in high post-thaw sperm quality and acceptable litter size requires identification of factors that markedly impact post-thaw sperm quality. The chapter four study aims to identify factors in boar sperm cryopreservation that significantly impacts post-thaw sperm quality using an efficient, cost-effective, and relatively rapid approach. The Plackett-Burman experimental design is ideal for the screening of factors at their extreme, greatly reducing the amount of time and resources needed for a follow-up, full factorial design. Using commercial semen, a 9 factor, 12 run Plackett-Burman design was used on 10 boars, two ejaculates per boar split between 12 treatments. Via this method, cooling rate, GameteGuard® supplementation, straw size, and sodium dodecyl sulfate were identified as highly influential factors that impact post-thaw sperm quality. Glycerol concentration, extender type, and starting osmolality were also influential for some but not all post-thaw sperm parameters ($P<0.05$). Equilibration time in the straws before freezing and stepwise addition of glycerol were determined to have no impact on post thaw sperm quality parameters. Using the Plackett-Burman design, it can be concluded that four of the nine factors warrant detailed investigation in a full factorial experiment to further develop a boar sperm cryopreservation extender that is competitive with cooled sperm.

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CHAPTER 1: LITERATURE REVIEW

1.1. Introduction

The commercial pork industry is largely a vertically integrated system. In this type of system, boars are kept in separate facilities from sows, and market hogs are moved out of the sow facilities after weaning to be finished in a separate offsite location. Vertically integrated systems are designed to reduce the spread of disease and minimize potentially catastrophic losses if disease outbreaks occur by maintaining animals with valuable genetics across multiple facilities. Because boars are kept isolated from the sows, the use of artificial insemination allows for the ejaculates of a smaller number of boars to be spread over many females. This has resulted in the development of a variety of technologies that aid in semen collection and processing.

Boars in stud facilities are trained to mount a collection dummy. This is a clean and safe method for semen collection as the dummies are disinfected between use. Once mounted on the dummy, the sperm is collected using the gloved hand technique or specialized collection devices designed to maintain pressure on the penis [3]. The spermicidal gel fraction is traditionally discarded before the sperm rich fraction is collected; however, collection bags have been developed to capture this gel fraction allowing the sperm rich fraction to drain into the collection bag. Because the ejaculation of boars can last up to 10 minutes, the semen being collected is often protected from cold shock by allowing the sperm rich fraction to pool in an insulated cup lined with a collection bag [3].

Computer assisted sperm analyzers (CASA) are widely used to assess sperm motility and morphology. There are industry standards for both motility and morphology of the sperm for it to be considered for further processing and distribution to sow farms [4]. Ejaculates are then

extended in a chemically defined medium, packaged into 75mL doses, then cooled and shipped at 15°C to 17°C to sow farms. Storing sperm at decreased temperatures slows down metabolic activity which aids in the preservation of cells over 3 to 5 days [5]. While the above is common practice, it necessitates the handling of sperm for collection, assessment, extension, packaging, and shipping which can impact sperm quality through induced sperm damage.

Pigs are polytocous and require a minimum number of fetuses for pregnancy to progress to term [6, 7]. Thus, multiple oocytes are ovulated over an extended period of time of about 2 hours which can occur within the 12 to 18 hour period of standing estrus [8]. This requires viable sperm to be present longer than monotocous species and results in sows receiving 2-3 insemination doses over a 24 hour period to insure that sperm is present for fertilization of many oocytes [8, 9]. This results in needing more sperm when inseminating sows (2 to 4 billion sperm/dose) compared to other livestock species such as bovine, caprine, and ovine (10 to 200 million sperm/dose) [10-12]. One way to potentially decrease the number of sperm per dose would be to use estrus synchronization or timed ovulation protocols followed by insemination, but these protocols are limited and often underutilized within the industry. Alternately, the shelf life of sperm can be increased so that sperm quality is maintained, and ROS induced damage is mitigated. This enables doses to be stored longer and allows for insemination when females are naturally coming into estrus.

Capacitation, the final sperm maturation step, must be prevented during processing and storage for sperm to retain fertilizing ability once in the female reproductive tract. If not prevented, premature capacitation and the subsequent acrosome reaction renders sperm non-viable. Even when diluted with commercial semen extenders, boar semen quality declines rapidly in the first 72 hours after collection and extension with respect to motility, acrosome

integrity, membrane quality, DNA integrity, and mitochondria membrane potential [13]. As a result, most cooled semen is used in the first 48 hours after semen collection and extension [14].

Boar sperm contains a high proportion of polyunsaturated fatty acids (PUFA) in the membrane relative to other species which are highly susceptible to lipid peroxidation leading to the production of the free radicals Malondialdehyde (MDA) and other aldehydes [15, 16]. Because of this, boar sperm experience an elevated incidence of DNA fragmentation from oxidative damage and loss of motility through exposure to reactive oxygen species (ROS). The decline in number of viable sperm over time, as well as the increased fragmentation of the DNA leads to decreased conception rates, pregnancy retention (farrowing rate), and litter size. Thus, the need to improve the shelf-life of extended semen by reducing oxidative damage during storage would decrease costs and allows boar studs to increase profitability per ejaculate.

An alternative to cooling semen is cryopreservation. The ability to cryopreserve semen provides several advantages including reduced disease transmission, genetic banking, and more precise timing of insemination with optimum female fertility. Although semen cryopreservation is routinely used in other livestock species, it is not popular in porcine due to poor cryotolerance. This is attributed to the elevated ROS production due to the high concentration and susceptibility of PUFA's to lipid peroxidation in the boar sperm membrane [1, 2, 17]. The increased oxidative stress also results in post-thaw sperm quality inconsistencies, poor fertilizing ability and decreased litter size [2] . Current studies with the use of frozen-thawed sperm not only demonstrate decreased farrowing rate of ~50% but also decreased number of piglets born per sow compared to cooled semen which is likely due to ROS generation during the cryopreservation and thawing process [2]. The current industry average litter size is 11.8 piglets/litter born using cooled semen but with frozen-thawed sperm this decreases to an average

of 9 piglets/litter and creates an economic disincentive [2]. This smaller litter size occurs despite higher numbers of cryopreserved sperm being used for insemination than when using cooled sperm, resulting in only ~20 doses/ejaculate versus ~40 doses/ejaculate using cooled stored sperm. For these reasons frozen-thawed semen is rarely used, yet the need for a viable frozen boar sperm technology is regularly discussed within the industry. An effective and easily reproducible method for freezing boar sperm would allow farms to further reduce disease transmission and enable them to continue operating and breeding in the face of a disease outbreak. Furthermore, genetics can be banked, and genetically valuable boars can potentially be resurrected.

Sperm and seminal plasma come equipped with a natural repertoire of antioxidants; however, they are diluted when processing semen for cooled storage and largely removed through centrifugation when processing for cryopreservation. In addition, there is variation in antioxidant concentrations between boars and between an individual boar's ejaculates across seasons [4, 18]. Because of this, supplementing boar semen extenders with antioxidants becomes an ideal solution to protect sperm from oxidative stress while restoring the natural balance of production and reduction of ROS. To better understand the benefits of antioxidant supplementation, it is necessary to understand the mechanisms of ROS.

1.2. Reactive Oxygen Species:

Reactive oxygen species can be both free radicals and non-free radicals derived from both endogenous and exogenous sources. Free radicals are defined as molecules that have one or more unpaired electron(s) in the shells surrounding the atomic nucleus. This odd number of electron(s) make free radicals markedly unstable, short lived, and reactive. Endogenous sources of ROS are normal byproducts of cellular metabolism in which increased amounts of oxygen are

consumed [19, 20]. Exogenous sources of ROS, that come in contact with ejaculated sperm, include exposure to pollutants, alcohol, heavy metals, transition metals, and radiation [19]. These contaminants are typically and inadvertently introduced by mishandling of samples post collection and from poor quality water sources used for extender preparation. Other sources of ROS may include mechanical damage of sperm such as agitation of samples during transport and improper temperature storage [21].

Perhaps the most important sources of endogenous ROS are free radicals derived from oxygen during metabolic reactions. An oxygen molecule, in itself, is a free radical due to the presence of unpaired electrons. Oxygen also forms the base for other free radicals which includes superoxide ($O_2^{\bullet -}$), oxygen radical ($O_2^{\bullet\bullet}$), hydroxyl ($OH^{\bullet}$), alkoxy radical ($RO^{\bullet}$), peroxyl radical ($ROO^{\bullet}$), nitrogen monoxide ($NO^{\bullet}$), and nitrogen dioxide ($NO_2^{\bullet}$). The unpaired electrons found among free radicals make them highly reactive which enables them to donate or extract elections from the nearest stable molecule to achieve stability. Non-radical ROS include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hyopbromous acid (HOBr), ozone (O_3), singlet oxygen ($^1O_2$), nitrous acid (HNO_2), nitrosyl cation (NO^+), nitroxyl anion (NO^-), dinitrogen trioxide (N_2O_3), dinitrogen tetraoxide (N_2O_4), nitronium cation (NO_2^+), organic peroxides (ROOH), aldehydes (NCOR), and peroxynitrite (ONOOH). While the above are not free radicals, they are precursors that may be converted to free radicals.

Superoxide is the most ubiquitous radical derived from electron exchange with oxygen. The majority of superoxide is produced by the mitochondria of cells. Within the electron transport chain, superoxide can be produced by NADH dehydrogenase (complex I) and ubiquinone cytochrome c reductase (complex III) [19, 22]. Superoxide production by complex I starts by transferring electrons to ubiquinone (Q) and is ultimately reduced to ubiquinol (QH_2) at

the ubiquinone reduction site (Figure 1.1A). Complex I can produce superoxide in both the flow and reverse directions of the electron transport chain; however, far more superoxide is produced in the reverse direction due to production of semiquinone in the quinone binding region of complex I [22].

Complex III reduces QH_2 produced by complex I then transfers elections to cytochrome *c* and cytochrome *c* oxidase which will be further reduced to water. This occurs by means of the Q-cycle described by Osyczka [23]. Semiquinone anion ($^{\bullet}Q^{-}$) produced in the Q-cycle will remove a single election from oxygen to give rise to superoxide (Figure 1.1B). This final step in superoxide production is non-enzymatic and links the production of ROS to metabolic rate. As a result, the high reactivity of newly formed free radicals leads to a cascade of free radical formation that consequently causes oxidative stress through electron extraction from the nearest stable molecule.

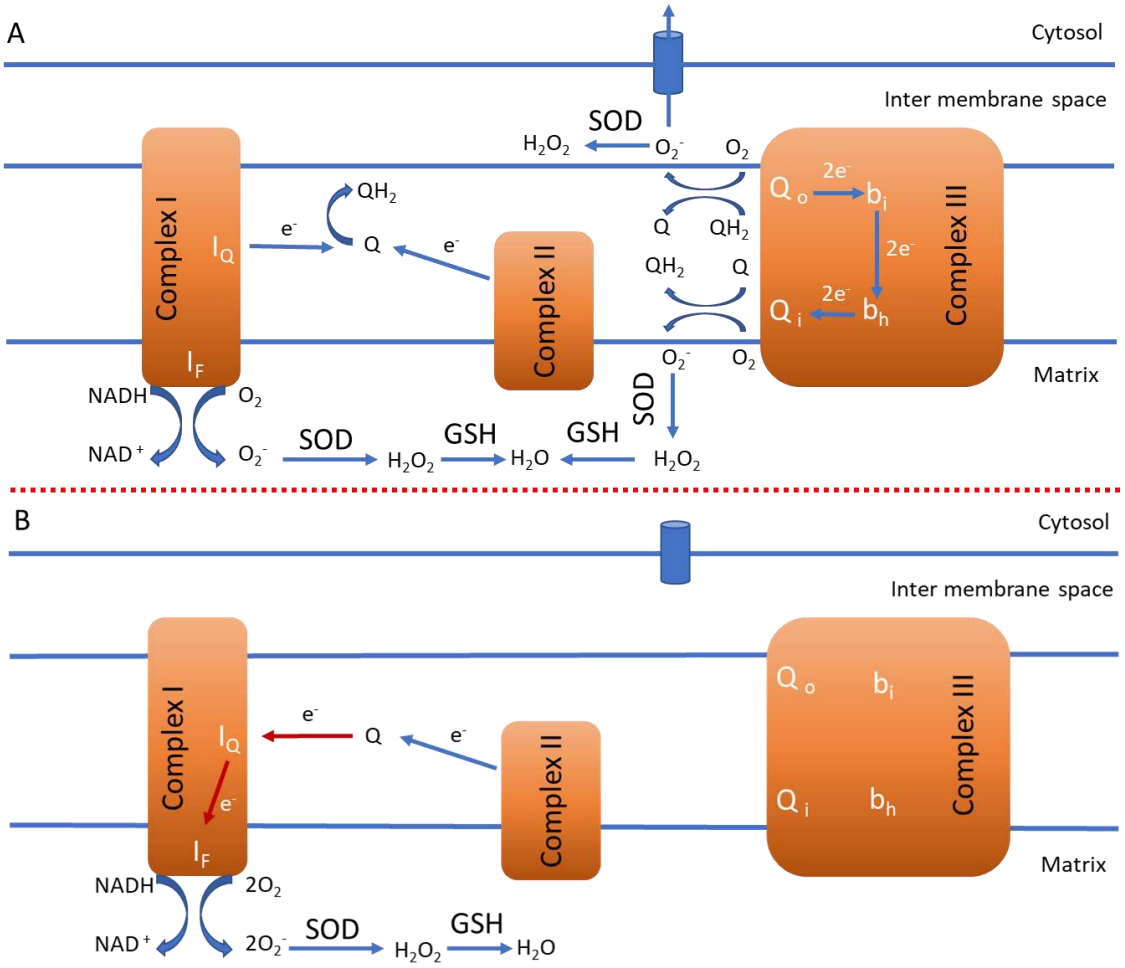

Figure 1.1: Superoxide (O_2^-) production by the electron transport chain where image A is the production of O_2^- in the forward direction and B is the in the reverse direction. When NADH is reduced to NAD^+ in the forward direction, elections are released and O_2 is converted to O_2^-. Electrons are also moved from complex II and the Q-binding site (I_Q) of complex I converting ubiquinone (Q) to ubiquinol (QH_2) which is then reduced back to Q by complex III. The electrons transferred from QH_2 by means of Q_O binding site are transferred to low cyt b heme (b_i) then the high cyt b heme (b_h) and the Q_i binding site to convert Q back into QH_2 in a process known as the Q-cycle. This process initiates the production of O_2^- in both the matrix and the inter membrane space of the mitochondria. In the reverse direction, Q becomes overwhelmed with elections, forcing the elections back to I_Q and the flavin mononucleotide (I_F) which leads to the production of two O_2^- molecules. The O_2^- can be reduced to H_2O_2 by superoxide dismutase (SOD) and further reduced to H_2O by Glutathione peroxidase (GSH).

1.3. ROS Sources:

Morphologically abnormal and immature sperm cells in vitro are known to cause overproduction of ROS [16, 24, 25]. One morphological abnormality that causes problems are

cytoplasmic droplets. These cytoplasmic droplets are present in all spermatozoa following spermiation into the seminiferous tubule. The droplets move down the midpiece as the spermatozoa transverse through the epididymis until they are eventually lost. Thus, sperm with excessive proximal and distal cytoplasmic droplets are considered to be immature cells. These cytoplasmic droplets initiate the production of NADPH by the rate limiting enzyme glucose-6-phosphate dehydrogenase (found inside the cytoplasmic droplet) in the pentose phosphate pathway [24, 26]. The cascade of events surrounding the pentose phosphate pathway can also activate membrane bound NADPH oxidase which catalyzes the production of superoxide by extracting electrons from NADPH.

The presence of leukocytes in the seminal plasma is another source of ROS production [27]. Polymorphonuclear leukocytes and macrophages found in seminal plasma are considered to be peroxidase positive leukocytes which are derived from the prostate and seminal vesicles [27]. Peroxidase positive leukocytes generate ROS by means of membrane bound NADPH oxidase activation [24]. Furthermore, stress and active infections while sperm are traversing through the epididymis increases proinflammatory cytokines resulting in increased peroxidase positive leukocytes present in seminal plasma. This generation of ROS eventually overwhelms these intra- and extracellular sources of antioxidants leaving sperm exposed to oxidative stress.

1.4. Oxidative damage:

Damage to lipids by ROS occurs through lipid peroxidation and is accompanied by loss of healthy membrane function. This ROS induced damage can manifest in alterations of membrane fluidity, inactivation of membrane bound enzymes, inactivation of receptors and increased permeability to ions [19]. Phospholipids contain PUFA's that are prone to lipid peroxidation from free radicals in a self-propagating reaction. Lipid peroxidation can be

described as a two-step process of initiation and propagation. In the initiation step, free radicals attack and obtain hydrogens from unsaturated fatty acid methylene groups to produce a carbon centered lipid radical. In the propagation step, lipid radicals react with oxygen molecules to produce lipid peroxyl radicals enabling the abstraction of a hydrogen from another unsaturated fatty acid. This results in an additional lipid radical and newly formed lipid hydroperoxide (ROOH). Due to the production of another lipid radical, propagation of lipid peroxidation will continue until the substrate is consumed. Malondialdehyde (MDA) is the pernicious and final product of lipid peroxidation responsible for ROS damage of sperm DNA and proteins (Figure 1.2). Formation of MDA occurs when peroxyl radicals rearrange to form endoperoxides. Moreover, the decomposition of hydroperoxides results in the formation of cytotoxic aldehydes that plays a role in releasing additional MDA from low density lipoproteins. Other products of lipid peroxidation may include ketones, oxo hydroxy acid, hydroxy acid, ethane, pentane, saturated and unsaturated aldehydes.

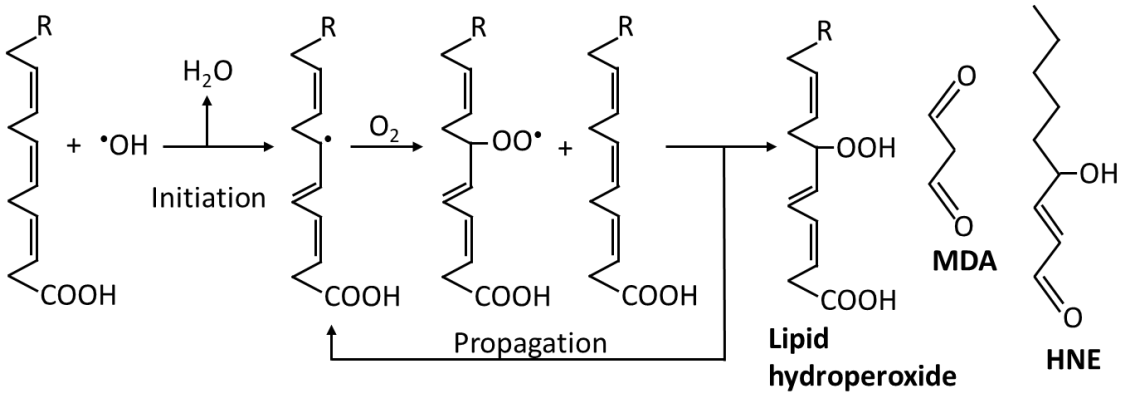

Figure 1.2: Lipid peroxidation initiation and propagation ultimately resulting in the production of Malondialdehyde (MDA), 4-hydroxynonenal (HNE), and lipid hydroperoxides capable of damaging DNA.

The lipid bilayer of somatic cells normally has an asymmetrical arrangement, but sperm have a decidedly different arrangement [16]. The elevated lipid content of mammalian sperm

such as PUFAs, plasmalogens and sphingomyelins enables the flexibility needed for normal flagellar movement [1]. These lipids are also important for regulation of sperm function such as motility, capacitation, and the acrosome reaction, but the elevated lipid content leaves sperm highly susceptible to lipid peroxidation. Furthermore, spermatozoa have a limited ability to maintain an appropriate balance of ROS production and reduction when lipid peroxidation propagates [19]. Thus, alterations to the sperm lipid bilayer by lipid peroxidation decreases motility, induces premature capacitation and acrosome reaction by changing the fluidity and elasticity of lipids and membrane permeability to calcium [1, 26].

Capacitation is the final maturation process sperm must undergo in the female reproductive tract to be capable of fertilization. Under normal conditions, this would happen in the ampulla of the oviduct but premature capacitation and the subsequent acrosome reaction of sperm renders them non-viable. It is widely accepted that capacitation and acrosome reaction are characterized by effluxes in cholesterol, and influxes of bicarbonate, calcium and other ions which leads to the hyperpolarization of the cell. This results in rising pH, cAMP, and the activation of the PKA pathway resulting in tyrosine phosphorylation. ROS, such as hydrogen peroxide, activates adenylyl cyclase to increase intracellular cAMP resulting in the depletion of membrane bound cholesterol, inhibition of phosphotyrosine phosphatases, and initiation of tyrosine phosphorylation [1, 27, 28]. Superoxide is also responsible for activation of adenyl cyclase through oxidation of its thiol group [27].

After spermiation, damage to DNA can become permanent [29]. While small amounts of DNA damage arising from sperm can be repaired during early embryonic development, this can lead to delays in development or decreased embryo quality [27, 30]. Mature spermatozoa lack the ability to repair DNA damage caused by oxidative stress because transcription and translation

capabilities are lost after spermatogenesis [29]. The DNA of spermatozoa is protected from ROS by its tightly compact nature [29]. This is accomplished by replacing histone beads with characteristically small protamines during spermatogenesis [29]. These protamines contain cysteine residues that create crosslinked disulfide bond development resulting in condensed and stabilized DNA [29]. Despite this natural barrier, oxidative stress is still capable of causing both single and double stranded DNA fragmentation where the latter is known to be more deleterious. Hydroxyl radicals produced by the reduction of H_20_2 can cause oxidative damage to DNA. These hydroxyl radicals and aldehydes such as MDA, react directly with purines, pyrimidines, and deoxyribose sugar backbones resulting in alterations such as single and double stranded DNA breaks [19]. The percent of sperm with DNA fragmentation can be determined by using a sperm chromatin structure assay (SCSA) which leverages the use of flow cytometry to measure the DNA quality of many sperm cells. Other assays used to measure DNA fragmentation include the terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labelling (TUNEL) test, comet assay, and sperm chromatin dispersion (SCD also known as HALO) [31]. Sperm with high degrees of DNA damage are known to result in decreased litter sizes in sows [27], and early embryonic mortality in cows and humans [27, 30].

Aside from DNA, ROS negatively affects other cell proteins. Protein oxidation can arise from both free radical and non-free radical species, but hydroxyl radicals are particularly problematic as they are known to covalently bind protein aggregates [32]. This linkage leads to increased proteolytic denaturing in sperm resulting in the loss of protein function of enzymes, cell receptors, and transporters [24, 32].

A major source of intracellular ROS is the mitochondria which has a negative impact on the electron transport chain. The high mitochondrial membrane potential is maintained by the

relationship of electron transport to oxidative phosphorylation for the production of ATP [33]. Hydrogen peroxide, produced by the reduction of superoxide from mitochondrial and cytoplasmic superoxide dismutase, is often the culprit for the depolarization of sperm mitochondria [34]. It is proposed that depolarization is due to the high membrane permeability of hydrogen peroxide [34]. Concurrently, the mitochondria has a membrane permeability transition pore that can respond to a threshold of ROS [35]. When ROS reaches the threshold capacity of the mitochondria, the pore opens causing a loss of mitochondrial membrane potential and signaling apoptotic events [36]. Little is known about the full mechanism resulting in ROS induced changes in membrane potential of boar sperm; however, the understanding of these events have been characterized in the somatic cell and found to have similar function in human and stallion sperm [36].

An increase in hydrogen peroxide is also believed to inhibit enzymes within oxidative phosphorylation and glycolysis resulting in limited production of ATP [37]. Yet the loss of motility by ROS exposure can be independent of mitochondria function as indicated by Guthrie et al. [34]. In this study, the disruption of motility occurred independent of ATP being produced. This is possible by hydrogen peroxide exposure negatively affecting the sperm axonemal protein phosphorylation required for flagellar movement [1, 34]. In some cases, this allows sperm to maintain high viability but will have a notable loss in motility when exposed to hydrogen peroxide [1, 26].

ROS is not completely deleterious and is often necessary for various physiological functions. In small amounts, ROS is known to play a role in physiologically normal signaling pathways [16]. In sperm, a modest amount of ROS at the time of fertilization plays an important role in capacitation, hyperactivation, acrosome reaction, and sperm/oocyte fusion [16]. For

example, ROS at low concentrations is necessary for the initiation of the tyrosine phosphorylation cascades affiliated with capacitation and the acrosome reaction. But when ROS is present at higher concentrations, the premature damage to biomolecules by oxidative stress ensues [16, 19].

1.5. Sperm natural antioxidant systems:

Several studies have indicated the positive roles of seminal plasma in reducing oxidative stress and cryo-induced capacitation of boar sperm [20, 38, 39]. Sperm and seminal plasma contain their own antioxidants, but these can vary between males, diets, breeds, health status, season, and living conditions [4, 18]. Seasonality is known to influence cryotolerance and longevity of cooled stored semen. Findings suggest that ejaculates collected in autumn and summer have improved preserved sperm quality compared to ejaculates collected in the winter and spring because they contain increased antioxidant content [18, 40]. Enzymatic antioxidants commonly found in sperm and seminal plasma includes catalase, SOD, and glutathione peroxidase [4, 26]. Low molecular weight components of seminal plasma that also have antioxidant activity include vitamin E, vitamin C, urate, albumin [4], taurine, and hypotaurine [26]. Little is known about the variation seen between ejaculates and breed with respect to low molecular weight antioxidants. The enzymatic antioxidants have been demonstrated to have significant differences between breeds [4]. Žaja et al. (2016) studied the ejaculates of four pure bred lines and one hybrid line of commercial boars and their seminal plasma concentration of GSH and SOD [4]. Statistically significant differences in GSH (70.61, 44.43, and 24.51 U/g) and SOD (1950.98, 1191.00, and 619.60 U/g) were observed in Swedish Landrace, Pietrain, and hybrid boars respectively [4]. These data demonstrate the great variation that can be seen between breeds where the hybrid boars, in this case, underperformed in seminal plasma

antioxidant concentration. This variation potentially accounts for decreased ability for sperm cells to tolerate both cooled and frozen preservation because the essential balance of the production and reduction of ROS is greatly altered.

The first line of defense against oxidants is SOD which serves as the catalyst for converting superoxide to H_2O_2 and O_2 which is further enzymatically dissociated to H_2O and O_2 by catalase [41]. Boar sperm and seminal plasma are characteristically low in catalase concentrations resulting in reduced ability to dissociate H_2O_2 into its products [1]. GSH peroxidase is another enzyme with the ability to catalyze the reduction of H_2O_2 and other hydroperoxides into H_2O and alcohols [26, 42]. GSH, itself, is a cofactor to GSH peroxidase and is characterized as a thiol tripeptide (γ-L-glutamyl-L-cysteinylglycine) which functions as an antioxidant in a reversible redox reaction (Figure 1.3) [43]. GSH in normally released into the lumen of the epididymis and believed to play a vital role in maintaining membrane integrity as the sperm transverse through the epididymis [42]. Inflammation, environment, and seasonality also plays a role in the concentration of GSH, SOD, catalase found in the sperm cells and seminal plasma.

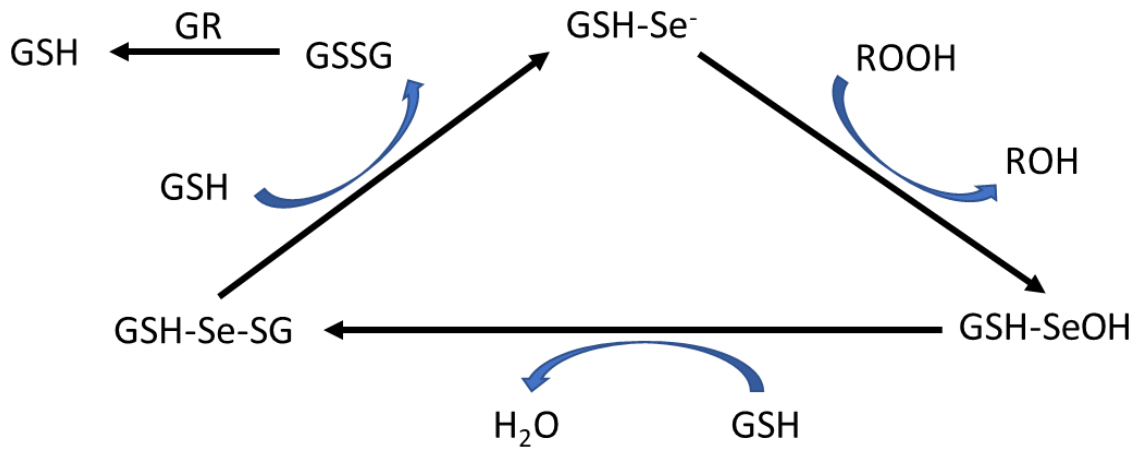

Figure 1.3: The catalytic cycle of GSH peroxidase begins when the selenol portion becomes oxidized to selenenic acid by hydroperoxides (ROOH). Selenenic acid is then converted into Selenadisulfide to release the oxygen in the form of H_2O. Selenadisulfide is then reduced through thiol-disulfide exchange (GSSG) to regenerate selenol and enabling GSH to start the next cycle [42]. At the same time GSH reductase (GR) converts GSSG back to GSH.

1.6. Challenges associated with boar sperm preservation:

Boar sperm used for reproductive technologies are subject to selection criteria at the stud which include motility and morphology assessments prior to extension in chemically defined extender for storage at 15° to 17°C. [4]. Although commercial extenders are labeled for sperm shelf life of about 5 days, the fertilizing potential of boar sperm stored at 15° to 17°C declines over the first 72 hours of storage [13].

While artificial insemination enables one ejaculate to be spread across many sows, single sire mating gets very little attention in the swine industry [44, 45]. Knox et al. (2008) states that approximately 60% of boar studs pool semen using 2-6 boar ejaculates [10]. The popular use of pooled semen was introduced to minimize the lower conception and litter sizes seen by boars with reduced fertility [45]. However, when boars are mated in these pooled systems, it is often observed that a single boar will sire the bulk of the litters [46, 47]. Boars with high genetic merit and low to moderate fertility will often be underrepresented in the litters of pooled matings. To

leverage the genetic progress these high merit boars provide, methods to improving the sperm quality and shelf life can make single sire matings a reality.

Routine analysis of total motility is often not powerful enough to detect the sublethal changes in sperm quality which may impact sow conception and farrow rates [13]. Computer assisted sperm analyzers (CASA) and flow cytometers are powerful instruments for sperm assessment but their capabilities are often underutilized or limited by machine operator capabilities [13]. This not only warrants the need for more rigorous semen evaluation but also highlights the need to protect aged sperm from lethal and sublethal damage. Assessment of the kinematic parameters generated by CASA to detect sublethal damage to sperm is but one example of enhancing sperm analysis [13].

Additionally, the use of other fluorescent probes to evaluate acrosome integrity, membrane stability, and DNA integrity parameters further bolsters the ability to detect damage to liquid cooled sperm by oxidative stress but it may be possible to utilize the mean fluorescent intensity of these probes to obtain information on sublethal modifications. Work performed by D'Cruz et al. (1995) has previously demonstrated that the mean fluorescent intensity of lectin conjugated fluorochromes are useful in determining the degree of acrosomal cap degradation rather than a simple binomial assessment of non-reacted versus reacted acrosomes which has been standard for decades [48]. Similarly Treulen et al. (2018) utilized the mean fluorescent intensity of tetramethylrhodamine methyl ester perchlorate accumulation in the mitochondria as a method of determining overall mitochondrial membrane potential of the population rather than a binomial polarized or non-polarized assessment [49]. Because these methods are used to assess the quality of the population of interest, they may be useful as markers in determining the presence of sublethal sperm modifications that could impact male fertility.

The training of boar stud staff as well as the equipment supplied to them account for great variability in sample quality leaving a genetic center. It is not surprising to find that morphological abnormalities often slip past the initial visual assessment resulting in some semen doses containing high cytoplasmic droplet counts. A study performed by Waberski et al. [50] illustrated the negative correlation between both proximal and distal cytoplasmic droplets numbers with conception rate and liter size when semen was stored and inseminated 2 and 4 days post-collection. These challenges not only impact cooled stored semen but can alter the success of cryopreservation.

Cryopreserved boar sperm has been available since the 1970's but the technology is underutilized due to the decrease in conception rate and litter size accompanying the increase cryodamage to sperm cells [2, 20, 51]. In fact, <1% of sow insemination is performed with frozen-thawed sperm worldwide [51]. The increased cryodamage is largely attributed to the boars sperm increased sensitivity to cold shock [51] and the high concentration of PUFA's found in the sperm membrane that contributes to increased ROS production through lipid peroxidation. Consequently, boar sperm that undergoes the cooling, cryopreservation, thawing process experiences membrane destabilization that alters the calcium permeability and acrosome integrity [2, 51-53]. Additionally, freezing and thawing sperm disrupts the disulfide bridges necessary to protect DNA from damage. DNA damage is often not visualized immediately post-thaw [54, 55] but varying degrees of DNA damage can be observed when thawed sperm is allowed to incubate at 37°C for 2 to 4 hours [40, 55].

When cryopreserving boar sperm, the seminal plasma is often removed by centrifugation, dilution by extender, or a combination thereof. This removes the naturally occurring antioxidant defense found in seminal plasma. To compound the issue, centrifugation induces mechanical

damage to the sperm membrane and extracts proteins and lipids, resulting in altered membrane permeability and increased rate of ROS formation [2, 51, 56, 57]. Yet, centrifugation is necessary to concentrate sperm for cryopreservation.

Knox et al [2] states that there is an average loss of boar sperm viability of about 60% post-thaw. While some studies may achieve >60% post-thaw viability, there is still great variability in successfully cryopreservation of boar sperm. Due to the complex nature of combined factors that impact cryotolerance, a multifaceted approach to improve the post-thaw sperm quality of boars should be considered. This does not limit the development of cryopreservation medium to tactical use of additives such as antioxidants and sodium lauryl sulfate, but rather, it should also focus on other factors known to influence cryopreservation success such as the base extender used, base extender starting osmolality, sperm cooling rate to 4°C, cryoprotectant concentration, straw size, and time of equilibration after cryoprotectant addition.

The primary goal of any screening experiment is to identify the factors influencing the outcome. However, with the factors listed above in a full factorial design (9 factors or treatments each in multiple aggregations) would require >1600 treatment combinations. To avoid time consuming and cumbersome task of screening the many factors in full factorial designs, screening methods such as the Plackett-Burman experimental design provides an effective and rapid approach to identifying those factors that require further investigation. The use of a Plackett-Burman experimental design aids in extracting information on single factors that influence post-thaw sperm quality of boars without focusing on the factor interaction. This is made possible by looking at each factor at two extremes in a variety of combinations with other factors. In this manner, it is possible to move forward in optimization of sperm cryopreservation

factors, such as antioxidant supplementation, in future studies thereby reducing the variability of post-thaw boar sperm viability [58].

1.7. Antioxidant supplementation:

Because the PUFA's of boar sperm are highly susceptible to oxidative stress, it comes as no surprise that studies show striking improvements in cooled, aged, and post-thaw sperm quality when supplemented with antioxidants. Thus, antioxidant supplementation becomes an attractive solution to reestablish protection against oxidative stress. Some of the common antioxidants used to protect boar sperm from oxidative stress include butylated hydroxytoluene (BHT) [2, 59], estradiol 17β [60], α-tocopherol [61], glutathione peroxidase (GSH) [25], ascorbic acid [61], superoxide dismutase (SOD) [25], catalase [25], and polysaccharides [62]. These antioxidants have been shown to improve boar post-thaw sperm quality, reduced lipid peroxidation and increased fertilizing capacity [2, 20].

Vitamins such as α-tocopherol (vitamin E) and ascorbic acid (vitamin C), have long been known for the oxidant scavenging abilities. Vitamin E, a lipid soluble vitamin, is capable of donating one or two protons from hydroxyl groups [61]. This prevents lipid peroxidation by interfering with the propagation step of lipid radical production [63]. The donation does not alter the structure of vitamin E, and its free radical form remains unreactive and will regain its scavenging ability when it is regenerated by Vitamin C [61, 63]. The synergistic relationship between vitamin E and C suggests that they are best used in combination when supplementing semen extenders. When vitamin E donates its second proton without regeneration of the first proton, irreversible damage to the vitamin E structures renders it useless [61]. Vitamin C, a water soluble vitamin, is also capable of donating one or two protons when scavenging oxidants[61]. Vitamin C interacts with free radicals directly to prevent oxidative stress to cells [63]. The first

proton donation results in a semi-dehydroascorbate radical and the second donation results in dehydroascorbate. These are unstable free radicals but can be converted back to vitamin C by GSH [61]. Like vitamin E, the lack of replenishing the vitamin C protons results in inactivation of the vitamin as an oxidant scavenger [61]. Fortunately, there are several replenishing compounds capable of donating protons to the vitamin E and C without becoming free radicals themselves. These replenishers include carotenoids, flavonoids, coenzyme Q, and GSH. Carotenoids and coenzyme Q most commonly interact with Vitamin E due to their lipophilic nature while flavonoid and GSH interact with vitamin C in aqueous conditions [61, 63]. Butylated hydroxytoluene , a synthetic analogue of vitamin E, has been extensively used in food preservation and researched in protecting sperm cells from oxidative stress by reducing radicals and hydroperoxides [61, 64].

While not classified as antioxidants, estrogens have been found to enhance the antioxidant activity of enzymes such as SOD, GSH, and catalase [60, 65]. The estrogenic effects of estradiol and bisphenol A have been studied in zebra fish which demonstrated a significant (P<0.05) increase of enzymatic antioxidant activity in hepatic and gonadal cells [60]. The use of estradiol is a subject of debate as it is known to increase cellular metabolism of human sperm [66] and has been observed to enhance sperm motility in bucks in vitro [67]. This suggests that oxidative stress would increase and perhaps the increase in enzymatic antioxidant activity is a secondary side effect.

Supplementation with amino acids also demonstrates pro-antioxidant abilities. L-cysteine, a precursor to GSH, has been well studied and found to enhance the intracellular GSH profile of sperm [20]. Polysaccharides have been demonstrated to enhance the activity of enzymatic antioxidants while also having the ability to scavenge free radicals [65]. A study

looking at the cryosurvival of buck sperm supplemented with polysaccharides demonstrates a significant (P<0.05) increase in the activity of SOD, GSH, and catalase while also showing significant (P<0.05) improvement in sperm post-thaw motility and mitochondrial membrane potential [65].

1.8. Plant extract supplementation:

Antioxidant supplementation shows promise, especially when considering lowering the concentration of sperm in insemination doses [13]. Processing sperm at lower dose concentrations further decreases any natural protection from oxidative stress. This makes plant extracted antioxidants an attractive choice as extender additives because they often contain a blend of naturally occurring antioxidants and reduces the sperm quality variation seen in single antioxidant supplementation [68]. In fact, the antioxidant and other pharmaceutical properties of plants are of great interest as remedies in biomedical science due to their relatively safe nature [69].

A study performed by Ros-Santaella et al. using 12.5µg/mL Honeybush (*Cyclopia intermedia*) extract in cooled stored boar semen demonstrated a significant improvement (P<0.05) in motility, membrane and acrosome integrity at 120 hours post semen extension when held at 17°C [14]. These data also showed reduced lipid peroxidation when induced by Fe^{2+}/ascorbate (P<0.05) [14]. When taking a closer look at sperm kinematics, a significant improvement (P<0.05) was also observed with respect to VAP, VSL, ALH, BCF, and LIN [14]. This supports the use of kinematics to detect sublethal sperm parameters which, in turn, increases the semen shelf-life and positively impacts conception rate, farrowing rate and litter size.

Another benefit to plant extract supplementation is delaying capacitation by decreasing the concentration of ROS. Activation of adenylyl cyclase by ROS initiates the cascade that leads

to tyrosine phosphorylation and capacitated hyperactivation [28]. A study using blueberry (Vaccinium corymbosum) fruit and leaf extract supplementation in extended boar semen stored at both 5°C and 15°C for 5 days supports a plant extract's ability to significantly (P<0.05) delay capacitation and the acrosome reaction in vitro [70].

Cryopreservation extenders often contain egg yolk which has membrane stabilizing phospholipids as well as some antioxidants to protect against oxidative stress [71]. Yet, these egg yolk derived antioxidants are often not enough to protect boar sperm from oxidative stress [71]. This makes the addition of plant extracts to cryopreservation extenders beneficial in the role of returning the balance of antioxidants to sperm. The use of fennel (*Foeniculum vulgare*) extracts in cryopreservation extender has been shown to improve post-thaw boar sperm DNA integrity [71]. While there was no significance impact on motility, viability or acrosome integrity immediately post-thaw, significant improvements (P<0.05) were observed after 2 hour incubation at 37°C [71]. This underscores the ability of plant extracts to improve the longevity of post-thaw sperm making it possible for sperm to be available when ovulation occurs.

1.9. Summary

Artificial insemination is widely used within the swine industry where cooled semen accounts for most of the inseminations. Preservation of boar sperm is most commonly accomplished by cooling and storing sperm at 15° to 17°C using chemically defined extenders. The use of cryopreservation is also available but less common with <1% of the total insemination being performed with frozen-thawed sperm. The increased polyunsaturated fatty acid content of boar sperm is highly susceptible to lipid peroxidation and free radical formation which increases oxidative damage during sperm cooled storage, cryopreservation, and thawing.

Thus, supplementation with antioxidants, such as those extracted from plants, can be used to protect sperm from the negative impacts of oxidative stress.

This chapter has served as the foundation to the challenges associated with the work presented in this dissertation. The following chapters will focus on supplementing cooled aged boar semen with novel plant extracted antioxidants (GameteGuard®; Membrane Protective Technologies, Inc., Fort Collins, CO) and evaluating the impacts on sperm quality, conception rate farrowing rate, and litter size. This work will also discuss the assessment of sublethal sperm modifications using mean fluorescent intensity of fluorochromes. Finally, the screening of cryopreservation factors that impact post-thaw boar sperm quality using the Plackett-Burman experimental design will be discussed. Taken together, the work of this dissertation focuses on reduction of ROS through antioxidant supplementation, improving sperm quality evaluation for boars, and techniques that aid in understanding how to overcome oxidative stress when preserving boar sperm.

CHAPTER 2: MULTI-STAIN FLOW CYTOMETRIC IDENTIFICATION OF BOAR SUBFERTILITY USING MEAN FLUORESCENT INTENSITY

2.1. Introduction:

Artificial insemination (AI) of sows in the commercial pork industry using cooled semen stored at 15°C to 17°C accounts for 99% of the artifical inseminations world-wide [72-75]. The use of AI with liquid semen offers several advantages such as dispersal of superior genetics across herds, and increases profitability per boar ejaculate [75-77]. To reduce incidence of male infertility such as low conception rate, routine analysis of sperm is performed using conventional methods such as assessing motility, morphology and sperm dose concentration [74, 78].

The only indisputable proof of sperm fertilizing ability is the generation of a pregnancy [79] because the information obtained from the conventional analysis of boar sperm yields unreliable results as it relates to in vivo fertility [74, 78]. Other methods of sperm evaluation elucidate information about structural integrity and functionality such as viability, DNA intactness, membrane stability, and mitochondrial membrane potential. While these tests provide valuable data, they also yield little to no relationship to sperm fertilizing ability in single assays [73, 78, 80].

To improve the reliability of sperm assays as it relates to fertility, a two to three assay approach to sperm analysis should be employed [74, 78, 79]. Yet, the evaluation of sperm using multiple flow cytometry assays is often expensive and labor intensive. Thus, the evaluation of sperm would benefit from the ability to collect more information using a single assay approach such as the combination of cell viability, acrosome integrity, and mitochondrial membrane potential [79, 81-83].

Building off the multi-assay approach and maximizing the potential of instruments such as flow cytometers, the mean fluorescent intensity (MFI) of fluorochromes may be obtained. The use of MFI was traditionally utilized in early validation of fluorochromes that label exposed acrosomes and populations with high mitochondrial membrane potential; however, they are not routinely utilized today despite their potential in identifying shifts in sperm quality [48, 49].

Advances in flow cytometry tools such as the emergence of temperature-controlled stages with automated plate readers enables sperm samples to be stained and evaluated in larger quantities. Several flow cytometers are also available with multiple lasers and bandpass filters enabling the use of larger co-stain panels. In turn, these flow cytometry capabilities facilitate the incorporation of a multi-assay approach to evaluating the fertilizing potential of many sperm samples in a short period of time.

The sensitivity of sperm to their immediate environment may lead to sublethal modifications [84]. Environmental temperatures that increase testicular temperature outside of physiological norms are capable of inducing irreversible damage to sperm motility, membrane integrity, and membrane stability [85]. The relationship between decreased reproductive performance and increasing in vitro temperature is well understood in boar sperm [86], yet the impacts on sperm quality throughout spermatogenesis and during transit in the epididymis are not well studied. Seasonality and changes in temperature are known to influence epididymal and seminal plasma antioxidant concentrations which may leave sperm exposed to oxidative stress and subfertility [25, 87, 88]. Unfortunately, sublethal damage often goes undetected using conventional motility and viability analysis [85, 89].

The present study aimed to incorporate MitoTracker® Orange, a mitochondrial membrane potential fluorochrome, into a commonly utilized co-stain protocol utilizing

SYBR-14, propidium iodide, and PNA conjugated Alexa Fluor® PNA 647 [90]. Because this study employed the use of a flow cytometer equipped with a heated stage and automated plate reader, the optimum temperature and staining time was investigated. Finally, the present study addresses potential identification of sublethal sperm parameters by utilizing flow cytometric MFI, and total antioxidant reactivity (TAR) of sperm doses. Accordingly, determination of potential sublethal parameters was obtained by evaluating cooled aged boar sperm and deriving correlations between the temperature of boar housing facilities and sperm parameters. From these objectives, we hypothesize that MitoTracker® orange can be added to create a 4-panel co-staining protocol to obtain mitochondrial membrane potential while simultaneously demonstrating that 22°C staining temperature is ideal or cooled boar sperm evaluation. In addition, we hypothesize that potential identification of subfertile ejaculates can be achieved with the use of MFI.

2.2. Methods and Materials

2.2.1. Animal handling

Boar handling and semen collection was performed according to the National Institutes of Health guide for the care and use of animals and under Colorado State University IACUC approval (CSU IACUC #: 18-7948A). The 21 commercial Duroc boars 10-12 months of age used for this study were housed in the same facility and fed a commercial pelleted diet.

2.2.2. Chemicals, Reagents and Disposables

All research grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Analytical reagents such as flow cytometry fluorophores were purchased from Thermo Scientific (Carlsbad, CA) unless otherwise stated. Disposables were purchased from Life Science Products (Frederick, CO).

2.2.3. Boar collection:

AndroStar Plus® extender (Minitube USA, Inc.) was prepared according to manufacture recommendation by adding 47 g/L in nanopure water, then mixed and warmed to 35°C on the day of use. A total of 139 ejaculates were collected from the above boars using the gloved-hand collection method to extract the sperm rich fraction [91]. Initial motility, morphology and sperm concentration were assessed at the boar stud with minimum passing requirements set at 75% motility and 75% normal morphology on the day of collection to be used in the study. Ejaculates were extended to 1.5 billion sperm/mL and loaded into 75 mL doses. Samples were cooled to 17°C and shipped to the lab for sperm analysis on day 1post-collection.

2.2.4. Temperature collection

All boars utilized for this study were housed in the same enclosed environmentally controlled facility. The facility temperature the boars were housed in was recorded starting from 55 days prior to collection and ending on the day of collection to assess its impact on early spermatogenic and transportation through the epididymis. Thus, the temperature log for the collections above started in April and ended with the last collection in August.

2.2.5. Sperm motility evaluation

Sperm motility was assessed on both day 1 and 4 post collection for total percent motile. Semen aliquots of 200 µL for each sample were incubated at 37°C for 10 min. Each sample was analyzed on a four chamber 20 µM depth slide (LEJA; Spectrum Technologies, Healdsburg, CA) using computer assisted sperm analysis (CASA, IVOS II, Hamilton Thorne Ltd, Danvers, MA). For each sample, >800 cells were examined in duplicate. The CASA system captured 60 frames/sec and settings included 30 frames acquired; max and min head size at 50µm^2 and 10 µm^2 respectively; default cell intensity at 150; progressive cells threshold straightness at 67%;

velocity along average path (VAP) cut-off at 40 μm/s; slow cell VAP cut-off at 15 μm/s; and slow straight-line velocity (VSL) cut-off at 10 μm/s.

2.2.6. Flow cytometric evaluation

Flow cytometry assessment of sperm occurred using a ZE5 flow cytometer (5 laser system; Bio-Rad Laboratories, Hercules, CA) operating at 0.5 μL/s, to acquire >10,000 DNA positive events for each assay performed. Calibration of the instrument prior to every use was performed with flow cytometry calibration beads. Using the flow cytometer, each extended sample was analyzed in on a 96-well-plate in 125 μL volumes for each assay performed ($2x10^6$ sperm/mL). All assays below were analyzed using FlowJo software (BD Biosciences, San Jose, CA) by utilizing gating schemes set in logarithmic scale (gating described below for each assay).

Five boar ejaculates extended to $26x10^6$ sperm/mL were used for the validation of a 4-panel co-stain protocol and investigation of optimal staining time and temperature when evaluating samples on a plate reading flow cytometer. As is true for all flow cytometric evaluation in the present study, a killed sperm sample by snap freezing was utilized as a negative control. The 3-panel co-stain protocol used was similar to that described by Nagy et al. [90]. Briefly, sperm samples of 12.5 μL were added to 112.5 μL of HEPES buffer (HEPES 10 mM, bovine serum albumin 1.5 mM, and sodium chloride 150 mM) containing SYBR-14 (0.1 μM, 488 nm; 525/35 bp), propidium iodide (12 μM, 561 nm; 615/24 bp), and PNA conjugated Alexa Flour 647® (6.5 μg/mL, 640 nm; 670/30 bp). The 4-panel being evaluated was the 3-panel as described above with the addition of MitoTracker® orange (40 nM, 561 nm; 577/15 bp). For panel validation, samples were incubated for 10 min at 22°C in the dark then subject to acquisition on the flow cytometer. For evaluation of staining time and temperature, samples were prepared and stained at 17°C, 22°C, and 37°C. Then each stain temperature was subject to flow

cytometric data collection at 10-, 20-, 40-, and 60-minute staining times. Sperm populations were considered membrane impermeable when SYBR (+), propidium iodide (-), and acrosome intact when PNA (-) [90]. Populations with high mitochondrial membrane potential were identified by SYBR (+) and MitoTracker® orange (+) events. In addition to the percent of the populations collected, the mean fluorescent intensity of SYBR-14, PNA conjugated Alexa Flour 647®, and MitoTracker® orange was collected from the viable population (membrane impermeable).

For the second part of this study, the above 4-panel was used after staining sperm for 10 minutes at 22°C. In addition, the stability of the cell membranes was evaluated using a M540 co-stain protocol [92]. Thus, membrane stability was queried using 12.5 µL of extended semen added to 112.5 µL of HEPES buffer mentioned above containing final concentrations of Hoechst 33342 (80 µg/mL, 375 nm; 447/60 bp), YOPRO-1 (0.4 µM, 488 nm; 525/35 bp), M540 (5.12 µM, 561 nm; 577/15 bp). Samples were stained for 15 min at 22°C in the dark then data was collected using the flow cytometer. Membrane stability was defined by YOPRO-1 (-) and low fluorescent intensity of M540 [92]. In addition to the percent of membrane stable populations recorded, the mean fluorescent intensity of M540 was collected.

2.2.7. Fluorescent microscopy

Sperm cells were stained using the 4-panel described above and immunofluorescent images were captured on a fluorescent microscope (BX53; Olympus Scientific). Differential interference contrast images were overlayed with fluorescent images using cellSens software (Olympus Scientific) to demonstrate MitoTracker® orange specific staining of the sperm midpiece.

2.2.8. Total antioxidant reactivity

A 4mL aliquot of each boar's extended sample was frozen on day 4 post-collection at -80°C to be utilized on a later date for evaluation of total antioxidant reactivity (TAR). Using a microplate luminometer (Promega, Madison, WI) a luminol-based assay was used to detect the luminescence of antioxidants abstracting electrons from hydrogen peroxide. A TRIS buffered solution (263 mM Trizma Base, 86.3 mM Citric Acid), detection solution (1mM luminol in 50% DMSO: H2O), catalyzing solution (50 mL of 300 mM hydrogen peroxide in TRIS buffered solution), samples being tested, and a positive control (0.01 µg/mL Trolox) were used in this catalyzed reaction. On a 96-well plate, samples (100 µL) were prepared in a dilution series, the catalyzing solution added to each well (75 µL), then the luminometers autoinjector administered the detection solution (25 µL). The positive control used in this assay was Trolox which is needed to calculate the TAR value by Trolox equivalent reactivity [93]. The amount of reactive oxygen species (ROS) removed is determined by finding the percent difference in relative luminance from the sample compared to a negative control containing nanopure water and catalyzing solution. From these data, TAR value was computed using the following equation [93],

$$TAR = \left(\sum ki\ [xi]\right) / k_{Trolox}$$

where ki is the amount of ROS removed at the i-th position, xi is the concentration of the sample being tested at the i-th position and k_{Trolox} is the total reactivity of Trolox using equation $(\sum ki\ [xi])$.

2.2.9. Statistical analysis

All statistical analysis was performed using R version 3.5.1 through RStudio [94, 95]. Data was first subject to a normality test before proceeding with other statistical analysis.

A two-tailed student t-test was used to detect differences between the 3- and 4-panel co-staining protocols. To detect the pairwise comparison of staining time and temperature in repeated measures of time, an ANOVA adjusted with a TUKEY HSD was used [96]. Due to lack of normal distribution of the data for the second part of the experiment (n = 139 ejaculates), sperm one day post-collection was used to generate Spearman correlation coefficients for assessment of the correlation between the measured sperm quality parameters and average temperature of the boar facility during sperm development and storage in the epididymis. Coefficients >0.7 or <-0.7 were considered high correlation while coefficients between 0.4 to 0.69 or -0.4 to -0.69 were considered moderate correlation [97].

2.3. Results

2.3.1. Four-panel flow cytometry validation

When co-staining MitoTracker® orange with SYBR-14, propidium iodide, and PNA conjugated Alexa Fluor® 647 in a new 4-panel protocol, there was not enough evidence to conclude that a difference exists between the panels for membrane permeability and acrosome intactness (Table 2.1), yet a population with high mitochondrial membrane potential was successfully obtained. Figure 2.1 demonstrates that there is indeed specific labeling of the mitochondria for MitoTracker® orange.

Table 2.1: Validation of 4-panel co-staining protocol that includes MitoTracker® orange

	3-Panel	4-Panel	*P*-value
Membrane impermeable (%)	89.5±1.7	88.8±1.2	0.76
Membrane permeable (%)	10.5±1.7	11.2±1.2	0.75
Intact acrosomes (%)	83.2±2.6	82.5±1.3	0.80
Reacted acrosomes (%)	16.7±2.6	17.5±1.3	0.79
Mitochondrial membrane potential (%)	NA	84.6±1.3	NA

Table 2.1 demonstrating that there is no difference detected for sperm quality parameters between the 2 panels evaluated ($P<0.05$; $n = 5$ boars).

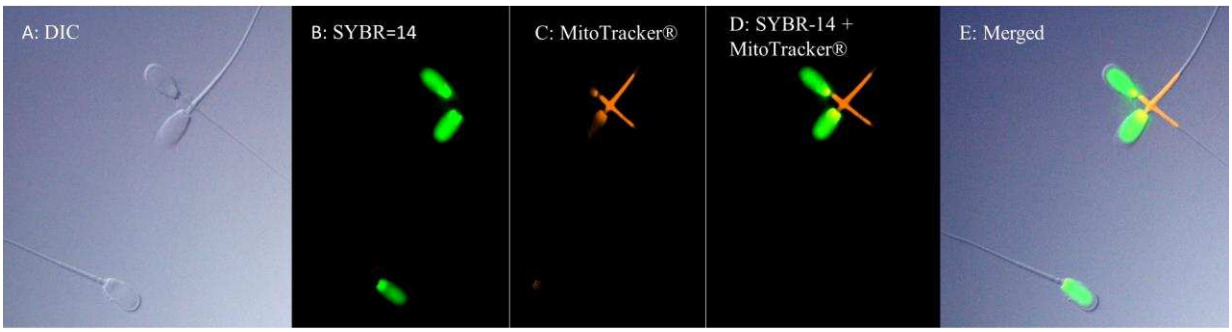

Figure 2.1. Fluorescent microscopy images of boar sperm stained with SYBR-14 and MitoTracker orange; A) differential interference contrast (DIC) image of sperm; B) sperm DNA stained with SYBR-14; C) specific staining of sperm with high mitochondrial membrane potential within the midpiece coupled with a sperm lacking mitochondrial membrane potential; D) merged fluorescent images of SYBR-14 and MitoTracker orange showing specific staining; and (E) DIC and fluorescent images merged.

2.3.2. Flow cytometer temperature and staining time

The temperature of staining and the stage temperature of analysis were evaluated during this study to determine the optimal temperature to evaluate cool stored boar sperm using flow cytometry. Sperm can be stained at both 17°C and 22°C and enables the sperm to be evaluated without a significant decline in sperm quality parameters over time (Table 2.2). When staining and evaluating sperm at 37°C, there is a significant decline in membrane impermeability, mitochondrial membrane potential, and acrosome intactness following 20 minutes of staining time ($P<0.05$).

Table 2.2: Comparison of sperm parameters at different staining and evaluation temperature over time using 4-panel co-staining protocol

Temperature	Sperm parameter	10 (min)	20 (min)	40 (min)	60 (min)
17°C	Membrane impermeable (%)	89.1±3[a]	90.1±1.4[a]	89.9±2[a]	88.3±2.5[a]
	Mitochondrial membrane potential (%)	84.6±4.5[a]	85.7±1.9[a]	86.0±2.4[a]	84.2±2.4[a]
	Acrosome intactness (%)	81.4±4.9[a]	83.5±2.4[a]	83.2±3.3[a]	81.6±2.7[a]
22°C	Membrane impermeable (%)	88.8±2.5[a]	88.8±1.9[a]	89.3±1.8[a]	89.3±1.3[a]
	Mitochondrial membrane potential (%)	84.6±2.7[a]	85.4±2[a]	86.3±1.8[a]	86.1±1.6[a]
	Acrosome intactness (%)	82.6±2.7[a]	82.4±2.6[a]	83.5±2.3[a]	82.5±2.9[a]
37°C	Membrane impermeable (%)	87.5±1.5[a]	86.7±1.1[ab]	83.9±0.7[bc]	80.2±0.4[c]
	Mitochondrial membrane potential (%)	85.1±1.7[a]	84.8±1.2[ab]	82.5±0.7[bc]	78.4±0.7[c]
	Acrosome intactness (%)	83.6±1.8[a]	83.1±1.2[ab]	78.9±1.7[bc]	73.9±1.5[c]

Table 2.2 demonstrates sperm quality for percent membrane impermeable, percent mitochondrial membrane potential, and percent acrosome intactness ± SD for the different staining and evaluation temperatures over time. Note that superscripts [a-c] signify difference detected within a staining and evaluation temperature ($P<0.05$; $n = 5$ boars).

The decline in quality sperm over time is apparent in Figure 2.2. These data confirm the ability to obtain sperm quality data on samples stained at 17°C and 22°C over a longer period (60 minutes). The samples stained at 37°C have a significant decline in percent membrane impermeable cells after 20 minutes and a decline in mitochondrial membrane potential after 40 minutes ($P<0.05$).

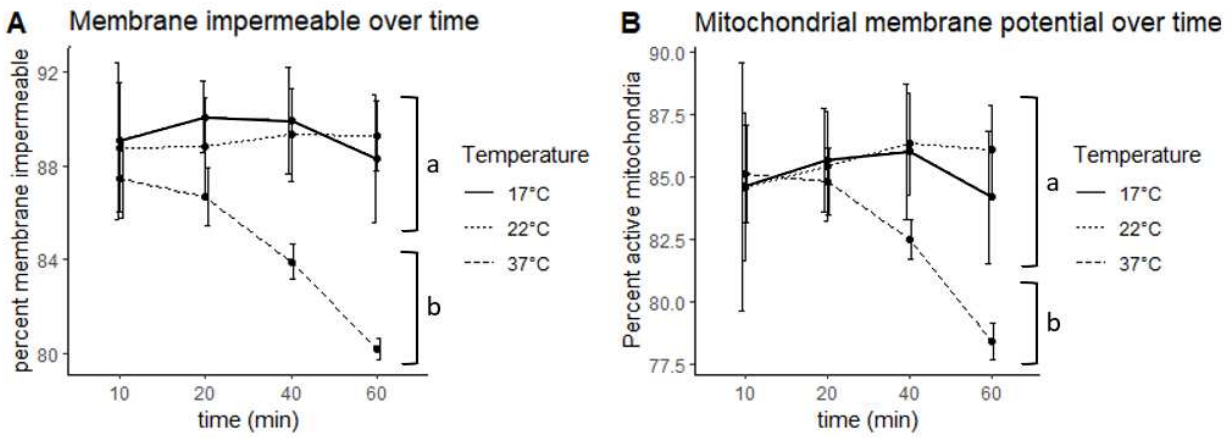

Figure 2.2. Comparison between the different staining and evaluation temperatures over time where difference is signified by [a-b] (P<0.05). Graph A demonstrating shifts in MFI membrane impermeable cells. Graph B demonstrating shifts in MFI for mitochondrial membrane potential.

It was recognized that the different temperatures assessed above may impact cellular activity such as aerobic respiration and activity of channels that enable fluorochrome cell penetration and labeling. Thus, the MFI of each fluorochrome for the viable population was evaluated for each staining temperature over time. Interestingly, there is no difference detected in MFI of PNA conjugated Alexa Fluor® 647 for the three staining temperatures tested except for an increase in MFI after 60 minutes of staining at 37°C (Table 2.3; P<0.05). The MFI of SYBR-14 stained at 37°C does not differ between 10 and 40 minutes, but there is a difference detected after 60 minutes (P<0.05). Samples stained at 17°C start with a significantly lower MFI for SYBR-14, MitoTracker® orange, and propidium iodide which increase in intensity over time (P<0.05). Both MitoTracker® orange and propidium iodide increase in MFI over 60 minutes of staining for all temperatures evaluated (P<0.05).

Table 2.3: Comparison of MFI at different staining and evaluation temperature over time

		MFI			
Temperature	Fluorochrome	10 (min)	20 (min)	40 (min)	60 (min)
17°C	SYBR-14	1290.5±100.2^{a}	1838.7±93.3^{b}	2264.8±143.7^{cd}	2273.9±87.4^{d}
	MitoTracker	5488±505.8^{a}	7285.1±479.4^{b}	9079.4±270.2^{cd}	9693.9±289.9^{d}
	Propidium Iodide	1006.9±155^{a}	1330.8±110.9^{b}	1640.9±83.4^{cd}	1725.3±120.5^{d}
	PNA Alexa Fluor 647	89±26.5^{a}	87.2±10^{a}	85.5±17.8^{a}	81.1±10.8^{a}
22°C	SYBR-14	2275.1±191.3^{a}	2448.3±115^{a}	2507.1±129^{a}	2293.2±103.4^{a}
	MitoTracker	9032.1±779.4^{a}	10128.5±438.4^{ab}	11475.1±249.4^{bc}	11475.1±249.4^{c}
	Propidium Iodide	1638.5±176.4^{a}	1819.6±106.6^{ab}	1965.9±84.1^{b}	2022.7±64.6^{b}
	PNA Alexa Fluor 647	85.9±9.6^{a}	87.9±3.3^{a}	86.1±12.1^{a}	86.3±22^{a}
37°C	SYBR-14	2107.2±116.7^{a}	2011±87.2^{ab}	1849.7±63.4^{ab}	1775.6±43.8^{b}
	MitoTracker	10063.9±516.7^{a}	11601.7±494.9^{b}	13749.6±343.5^{c}	15619.5±242.8^{d}
	Propidium Iodide	1815.9±112.5^{a}	2074.3±106^{b}	2406.3±61.3^{c}	2696.7±58.6^{d}
	PNA Alexa Fluor 647	103±11^{a}	103.3±8.8^{a}	104.3±9^{a}	116.3±13.8^{a}

Table 2.3 depicting fluorochrome MFI ± SD for the different staining and evaluation temperatures over time. Note that superscripts $^{a\text{-}d}$ signify difference detected within a staining and evaluation temperature (P<0.05).

Figure 2.3 illustrates the impact evaluation temperatures had on MFI within a staining time. After 10 minutes staining, SYBR-14, MitoTracker® orange and propidium iodide had a significantly lower MFI when samples are stained at 17°C (P<0.05). The MFI of 17°C samples remained lower than 22°C and 37°C for 20, 40 and 60 minutes (P<0.05); however, there was no difference detected between 17°C and 37°C at 20 minutes or 17°C and 22°C after 40 minutes. Differences between sperm samples stained at 22°C and 37°C were not detected at 10 minutes for SYBR-14, MitoTracker® orange, and propidium iodide, but a difference between these two stain times was detected at 20-, 40-, and 60-minute stain times (P<0.05). There was no difference

detected at 10, 20, and 40 minutes for MFI of PNA Alexa Fluor® 647 for all staining temperatures apart from an increase detected for 37°C at 60 minutes compared to 17°C and 22°C staining (P<0.05).

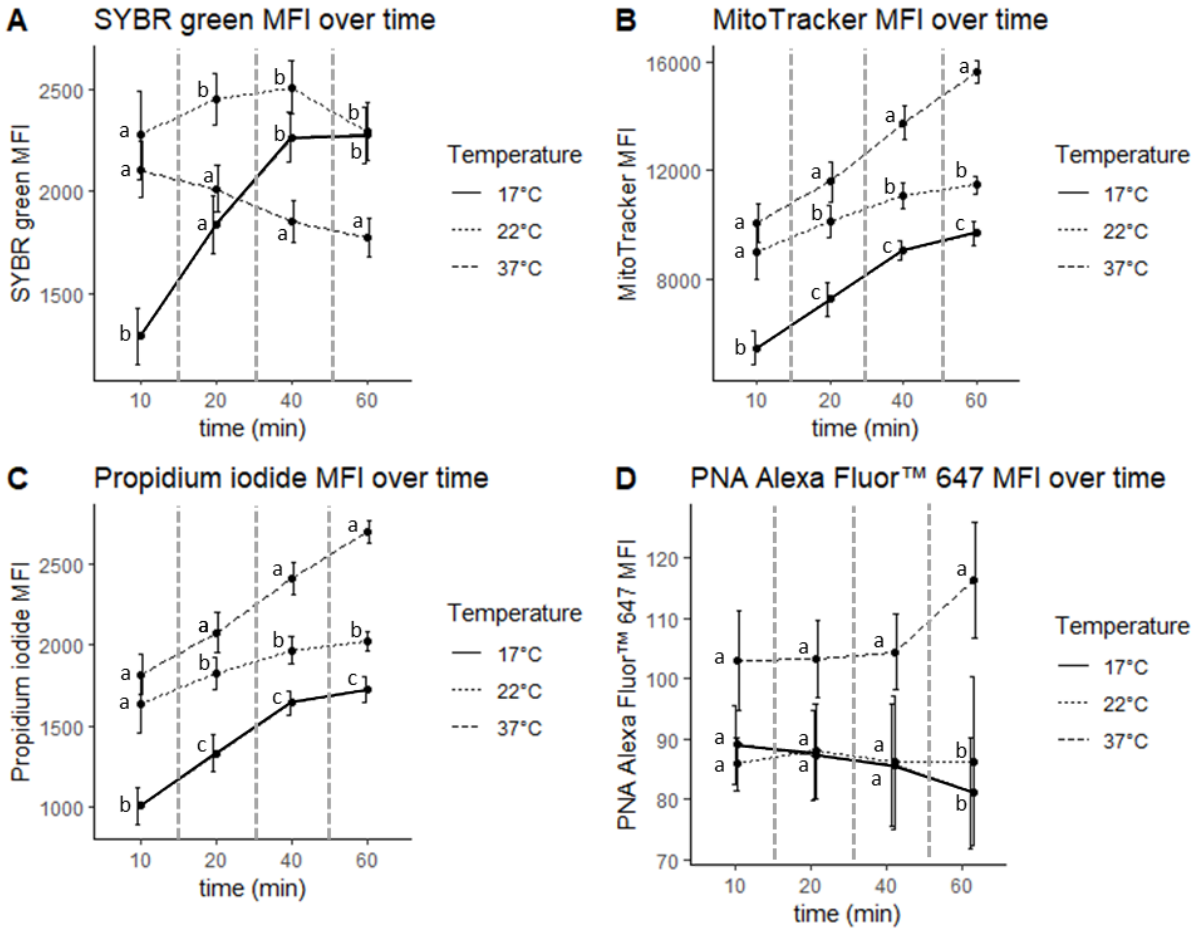

Figure 2.3. Comparing the MFI of fluorochromes over time at the staining and evaluation temperatures of 17°C, 22°C, and 37°C. Note that error bars are SD and [a-c] identifies difference between temperatures but within a time point as separated by the dashed lines (P<0.05).

2.3.3. Sublethal parameter identification

The first effort in identifying sublethal parameters was to assess conventional sperm assessment parameters and MFI from day 1 to day 4 post-collection (n = 134). There is a difference detected from day 1 to 4 in all the MFI parameters collected suggesting that they indeed change as sperm is stressed over a long period of time (Table 2.4). As expected, the

conventional sperm parameters such as percent total motility, membrane impermeability, mitochondrial membrane potential, and membrane stability have a significant decline from day 1 to 4.

Table 2.4: Sperm assay parameters on day 1 and 4 post-collection

	Day 1 post-collection	Day 4 post-collection
Total motile (%)	70.3 ± 3.6^{a}	59.6 ± 3.6^{b}
SYBR-14 MFI	2521.4 ± 387^{a}	2272 ± 348^{b}
Membrane impermeable (%)	87.6 ± 1.6^{a}	80 ± 1.4^{b}
Mitotracker MFI	4565.9 ± 379^{a}	3979.5 ± 329^{b}
Mitochondrial potential (%)	83.4 ± 2.2^{a}	72.4 ± 1.8^{b}
PNA MFI	946.1 ± 65.6^{a}	1391.1 ± 96^{b}
Acrosome intactness (%)	83.8 ± 1.5^{a}	77.2 ± 1.4^{b}
M540 MFI	587.8 ± 24^{a}	615.9 ± 25^{b}
Membrane stability (%)	72.9 ± 2^{a}	65.2 ± 1.7^{b}
Sample TAR value	41.7 ± 1.6^{a}	40.3 ± 1.5^{a}

Table 2.4 compares sperm quality parameters, fluorochrome MFI, and TAR value one both day 1 and 4 post-collection and cooled storage at 17°C. Results are the mean values ± SEM; $^{a-b}$ Different superscripts in the same row indicate differences between age day ($P<0.01$; $n = 134$).

The temperature within the boar housing facility ranged between 18.7° and 27.8°C from June to August of this study. Mean MitoTracker® fluorescent intensity and TAR were found to be correlated to the average temperature 55 days prior to collection (Table 2.5). A negative correlation between progressive motility with average temperature on day 15 prior to collection was observed. Meanwhile, there is positive correlation with mean MitoTracker® fluorescent intensity and the temperature on day 15 prior to collection. Mean fluorescent intensity of M540 is negativity correlated with the average temperature 10 days prior to semen collection. The average temperature on day 5 post collection had a moderate negative correlation to SYBR mean

fluorescent intensity. On the day of semen collection, there is very little relationship to average temperature and sperm analysis parameters.

Table 2.5: Correlation between average temperature on days prior to sperm collection and sperm health parameters measured one day post-collection.

	Day prior to collection					
	0	1	5	10	15	55
Total motility	-0.20	-0.25	-0.31	-0.30	-0.38	-0.12
Mitochondrial Potential	0.01	0.07	0.01	0.05	-0.07	0.07
Viability	0.07	0.13	0.05	0.18	-0.03	0.18
Acrosome intactness	-0.10	0.00	0.08	0.04	-0.07	0.11
Stable membrane	-0.09	0.09	0.05	0.00	-0.08	0.15
Mean SYBR	-0.16	-0.29	-0.48	-0.20	-0.13	-0.06
Mean Mitotracker	-0.04	0.01	0.03	0.30	0.50	0.57
Mean PNA	-0.18	-0.29	-0.22	-0.22	-0.06	-0.26
Mean M540	-0.38	-0.21	-0.15	-0.42	-0.39	-0.35
TAR	-0.06	0.06	-0.06	0.15	0.31	0.59

Spearman correlation coefficients generated for this table where >0.7 or <-0.7 represent high correlation. Moderate correlations where between 0.4 to 0.69 or -0.4 to -0.69 for positive and negative correlations respectively.

2.4. Discussion

2.4.1. Four-panel flow cytometry validation

The first objective of this study was to determine if a mitochondrial membrane potential labeling fluorochrome (MitoTracker® orange) could be incorporated into a co-stain protocol which includes SYBR-14, propidium iodide, and PNA conjugated Alexa Fluor® 647. To date, minimal effort has been given to the development and implementation of multiple fluorochrome assays that incorporate four or five fluorochromes such as the inclusions mitochondrial membrane potential probes overlaid with those that evaluate membrane permeability and acrosome integrity [82, 83]. The present study demonstrated that a mitochondrial membrane potential fluorochrome such as MitoTracker® orange can be incorporated into the commonly

used 3-panel as described above. With that said, there are many combinations of fluorochromes that can be used in co-staining protocols, but their exploration was outside of the scope of this study. Thus, there is potential for further work in designing and validating larger co-stain protocols for sperm.

It is likely that this 4-panel was made possible by the number of lasers and bandpass filters in the ZE5 flow cytometer used in the present study. On a flow cytometer with fewer lasers and bandpass filters, the 4-panel presented may have some compensation interference due to some overlap in the emission of propidium iodide and MitoTracker® orange. Lack of access to instruments like the ZE5 may be one of the reasons why larger co-stain protocols are not frequently explored because addition lasers also come at an additional cost. The use of 4-panel co-staining protocols using flow cytometers equipped with only 3 lasers have been developed for simultaneously assessing membrane permeability, acrosome integrity, and mitochondrial membrane potential using SYBR-14, propidium iodide, PNA conjugated fluorochromes, and MitoTracker® deep red and validated for use in bulls, bucks, and rams [83, 98, 99]. Because the present study had more acceptable lasers, we were able to avoid the need to excite fluorochromes using the same laser such as 488nm laser being used to exit both SYBR-14 and PNA above.

2.4.2. Flow cytometer temperature and staining time

The staining temperature was found to impact the flow cytometric evaluation of sperm membrane permeability, acrosome integrity, mitochondrial membrane potential, and fluorochrome MFI. When staining sperm at 37°C, degradation in acrosome integrity and mitochondrial membrane potential was observed over time while there was a simultaneous increase in membrane permeability. This was likely due to increased sperm metabolic activity leading to increased ROS production resulting in premature capacitation [100]. It appears that the

increased metabolic activity of higher temperatures also impacts the MFI of the fluorochromes where the MFI increases over time, particularly with respect to 37°C staining. At the same time, sperm staining at 17°C and 22°C was able to yield similar sperm quality parameters over time. These findings indicate that cooled boar sperm should not be rewarmed for flow cytometric evaluation.

While the use of a multi assay approach by evaluating motility, membrane permeability, acrosome integrity, and mitochondrial membrane potential bolsters the ability to detect potential fertilizing issues [79, 82, 83], it may be possible to utilize the mean fluorescent intensity of these probes to obtain information on sublethal modifications. Early work performed by D'Cruz et al. (1995) has previously demonstrated that the mean fluorescent intensity of lectin conjugated fluorochromes are useful in determining the degree of acrosomal cap degradation rather than a simple binomial assessment of non-reacted versus reacted acrosomes [48]. Similarly Treulen et al. (2018) utilized the mean fluorescent intensity of tetramethylrhodamine methyl ester perchlorate accumulation in the mitochondria as a method of determining overall mitochondrial membrane potential of the population [49]. The family of MitoTracker®'s are also known to accumulate in active mitochondria which offers an opportunity to assess the level of activity through MFI evaluation. Because these methods are used to assess the quality of the population of interest, they may be useful as markers in determining the presence of sublethal sperm modifications that could impact male fertility. Further work will need to be performed to evaluate their implications on actual fertility.

When evaluating the MFI of sperm populations over time at the different staining temperatures, it is clear that 17°C was at a disadvantage over other staining temperatures. The 17°C samples started at a significantly lower MFI for all fluorochromes except PNA conjugated

Alexa Fluor® and it toke up to 40 minutes to reach the same MFI that 22°C and 37°C stained samples start with at 10 minutes. The significant increase in mean MFI for PNA conjugated Alexa Fluor® after 60 minutes staining would suggest that 22°C staining and evaluation of cooled boar sperm may be ideal. This conclusion incorparates the suggestion that elevated temperatures during evaluation may induce premature capacitation and subsequent acrosome reaction.

Because MFI is in arbitrary units, to employ its use in the routine analysis of sublethal parameter evaluation may require a set of reliable testing standards. This may be in the form of frozen-thawed semen where the quality parameters are known to be within five percent from straw-to-straw. A killed sample may also prove useful as an MFI standard but would likely only confirm the MFI in the extremes such as very high propidium iodide or very low MitoTracker®. These standards may also be necessary to account for the drifting of laser voltages over the life the instrument and changes in fluorochrome lot numbers. As such, standards should be routine practice for any flow cytometry assay regardless of the use of MFI.

2.4.3. Sublethal parameter identification

The present study evaluated the MFI of fluorochromes for healthy populations on both days 1 and 4 post-collection to confirm that these values will change when under stressful conditions. Sperm are susceptible to damage by reactive oxygen species over several days of liquid cooled storage which may contribute to the sublethal modifications over time making this an ideal method for determining shifts in MFI [88, 101]. The results demonstrate that there were significant shifts in MFI for each fluorochrome evaluated which suggests that the MFI will indeed shift with changes in sperm quality. As mentioned above, more work in quantification of ideal values may need to be explored with the use of reliable standards for MFI.

The sensitivity of sperm to their immediate environment may lead to sublethal modifications [84]. These modifications can occur in the male reproductive tract, during in vitro handling of sperm samples, and within the female reproductive tract [84, 102]. While in the above environments, sperm come into contact with fluids, epithelial cells, and immunocompetent cells from host regions of the epididymis, uterus, and oviducts [102]. These fluids are often meant to support the sperm up to the point of fertilization [102]; however, factors such as high or low temperatures may alter the functionality of sperm and ultimately impact fertility outcomes prior to ejaculation [85].

The relationship between in vitro temperature changes and sperm quality is well understood [85], but the present study aimed to determine what sublethal impacts the environmental temperature of the boar facilities have on sperm during spermatogenesis and transport through the epididymis. Accordingly, MFI of the fluorochromes was explored to determine if there is efficacy in utilizing this information to detect sublethal modifications induced by boar housing temperature.

Extreme environmental temperatures above 30°C are known to impact spermatogenesis which can lead to testicular degeneration, decreased libido, lower sperm concentrations, decreased sperm volumes, and increased sperm morphological abnormalities [86, 103]. Furthermore, tolerance to heat decreases as boars age which likely makes them susceptible to negative reproductive performance between the temperatures of 20°C and 30°C [86, 103]. In the present study, the average temperature of the climate-controlled facility fluctuated between 18.7° and 27.8°C for the duration of the study. This range is considered to be within optimal temperature conditions for housing boars [86].

Using sperm data from 134 ejaculates measured for motility, membrane permeability, acrosome integrity, mitochondrial membrane potential, MFI of fluorochromes, and TAR, correlations with the average temperature of the boar housing facility from 55 to 0 days before semen collection were generated. Interestingly, sperm quality parameters such as membrane permeability, acrosome integrity, membrane stability, and mitochondrial membrane potential had little to no correlation to boar housing temperature. However, TAR and the MFI of SYBR-14, MitoTracker® orange, and M540 were found to show a relationship to housing temperature on different days. This may be due to the fact that percent results are qualitave measure where MFI may be viewed as a quantative measure for the fluoraphore being utlized. Thus, correlations differed in the MFI were they did not in rest of the sperm quality parameters because there was more or less staining activity.

Assessment of membrane stability is used in a research setting to determine the capacitation status of sperm and, in turn, offers insight into the surface modifications of sperm [78, 84]. The sublethal instability of the sperm membrane is characterized by the scrambling of phosphatidylserine of the sperm membrane which initiates premature capacitation and alters sperm longevity both in vitro and within the female environment [74]. Because M540 intercalates into the lipid bilayer of the sperm membranes [104], mean fluorescent intensity of M540 was used to determine the overall stability of the viable population. A viable population that has a mean shift in M540 may be an indicator of sublethal modifications.

There was a moderate negative correlation between decreasing M540 MFI and increasing average temperature that intensifies from day 55 to day 10. The correlation between these two parameters decreses abruptly between day 5 and the day of collection. A decreased MFI of M540 indicates that the sperm population tested has more stable membranes. The relationship between

increased temperature and decreased M540 fluorescence may suggest that temperature induces beneficial surface modifications that will aid in membrane stability maintenance until ejaculation. Thus, sperm coming into contact with proteins of the male tract released in response to temperature may aid in maintaining membrane stability [84]. While this finding is not considered to be sublethal, the understanding of this relationship may aid in boar stud management practices and may warrant further investigation.

The mean fluorescent intensity of Mitotracker® and TAR were both found to be positively correlated with average temperature 55 days prior to sperm collection (Table 2.5). Upon further investigation, these two parameters were found to be correlated to each other at 0.70 (P<0.05) which may suggest antioxidant induced relationship to early mitochondrial health. Like M540, the magnitude of the correlation decreased as it reached day 10 prior to collection.

Changes in seasonality and temperature are known to impact the natural occurrence of antioxidants within the epididymis and the seminal plasma at the time of ejaculation which may include catalase, superoxide dismutase and glutathione peroxidase [25]. Without adequate concentrations of antioxidants within the epididymis and in seminal plasma, sperm production of reactive oxygen species is not balanced with the reduction by antioxidants [88, 101]. This may lead to oxidative stress and alterations to sperm motility, membrane stability, and mitochondrial membrane potential [87, 88]. While the present study demonstrates that TAR and the MFI of MitoTracker® may be important markers for subfertility, further work is required to understand the role their relationship has on the early maintenance of sperm mitochondria.

In the present study, the lectin PNA conjugated Alexa Fluor® 647 was used to label exposed beta-galactose moieties on the surface of the acrosomal cap [105]. According to these results, the MFI of Alexa Fluor® did not appear to be an important marker for subfertility as it

relates to ambient temperature prior to ejaculation. A correlation was observed with the SYBR-14 MFI 5 days prior to collection. The use of SYBR-14 was employed for this part of the study rather than propidium iodide because the passage of these fluorochrome may be more illustrative as this fluorochrome labels and fluoresces viable sperm more brightly than damaged cells [106, 107]. With that said, the mechanisms for SYBR-14 entry into cells remains unclear making further discussion and investigation difficult at this time. Nonetheless, the remaining fluorophores show promise in future studies in identification of subfertility using MFI analysis.

2.5. Conclusion

To date, co-staining protocols with greater than 3 fluorochromes has been limited [83]. The present study was able to validate one such co-stain protocol that enables the evaluation of sperm flow cytometrically using four fluorochromes to simultaneously gather information on membrane permeability, acrosomes integrity, and mitochondrial membrane potential. In addition, advances in flow cytometer capabilities such as temperature-controlled stages with plate reading machinery, warranted investigation into proper staining time and temperature for cooled boar sperm. Thus, the findings of the present study suggest that cooled boar sperm is ideally stained and evaluated at 22°C for up to 60 minutes on a plate reader but similar data can also be obtained using 17°C for 60 minutes and for up to 20 minutes at 37°C.

The present study made progress in the initial understanding of how boar housing temperatures may impact sperm before ejaculation. However, further work assessing the boar housing temperature during spermatogenesis on antioxidant induction, and the relationship between high mitochondrial membrane potential and sperm membrane stability is needed. Furthermore, housing temperatures, even within suggested optimal ranges, impacts the MFI of fluorochromes and motility but appears to have little effects on the sperm quality parameters

such as membrane permeability, acrosome integrity, membrane stability, and mitochondrial membrane potential.

In conclusion, the routine assessment of total motility may not be powerful enough to detect boars or ejaculates that may be subfertile. Adopting a multi-assay approach coupled with assessment of fluorochrome MFI offers opportunities to improve potential fertility outcomes. Thus, larger flow cytometry panels, such as the one presented, provides researchers with more tools in one assay necessary for evaluating subfertility.

CHAPTER 3: THE IMPACTS OF BOAR SPERM ANTIOXIDANT SUPPLEMENTATION ON FERILITY

3.1. Introduction

Artificial insemination of sows using semen cooled at 15°C to 17°C and stored for up to 5 days is the preferred method used for commercial pork production [72, 73, 75]. In contrast to natural matings, insemination using liquid semen offers several advantages such as protection of nucleus lines in separate facilities, distribution of superior genetics across herds, and increase profitability of each boar ejaculate [75]. In recent years, the development of commercially available extenders has enabled lower sperm concentration per dose to around 2.75 to 3 billion sperm/dose while maintaining optimal sow fertility [75, 108]. Extenders are formulated to preserve sperm by using sugars, buffers, membrane stabilizers, and basic salts to maintain physiological conditions over time [88].

Sperm undergo a natural aging process which contributes to an increase in oxidative stress resulting in a decrease in viability and motility [88, 101]. This is likely due to the induction of tyrosine phosphorylation causing capacitation-like modifications such as lipid reorganization, and increases in calcium permeability during cooling and storage [101, 109-112]. These modifications are exacerbated when the production of reactive oxygen species (ROS) is not balanced with the reduction by antioxidant scavengers leading to loss of sperm motility, membrane stability, mitochondrial membrane potential, and DNA integrity [87, 88]. As a result of the above sperm damage, ROS concentration may increase without proper reduction by antioxidants.

Though some commercially available extenders are labeled with a 5 to 7 day shelf life, changes in aged sperm quality over time leads to decreased fertility, particularly when assessing

litter size [50, 88, 113]. Findings by Ratto and Jokinen (1991) demonstrate a decline in conception rate and litter size when using semen aged to day 5 post-collection compared to semen aged to 1 day extended in MR-A® [114]. Another study using Androhep demonstrated significantly reduced farrowing rate using semen aged to day 6 ($P<0.001$) and significantly reduced litter size when using semen aged to day 4 and beyond ($P<0.05$) [44]. Because of the variability in semen quality and fertility outcomes of aged semen, doses are often used by day 3 post collection [44, 75]. Therefore, improving the shelf life of boar semen remains a priority to the pork production industry and genetic centers.

Because the polyunsaturated fatty acids of boar sperm are highly susceptible to oxidative stress [17], improvements in cooled aged sperm viability supplemented with antioxidants have been observed [2, 25, 61]. Yet very few studies take these finding to mating trials. Antioxidants previously demonstrating boar sperm protection from oxidative stress include butylated hydroxytoluene, α-tocopherol, ascorbic acid, glutathione peroxidase, superoxide dismutase, catalase, and plant extracts [2, 25, 61]. The use of these antioxidants have been shown to reduce lipid peroxidation and increase fertilizing capacity of boar sperm [2, 20]. The use of plant extracted antioxidants are an attractive choice as extender additives because they often contain a blend of naturally occurring antioxidants and reduces the variation seen in single antioxidant supplementation. Plant extracted antioxidants also reduces the need to rely on animal products such as bovine serum albumin to protect against oxidative stress and cold shock [88, 115]. Another benefit to plant extract supplementation is delaying capacitation by decreasing the concentration of ROS [70].

GameteGuard®, a natural blend of plant extracted antioxidants, has been previously shown to maintain sperm quality of liquid stored stallion sperm [116]. GameteGuard® has also

been demonstrated to improve the post-thaw sperm quality of bulls [117], and bucks [118]. In bulls, the improvement of sperm quality when cryopreserved using GameteGuard® resulted in a 20% increase in pregnancy per insemination [117]. Thus, this study aims to assess the impact on semen shelf life, conception rate, farrowing rate and litter size by supplementing commercially available extender (Androstar Plus®) with GameteGuard®-CP (cooled porcine formulation) during a large field trial using semen aged to day 4 post-collection from commercial Duroc boars. We hypothesize that antioxidant supplementation will result in maintained sperm quality over 4 days of storage while also improving conception rate, farrowing rate and litter size when breeding sows with aged semen.

3.2. Materials and methods

3.2.1. Animal handling

Animal handling, semen collection, and sow insemination was performed according to the National Institutes of Health guide for the care and use of animals and under Colorado State University IACUC approval (CSU IACUC #: 18-7948A). Sixteen commercial Duroc boars 10 to 12 months of age were used for the duration of this study. All boars were housed in the same facility and fed a commercial pelleted diet. Commercial sows of parities ranging from one to four were enrolled in the study (n = 1476). All sows were fed a commercial pelleted diet and housed in one of two separate facilities.

3.2.2. Chemicals, Reagents and Disposables

All research grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Analytical reagents such as flow cytometry fluorophores were purchased from Thermo Scientific (Carlsbad, CA) unless otherwise stated.

3.2.3. Extender preparation

Extender (AndroStar Plus®, Minitube USA, Inc.) was prepared according to manufactures recommendation (47 g/L in deionized water). The extender was mixed and warmed to 35°C on the day of use. An aliquot of AndroStar Plus® was added to another 35°C vessel for treatment addition of GameteGuard®-CP (Membrane Protective Technologies Inc., Fort Collins, CO). GameteGuard®-CP was allowed to thaw in the dark for 30 minutes before 1g/L was added to the extender. Both extenders were held at 35°C for same day use.

3.2.4. Boar collection

The 16 boars were collected weekly over 10 total weeks using the BoarMatic automated collection technique to extract the sperm rich fraction as described by Mills et al. [119]. Initial motility, morphology and sperm concentration was assessed at the boar stud prior to splitting the ejaculate into control and treatment. Minimum standards were motility (75%) and normal morphology (75%). The split ejaculate was extended to 1.5 x 10^9 sperm/dose in control or treatment in 75 mL doses. Two doses from each boar and treatment were sent overnight at 17°C to the lab for sperm analysis on day 1 and 4 post-collection. The remaining doses were aged to day 3 post-collection at the boar stud in a 17°C room. Semen doses were turned once a day at the stud and then shipped to the sow farms for insemination on day 4 post-collection.

3.2.5. Sow insemination

Sows of parities ≥1 were subject to a day 4 post-weaning ovulation synchronization protocol using OvuGel® (United Animal Health, Inc., Sheridan, IN). Estrus detection was performed on day 4 post weaning using a teaser boar. Only sows exhibiting signs of estrus were given an intravaginal 2 mL dose of OvuGel®. Sows that did not receive OvuGel® were not enrolled into the present study. Insemination of sows was performed one time 24 hours post

OvuGel® administration using post cervical artificial insemination catheters (IMV Technologies, Maple Grove, MN). A single dose for each boar/treatment was balanced equally among sows of different parities and genetic lines. Sows that did not return to estrus after 21 days were examined for pregnancy by transabdominal ultrasound at 35 days post insemination. The pregnant sows remained enrolled in the study until farrowing where total litter size, born alive, stillborn, and mummies were recorded (n = 1476).

3.2.6. Motility evaluation

Sperm motility was assessed on both day 1 and 4 post collection. Semen aliquots of 200 µL for each sample were incubated at 37°C for 10 minutes to allow cells to equilibrate [44]. Each sample was analyzed on a 4-chamber 20 µM depth slide (LEJA; Spectrum Technologies, Healdsburg, CA) using computer assisted sperm analysis (CASA, IVOS II, Hamilton Thorne Ltd, Danvers, MA). For each sample, >400 cells were examined in duplicate (>800 total cells). The CASA system captured 60 frames/sec and settings comprised of 30 frames acquired; max and min head size at 50 μm^2 and 10 μm^2 respectively; default cell intensity at 150; progressive cells threshold straightness at 67%; velocity along average path (VAP) cut-off at 40 µm/s; slow cell VAP cut-off at 15µm/s; and slow straight-line velocity (VSL) cut-off at 10 µm/s.

3.2.7. Flow Cytometric evaluation

Simultaneous with CASA evaluation, samples were evaluated on a ZE5 flow cytometer (Bio-Rad Laboratories, Hercules, CA) operating at 0.5 µL/s, to acquire 10,000 events. Calibration of the instrument prior to every use was performed with flow cytometry calibration beads. Data were collected in duplicate on a 96-well-plate in 125 µL volumes ($2x10^6$ sperm/mL). This instrument was equipped with multiple lasers and bandpass filters for evaluating samples using a variety of fluorochromes in co-stained protocols. All assays below were analyzed using

FlowJo software (BD Biosciences, San Jose, CA) by utilizing gating schemes set in logarithmic scale (gating described below for each assay).

A 4-panel staining protocol which enables boar sperm to be analyzed for membrane permeability, acrosome integrity, and mitochondrial membrane potential was utilized. Briefly, sperm samples of 12.5 µL were added to 112.5 µL of 10 mM HEPES buffer containing 1.5 mM BSA, SYBR-14 (0.1 µM, 488 nm; 525/35 bp), propidium iodide (12 µM, 561 nm; 615/24 bp), PNA conjugated Alexa Flour 647® (6.5 µg/mL, 640 nm; 670/30 bp), and MitoTracker® orange (40 nM, 561 nm; 577/15 bp). Samples were stained at 22°C for 10 min in the dark then subject to acquisition on the flow cytometer. Sperm populations were considered viable with intact acrosome when SYBR (+), propidium iodide (-), and PNA (-). Herein, these populations will be used to define membrane and acrosome integrity. Populations with healthy functioning mitochondrial membrane potential were identified by PNA (-) and MitoTracker orange (+) events.

Membrane stability can be used to determine the capacitation status of sperm by assessing the stability of cell membranes using a co-stain merocyanine (M540) assay [92]. For this assay 12.5 µL of extended semen was added to 112.5 µL of HEPES mentioned above containing Hoechst 33342 (80 µg/mL, 375 nm; 447/60 bp), YOPRO-1 (0.4 µM, 488 nm; 525/35 bp), M540 (5.12 µM, 561 nm; 577/15 bp). Samples were stained at 22°C for 15 min in the dark then data was collected in duplicate on a 96-well-plate using the flow cytometer. Populations with stable membranes were defined by YOPRO-1 (-) and low mean fluorescent intensity of M540.

A sperm chromatin dispersion assay, created for measuring sperm cell chromatin intactness, was used to determine the percent sperm DNA fragmentation of a sample. Using the

protocol described by Ballachey et al. [120], samples were diluted to $8x10^6$ sperm/mL in a TNE solution then 400 µL of Triton X/HCl solution was added to each diluted sample and allowed to incubate for 30 seconds. Samples were then added to 1.2 mL of acridine orange stain solution containing 10 µL of acridine orange (20 µg/mL, 488 nm laser), disodium phosphate (200 mM), sodium chloride (150.6 mM), ethylenediaminetetraacetic acid (12.6 mM), citric acid (100 mM), and incubated for 5 minutes before data collection. A red long pass filter (750 nm bp) was utilized to identify events that have shifted towards red fluorescence which is indicative of fragmented DNA.

3.2.8. Total antioxidant reactivity

A 4 mL aliquot of each boar's treatment and control was utilized to determine the total antioxidant reactivity (TAR) on both day 1 and day 4 post collection. All samples were stored at -80°C then analyzed together. A luminol-based assay detected the luminescence of antioxidant reduction of ROS using a microplate luminometer (Promega, Madison, WI). A TRIS buffered solution (263 mM Trizma Base, 86.3 mM Citric Acid), detection solution (1 mM luminol in 50% DMSO:H2O), catalyzing solution (50 mL of 300 mM H_2O_2 in TRIS buffered solution), positive control (0.01 µg/mL Trolox), and samples being tested were used in this catalyzed reaction. Samples were added to a 96-well plate in a dilution series, the catalyzing solution was added, then the luminometers autoinjector administered the detection solution. The Trolox positive control was used to calculate TAR by deriving the equivalent reactivity value or percent ROS removed. The amount of ROS removed was calculated by the percent difference in relative luminescence from the sample compared to a negative control containing deionized water and catalyzing solution then TAR value was mathematically derived using the following equation [93]

$$TAR = \left(\sum ki\ [xi]\right) / k_{Trolox}$$

where ki is the amount of ROS removed at the i-th position, xi is the concentration of the sample being tested at the i-th position and k_{Trolox} is the total reactivity of Trolox using equation $(\sum ki\ [xi])$.

3.2.9. Statistical analysis

All statistical analysis was performed using R version 3.5.1 through RStudio [94, 95]. After subjecting the data to a normality and variance equality test, a linear mixed model was used to detect differences in sperm parameters where treatment was used as the fixed effect and boar was used as the random effects ($\alpha = 0.05$). Conception rate (CR) and farrowing rate (FR) were assessed using a chi squared method. A generalized linear mixed model with a Poisson regression was used to assess the differences in litter data (total born, born alive, stillborn, and mummies) where treatment represents the fixed effect and the random effects included boar, boar to treatment interaction, sow parity, sow genetic line, farm location (two farms used), and breed date ($\alpha = 0.05$) [96, 121, 122]. A chi squared method was also used to detect differences in litter data.

Ejaculate response to treatment was determined using a weighted ranking of parameters impacted by antioxidants (DNA intactness, mitochondrial membrane potential, and membrane impermeability). Ejaculates were assigned a group according to weighted ranking of antioxidant GameteGuard®-CP response (no response, low response, moderate response, high response to GameteGuard®-CP treatment) using the delta between control and treatment of intact DNA, mitochondrial membrane potential, and membrane impermeability. The litter data was then subject to the same litter generalized linear mixed model as described above ($\alpha = 0.05$).

3.3. Results

A total of 134 ejaculates originating from 16 boars were used for sperm analysis in this split ejaculate study. The sperm parameters on day 1 post-collection largely do not differ between treatments with the exception of total percent motility (Table 3.1, $P<0.01$). On day 4 post-collection, the day the sperm was used for mating, there was a significant difference detected between treatments for motility, membrane integrity, membrane stability, acrosome intactness, mitochondrial membrane potential, and DNA intactness. Difference between control and GameteGuard®-CP TAR values was also detected on both day 1 and 4 post-collection (27% and 28% improvement of each age day, $P<0.01$).

When comparing sperm between age (from day 1 to 4) within the GameteGuard®-CP treatments, there was only difference detected in total motility while the rest of the sperm parameters maintain quality over the age days (Table 3.1). This was not observed with the control as a difference was detected in control doses for all sperm quality parameters measured between age days ($P<0.01$).

Table 3.1: Sperm health assessment parameters on day 1 and 4 post-collection

	Day 1 post-collection			Day 4 post-collection		
	CO[a]	GG[b]	P value	CO[a]	GG[b]	P value
Total motile (%)	68.2 ± 5.9	53 ± 4.6	<0.0001	51.4 ± 4.4	41.6 ± 3.6	<0.0001
Membrane intactness (%)	87.6 ± 1.6	87.1 ± 1.6	0.966	79.0 ± 1.4	85.6 ± 1.5	<0.0001
Membrane stability (%)	72.9 ± 2	74.0 ± 2	0.929	65.2 ± 1.7	72.2 ± 1.9	0.0001
Acrosome intactness (%)	83.8 ± 1.5	83.9 ± 1.5	0.999	77.2 ± 1.4	82.2 ± 1.5	<0.0001
Mitochondrial potential (%)	83.4 ± 2.2	84.1 ± 2.2	0.968	72.4 ± 1.8	82.8 ± 2.1	<0.0001
DNA intactness (%)	94.1 ± 1.2	95.1 ± 1.3	0.746	90.4 ± 1.2	94.3 ± 1.2	0.0001
TAR value	41.9 ± 1.6	52.5 ± 2	<0.0001	40.3 ± 1.5	51.2 ± 2	<0.0001

a Control extender (AndroStar Plus®) represented by CO where mean values for sperm quality are ± SEM

b GameteGuard®-CP represented by GG where mean values for sperm quality are ± SEM

Conception rate and farrowing rate were significantly improved in sows inseminated with sperm treated with GameteGuard®-CP (Table 3.2, $P<0.05$). This translates to a 7.4% and 9.7% improvement in conception rate and farrowing rate, respectively. Pregnancy loss from conception to farrowing was reduced 21.2% using antioxidant supplementation with GameteGuard®-CP ($P<0.05$).

Table 3.2: Effect of GameteGuard®-CP on conception rate, farrowing rate and pregnancy loss.

	Treatment		
	Control	GameteGuard®-CP	P value
Total number of sows[a]	736	738	-
Conception rate (%)[b]	70.0	75.2	0.03
Farrowing rate (%)[c]	64.0	70.2	0.01
Pregnancy loss rate (%)[d]	9.3	7.1	0.30

a total number of sows inseminated for control and GameteGuard®-CP

b Percent of sows that positive for pregnancy by 35-day ultrasound for control and GameteGuard®-CP

c Percent of sows farrowed for control and GameteGuard®-CP

d Percent of sows that failed to farrow after being confirmed pregnant by 35-day ultrasound for control and GameteGuard®-CP

As a result of improved conception and farrowing rates using GameteGuard®-CP, the total number of piglets born also increased by 4.2% and represented 276 additional piglets ($P<0.05$, Table 3.3). The total piglets born alive increased by 5% and represented 293 more piglets born alive when using GameteGuard®-CP ($P<0.05$, Table 3.3).

Table 3.3: Chi-squared evaluation of GameteGuard®-CP on litter outcomes

	Treatment		
	Control	GameteGuard®-CP	P value
Total Born[a]	6596	6872	0.02
Born Alive[b]	5845	6138	0.007
Stillborn[c]	534	498	0.26
Mummies[d]	217	236	0.37

A
Total number of piglets born for control and GameteGuard®-CP
b
Total number of piglets born alive for control and GameteGuard®-CP
c
Total number of stillborn piglets for control and GameteGuard®-CP
d
Total number of mummified piglets for control and GameteGuard®-CP

GameteGuard®-CP did not increase mean litter size, or the mean number born alive (Table 3.4; $P<0.05$) in aged semen yet the average number of still born piglets was reduced using GameteGuard®-CP ($P<0.05$). No differences were detected among the number of mummy piglets born.

Table 3.4: Effect of GameteGuard®-CP on mean litter data when using aged semen doses

	Treatment	
	Control	GameteGuard
Sows Farrowed	471[a]	518[b]
Farrowing measures		
Total born	13.8±0.02[a]	13.1±0.02[a]
Born alive	12.2±0.02[a]	11.7±0.02[a]
Stillborn	1.20±0.07[a]	1.02±0.07[b]
Mummified	0.47±0.09[a]	0.46±0.09[a]

Results are the mean values; [a–b] Different superscripts in the same row indicate farrowing measure differences between treatment ($P<0.01$).

Table 3.5 demonstrates the mean litter size within response groups as assigned by ejaculate response to GameteGuard®-CP after in vitro semen evaluation. Ejaculates that respond

positively to GameteGuard®-CP (High GG response), were found to have a significant increase in litters size by 1.8 piglets/litter ($P<0.05$).

Table 3.5: Litter size data by ejaculate response after in vitro sperm evaluation (n=134 ejaculates).

	Treatment		
	Control	GameteGuard®-CP	P value
Ejaculate GameteGuard®-CP response			
High response[a]	9.2 ± 0.92	11 ± 1.04	0.003
Moderate response[b]	12 ± 1.41	11.8 ± 1.38	0.67
Low response[c]	14.3 ± 0.57	13 ± 0.52	0.002
No response[d]	13.9 ± 0.69	13.6 ± 0.68	0.50

a Mean total piglets born of control and GameteGuard®-CP for ejaculates with a high response to antioxidant supplementation ± SEM

b Mean total piglets born of control and GameteGuard®-CP for ejaculates with a moderate response to antioxidant supplementation ± SEM

c Mean total piglets born of control and GameteGuard®-CP for ejaculates with a low response to antioxidant supplementation ± SEM

d Mean total piglets born of control and GameteGuard®-CP for ejaculates with a no response to antioxidant supplementation ± SEM

3.4. Discussion

Cooled extended boar sperm have steady, but decreased, metabolic activity [112] resulting in continued ROS production at 17°C [72, 73, 101] leaving sperm membranes exposed to oxidative stress [123]. Oxidative stress leads to damage in sperm quality and as a result, boar semen doses are commonly used for artificial insemination prior to three days post-collection [88]. The relationship between sperm quality and age of semen has led genetic centers to focus on solutions to improving the shelf life of boar semen while maintaining conception rate and litter size. In this and previous studies, antioxidant supplementation using GameteGuard®-CP

showed promise in increasing the shelf life of commercial extender demonstrated by maintaining sperm quality over time [116].

Compared to other studies using plant extracted antioxidants, the present study was substantially larger than other trials where previous work utilized ≤10 boars and ≤300 sows [112, 124]. To date, single antioxidant and plant extracted antioxidant supplementation in cooled stored boar semen has been limited to in vitro sperm assessment [70, 125, 126] and in vitro fertilization trials [14, 68]. Even fewer antioxidant supplementation studies incorporated relatively small mating trials [112, 124]. The inconsistency of data from single antioxidant supplementation may have led to decreased incidence of mating trials, and underscores effectiveness of an antioxidant blend such as GameteGuard®-CP. The use of 16 boars and >1400 sows in the present study demonstrates the potential for commercial application of GameteGuard®-CP within the genetic centers.

A luminol assay was performed to assess the total antioxidant reactivity (TAR) of each dose on day 1 and 4 post-collection. Using this method, significantly higher TAR was observed in GameteGuard®-CP supplemented doses confirming the antioxidant properties of GameteGuard®-CP which were capable of reducing ROS. Limited antioxidant activity was detected in control doses. This was to be expected as residual antioxidants present in a seminal plasma and low levels of antioxidant compounds in commercial extenders may account for this activity. These data suggest that the natural blend of antioxidants found in GameteGuard®-CP may be able to maintain sperm quality during aging by mitigating the effects of ROS.

Overall viability (intact acrosome and membranes) was maintained in aged GameteGuard®-CP treatments. This improvement using plant extracted antioxidant supplementation is also in agreement with finds by Ros-Santaella et al. [14] using Honeybush

(*Cyclopia intermedia*) extract in cooled stored boar semen held at 17°C for 120 hours. In this study, 12.5 µg/mL Honeybush extract in cooled stored boar semen demonstrated a significant improvement ($P<0.05$) in motility, membrane, and acrosome integrity [14]. In general, when using compound antioxidants, there was a decrease in lipid peroxidation and production of malondialdehyde [16, 19].

When peroxyl radicals rearrange to form endoperoxides, malondialdehyde (MDA) production results [19]. Malondialdehyde is a pernicious and final product of lipid peroxidation and is responsible for damage of DNA [16, 19]. Hydroxyl radicals and aldehydes such as MDA, react directly with purines, pyrimidines, and deoxyribose sugar backbones causing single and double stranded DNA breaks [19]. Li et al. (2018) observed a decrease in malondialdehyde concentration ($P<0.05$) of boar semen doses aged 72 hours at 17°C when supplemented with Oligomeric proanthocyanidins, a naturally occurring antioxidant [112]. Thus, the presence of the appropriate antioxidants will decrease MDA production as evidenced by both the luminol assay and the decrease in percent of sperm with DNA fragmentation in GameteGuard®-CP treated samples aged to 4 days compared to control.

In this study, sperm membrane stability was maintained when aged 4 days in antioxidant supplemented extender. A membrane with increased presence of the fluorophore M540 intercalated into the lipid tails of the bilayer indicates a cell with an increased degree of lipid reorganization such as that commonly observed during capacitation events [104]. In the present study, there was decreased percentage of sperm displaying M540 fluorescence in the lipid bilayer of treated sperm suggesting reduced capacitation and capacitation-like modifications that negatively affect longevity of sperm quality. These findings are in agreement with that of Desroches et al. using blueberry (*Vaccinium corymbosum)* fruit and leaf extracts in cooled liquid

stored semen stored at both 5°C and 15°C for up to 5 days. This study demonstrated that a plant extracted antioxidants have the ability to significantly delay capacitation [70]. When semen is aged, the ability for ROS reduction is limited and accumulation of ROS ensues if not properly reduced by antioxidants. It is believed that the natural blend of antioxidants found in GameteGuard®-CP and other plant extracts were able to reduce the ROS which plays a role in capacitation [70]. In this case, hydrogen peroxide is being reduced before activation of adenylyl cyclase which decreases tyrosine phosphorylation and calcium influx characterized in a capacitation event [28, 127]. This is supported by the present studies luminol assay where hydrogen peroxide was used as the source of ROS to measure its reduction and the total antioxidant reactivity in GameteGuard®-CP treated samples.

A major producer of intracellular ROS is the mitochondria, which in turn, negatively impacts the electron transport chain [24]. The high mitochondrial membrane potential is maintained by the coupling of electron transport to oxidative phosphorylation for the production of ATP [33, 100]. The decrease in mitochondrial membrane potential is believed to be attributed to the increased production of ROS such as aldehydes from lipid peroxidation and ROS produced by the mitochondria [34, 35, 100, 128]. The present study found that the mitochondrial membrane potential of sperm supplemented with GameteGuard®-CP was maintained over 4 days and suggests that ROS production is being reduced by adequate antioxidant mediated reduction.

One possible disrupter of mitochondrial membrane potential is the opening of the mitochondria membrane permeability transition pore. This permeability transition pore opens in in response to a large quantity of ROS following a process known as (ROS)-induced ROS release which is an increase in ROS production during times of excessive oxidative stress [35]. When ROS reaches the threshold capacity of the mitochondria, the pore opens causing a loss of

mitochondrial membrane potential and signaling apoptotic events [36]. Apoptotic events in sperm are not synonymous to the process of apoptosis observed in the somatic cell; however, the one similarly is that ROS induction of apoptosis increase ROS production from the mitochondria and lipid peroxidation in a self-propagating manner [129]. Thus, the apoptotic events that occur in sperm also increase the production and presence of ROS which may have a negative impact on neighboring sperm. The increased mitochondrial membrane potential observed in GameteGuard®-CP samples may indicate reduced overall ROS, leaving the transition pore inactive, mitochondrial function intact, and decreased incidents of apoptotic events.

In the present study, a decrease in sperm motility was observed with treated samples. Boar sperm are known to be sensitive to factors that influence their environment such as temperature, solutes, pH, and osmolality [101, 130, 131]. The motility decrease may be an artifact of decreased pH in GameteGuard®-CP samples yet mating trial data suggest that total motility may not be an important measure of potential fertility [132, 133]. The evaluation of motility is meant to identify potentially high or low quality sperm samples but does not account for the sperm interactions to the environments it will encounter prior to insemination and within the female reproductive tract [132]. Recall, viability as expressed by acrosome integrity, membrane integrity and mitochondrial membrane potential remained high in treated sperm which suggests that decreased motility may have been artifactual in this study. This was further supported by an increase in conception rate and farrowing rate when supplementing extenders with GameteGuard®-CP.

GameteGuard®-CP supplementation significantly improved conception rate and farrowing rate when used on day 4 post-collection which affords genetic centers one more day of shelf life and increases the profitability of a single boar's ejaculate. As a result of increased

conception rate, the total number of piglets born, and total number of piglets born alive was significantly improved despite the mean litter size remaining similar to the control. In a similar study using Oligomeric proanthocyanidins, conception rate was also found to be significantly improved when extenders were supplemented with this antioxidant [112].

It was understood that the environmental factors and semen handling variability may cause boar ejaculate variation. This presented an opportunity to assess ejaculate response to antioxidant supplementation on litter outcomes therefore ejaculates were assigned response groups following completion of sperm quality evaluation. The results demonstrated a significant increase in litter size by 1.8 piglets/litter in ejaculates that had a high response in sperm quality when supplemented with GameteGuard®-CP. This suggests that the antioxidant supplementation with GameteGuard®-CP improves the fertility outcomes of boars that would otherwise be subfertile [4, 18]. In addition, the use of GameteGuard®-CP may also enable the widespread use of single sire matings by decreasing the risk of low conception rate, farrowing rate, and litter size among potentially subfertile sperm doses. This especially adds value to the subfertile boars that have a high genetic index making them ideal candidates for technologies that aids in maintaining sperm quality and increasing shelf life of sperm, thereby maintaining high fertility outcomes.

3.5. Conclusions

The present study demonstrates that the use of the antioxidant blend of GameteGuard®-CP maintains higher sperm quality over time, thereby improving the shelf life of semen doses. The use of GameteGuard®-CP improves fertility outcomes such as conception rate, farrowing rate. This indicates that the increased shelf life of GameteGuard®-CP treated sperm allows for semen to be used over longer periods of time without significantly sacrificing fertility outcomes. The present study did not improve average litter size, but there was an improvement in total

piglets as a result of improved conception and decreased pregnancy loss using GameteGuard®-CP.

Applying this technology to known subfertile boars may have the potential to improve fertility outcomes and increases the possibility of subfertile but high index boars to be retained in genetic centers. Further work is needed to elucidate the impact of known subfertile boar and their fertility outcomes when supplemented with GameteGuard®-CP. In conclusion, the present study was a large step in understanding the impacts on sperm quality and fertility when supplementing sperm with antioxidants in a large field trial.

CHAPTER 4: A NOVEL EXPERIMENTAL DESIGN FOR BOAR SPERM CRYOPRESERVATION

4.1. Introduction

Cryopreservation of sperm is common technology in several livestock species but is not often used in commercial swine (*Sus domesticus*) production in the U.S. due to poor cryotolerance. Boar sperm quality variation, reduced fertility, and low litter size make a cryopreserved technology commercially uneconomical despite offering the same advantages found in other species including improved biosecurity, disease reduction, genetic banking, and more precise timing with ideal female fertility. Importantly, commercial farm litter sizes using the current technology for cryopreserved boar sperm results in ~8.5 piglets/litter versus ~11 when using cooled sperm [2]. This may be due in part to poor post-thaw motility and viability (25% to 60% compared to 75% to 90% motility in cooled semen [134, 135]). This suggests that a different approach is necessary in the development of a cryopreservation system for boar sperm that results in improved sperm quality, fertility outcomes, and commercial success.

Although factors that may influence the successful cryopreservation of boar sperm have been investigated in various combinations, little has been done with respect to defining the factors that should receive prioritized focus. A number of factors have been tested individually which include cryoprotectants [136-139], cooling rate [2, 140], antioxidant supplementation [20, 71, 141], equilibration time in the straw (prior to freezing) [138, 142], cryopreservation extender encompassing salts, sugars and buffers [134, 143, 144], straw volume [134, 145-147], osmolality [139, 148, 149], detergent addition [150], and stepwise addition of glycerol [151, 152].

The development of a cryopreservation systems is necessarily multifaceted, but the use of full factorial design to study factor impact on a biological response results in impractically large

numbers of experiments [153], and so the process would ultimately benefits from the rapid identification of the factors that should receive focus in follow-up experiments [154].

The use of a Plackett-Burman experimental design is an effective method of rapid identification to determine which factors are influential because it screens several factors simultaneously but is currently underutilized in many fields of study including animal and reproduction science [153, 154]. The, primary advantage to Plackett-Burman methods is a decreased number of required experiments to estimate main effects [153]. For example, to study nine factors in a full factorial design requires hundreds of experiments while a Plackett-Burman requires 12. The lower number of experiments is possible because factors are set at high and low levels and interactions between factors are ignored. Plackett-Burman design typically operates on a 4n experimental design and, in some cases, is equivalent to some factorial designs [153].

Influential factors are identified by assessing the response within a factor set at high- and low-levels [154, 155]. The high- and low-level extremes are assigned for each factor. As an example, the present study utilizes glycerol concentration as a factor; therefore, the extremes in the Plackett-Burman design will be 2% [–] and 6% [+] glycerol. Setting these factors at their extremes is important for estimating the effect of these factors during analysis and assessing whether or not they are influential [153, 154]. The use of such experimental designs are well suited for screening factors that can affect sperm quality of frozen-thawed boar sperm.

The most commonly used Plackett-Burman design is the 12-factor and 12-run design [154].The number of factors evaluated can be set between 7 and 11 and will necessarily include at least 2 “dummy” factors. If factors are fewer than 7 or above 11, the design can be shifted to an 8-run or 16-run design respectively which will also necessarily includes “dummy” factors [153]. Dummy factors, which have no physical contribution to the runs, are necessary to properly

assign runs with the correct number of high- and low-level factors and are useful in the estimation of residual variation. More dummy factors improve the estimate of the variation necessary for statistical evaluation of factor influence [153, 154]. Therefore, it is not uncommon to design a Plackett-Burman study with at least ≥2 dummy factors but at least two are needed for proper design and analysis.

The Plackett-Burman design focuses primarily on the factors of interest while minimizing the factor interactions contributions to a factors main effect [153, 154]; therefore, the present study aims to utilize this method to discern the independent influence of these factors on post-thaw sperm quality. The following factors were tested in the Plackett-Burman design: glycerol concentration, cooling rate to 4°C, antioxidant supplementation with GameteGuard®, equilibration time in the straw (prior to freezing), cryopreservation extender, straw type/volume, extender starting osmolality, sodium dodecyl sulfate addition, stepwise addition of glycerol. The experiment also included two dummy factors to complete the design.

Two of the factors in the Plackett-Burman design herein are extender additives which include an antioxidant additive (GameteGuard®) and a detergent (sodium dodecyl sulfate). Knox [135] recently indicated "the use of specific additives under defined conditions" appears to provide the best opportunity for improving post-thaw boar sperm. He discusses that the use of such additives like antioxidants may be important in improving boar sperm cryopreservation practices. GameteGuard®, a plant derived antioxidant extract, previously demonstrated protection of sperm cells during freezing and thawing resulting in improved pregnancy per AI in bulls [156] and improved post-thaw sperm quality of bucks [118]. Accordingly, the present study assessed 9 factors in 12 runs with 10 boars using a Plackett-Burman experimental design to assist in the first stage of the development of novel boar cryopreservation extender. Thus, we hypothesize that the

proposed experimental design will result in the identification of the most influential factors impacting post-thaw boar sperm quality which provides support to the use of Plackett-Burman design in fields of study such as reproductive biology.

4.2. Materials and methods:

4.2.1. Boar handling

Handling of the boars followed the National Institutes of Health guide for the care and use of animals and ejaculates were collected under IACUC approval by Colorado State University (CSU; IACUC# 17-7177A). Two replicates were performed on the same boars owned and housed by the Maschhoffs LLC, Carlyle, IL and Acuity Ag Solutions, LLC, Carlyle, IL. All boars were housed in the same genetic center and fed a commercial pelleted diet.

4.2.2. Reagents and disposables

All research grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Analytical reagents such as flow cytometry fluorophores were purchased from Thermo Scientific (Carlsbad, CA) unless otherwise stated.

4.2.3. Experimental design

Factors utilized in the Plackett-Burman design were assigned two levels for each factor extreme. Table 4.1 was generated using software designed for assigning high- and low-level factors in a Plackett-Burman design (SPC for EXCEL Version 5.0.1.6; BPI Consulting, LLC). High-level factors were denoted with "+" and the low-level factors denoted with "–". From Table 4.1, the treatment runs were prepared with respective assigned high and low factors.

Table 4.1: Experimental design generated by Plackett-Burman design software. Factor extremes (A-K) at high and low levels are denoted by "+" and "–" respectively.

	Experimental Factors									Dummy Factors	
Run	A	B	C	D	E	F	G	H	I	J	K
1	+	+	–	+	+	+	–	–	–	+	–
2	+	–	+	+	+	–	–	–	+	–	+
3	–	+	+	+	–	–	–	+	–	+	+
4	+	+	+	–	–	–	+	–	+	+	–
5	+	+	–	–	–	+	–	+	+	–	+
6	+	–	–	–	+	–	+	+	–	+	+
7	–	–	–	+	–	+	+	–	+	+	+
8	–	–	+	–	+	+	–	+	+	+	–
9	–	+	–	+	+	–	+	+	+	–	–
10	+	–	+	+	–	+	+	+	–	–	–
11	–	+	+	–	+	+	+	–	–	–	+
12	–	–	–	–	–	–	–	–	–	–	–

Experimental factors are as follows: A = Glycerol concentration (2% or 6%), B = Cooling rate to 4°C (Slow or Rapid), C = GameteGuard® concentration (1% or 3%), D = equilibration time in the straw before freezing (1h or 4h), E = cryopreservation extender type (egg yolk TRIS or lactose yolk), F = straw type/volume (0.5 mL or 5 mL), G = Extender starting osmolality (240 or 380 mOsm), H = sodium dodecyl sulfate concentration (0% or 0.5%), and I = stepwise addition of glycerol (1-step vs 2-step extension).

4.2.4. Media preparation

Commercially available extenders were not used for extender base as they are already optimized and balanced. The chemical base of these commercial extenders will be used to for extender extremes. The two base extenders used were egg yolk-TRIS buffer [157] and lactose egg yolk extender [144]. TRIS buffer, used as the base solution for egg yolk TRIS, was prepared using Trizma Base (263 mM), citric acid (86.3 mM), and fructose (73 mM). The base solution for lactose egg yolk was alpha lactose (310 mM in nanopure water). Osmolality was adjusted to either 240 or 360 mOsm using nanopure water and 20% egg yolk. Extenders were filtered twice through a 20 µm filter to remove large yolk particles. GameteGuard® 1% or 3% (Membrane Protective Technologies, Inc. Fort Collins, CO), sodium dodecyl sulfate 0% or 0.5%, and

glycerol 2% or 6% (v/v % as final concentration), were added at the described concentration for each unique treatment (Table 4.1). Half of the freeze extenders (runs 2, 4, 5, 7, 8, and 9) were two-step extenders, which include fraction A for cooling with no glycerol and fraction B containing glycerol as the cryoprotectant (glycerol at 2% or 6% (v/v%) final concentration, part A + B).

4.2.5. Sperm processing and cryopreservation

Two ejaculates from each of ten commercial Duroc boars 10-12 months of age were used in this study. Ejaculates were collected using the gloved-hand collection method to extract the sperm rich fraction [158]. Initial motility, morphology and sperm concentration were assessed. Ejaculates were required to meet minimum cutoffs of 75% total motility and 75% normal morphology. At the boar genetics center, qualified raw ejaculates were extended in 37° base extender to 40 mL ($40x10^6$ sperm/mL) for two-step extension or $20x10^6$ sperm/mL for 1-step, then cooled to 17°C. Note, ejaculates were not subject to centrifugation to remove seminal plasma and resuspending in cryopreservation extender as is common in boar sperm cryopreservation protocols. Cooling rate was controlled in transport incubators where slow cooling rate was set at -0.1°C/min and rapid cooling rate was set -0.4°C/min. Once slowly cooled samples reached 10°C in water baths, they were removed and passively cooled to 4°C. Samples designated for two-step extension received fraction B (containing glycerol) upon reaching 4°C. Sperm was loaded into 0.5 mL (IMV Technologies; Maple Grove, MN) or 5 mL (Minitube; Verona, WI) straws. Straws were frozen over liquid nitrogen vapor in a MOFA box freezing system capable of accommodating 0.5 mL (-18°C/min) and 5 mL (-13.5°C/min) straws starting at 5cm above liquid nitrogen and the weight of the rack controlled the freeze curve for each

straw type as it sank into the liquid nitrogen over 20 minutes (MOFA Global, product# 15043/0038).

4.2.6. Motility evaluation

Total and progressive sperm motility was assessed using an IVOS II (Hamilton Thorne, Beverly, MA) Computer Assisted Sperm Analyzer (CASA). For each boar and replicate, three straws per treatment were thawed at 70°C for 10 sec, or 70°C for 1 min for 0.5 mL and 5 mL straws respectively then pooled. Samples were stained with Hoechst 33342 (0.2 mg/mL) for 15 min at 37°C before CASA analysis. The CASA system captured video at 60 frames/sec and settings comprised of: frames acquired = 30; default cell intensity at 90; progressive cells threshold straightness at 67%; velocity along average path (VAP) cut-off at 50; slow cell VAP cut-off at 20 μm/s; slow straight-line velocity (VSL) cut-off at 15 μm/s.

4.2.7. Flow cytometry evaluation

Flow cytometry evaluation of sperm occurred using a ZE5 flow cytometer (Bio-Rad Laboratories, Hercules, CA) calibrated using ZE-Series QC Beads (Bio-Rad Laboratories, Hercules, CA) then operating at 0.5 μL/s to acquire 10,000 DNA labeled events. Using the flow cytometer, duplicated data per sample was collected on a 96-well-plate in 125 μL volumes.

A tri-stain protocol was used to determine membrane permeability and acrosome integrity using SYBR-14, propidium iodide, and PNA conjugated Alexa Fluor 647 respectively. For each boar and replicate, three straws per treatment were thawed as above. Sperm samples of 12.5 μL were added to 112.5 μL of 10 mM HEPES buffer containing 1.5 mM BSA, SYBR-14 (0.1 μM, 488 nm; 525/35 bp), propidium iodide (12 μM, 561 nm; 615/24 bp), PNA conjugated Alexa Flour 647 (6.5 μg/mL, 640 nm; 670/30 bp). Samples were stained at 37°C for 10 min in the dark on a 96-well plate before collecting data in acquisition on the flow cytometer.

Populations were analyzed using FlowJo Software (Becton, Dickinson and Company, Franklin Lakes, NJ) in logarithmic scale. Membrane impermeable cells were defined by populations that were SYBR [+] and propidium iodide [-] [159]. Acrosome intactness was identified by cell populations that were PNA [-] [159].

4.2.8. Statistical analysis

Plackett-Burman data is commonly subject to the *F*-test using calculated estimated effect to detect the significance of each factor [153]. The mean value for each run and for each sperm parameter (percent total motile, progressively motile, membrane impermeable, and acrosome integrity) was used to calculate the *F*-value. The estimated effect of each factor was computed in excel using the following equation [153]:

$$\frac{2[\sum(Y+) - \sum(Y-)]}{N}$$

where N is the total number of runs (N =12), Y+ is the mean value for high-level factors and Y– is the mean value for the low-level factors. The sum of squares (SS) was computed using the following equation [153]:

$$SS = \frac{(estimated\ effect)^2}{df(number\ of\ factors)}$$

where "df" is the degrees of freedom designated in the Plackett-Burman design. For factors df = 11 (N - 1) and for dummy factors df = 2. After determining the estimated effect and SS for each factor and sperm quality parameter, the *F*-values were calculated by dividing SS with mean sum of the dummy factors SS. A one-tailed *F*-test with alpha at 0.05 was used to determine the critical *F*-value using RStudio. Factor *F*-values larger than 18.5 ($F_{1,2}$) were identified as having a significant influence on post-thaw sperm parameters ($P<0.05$).

All statistical analysis was performed using R version 3.5.1 through RStudio [94, 95]. Data was checked for normality before further statistical analysis. To detect differences within factor extremes, ANOVA was performed using methods according to Waters and Dovletoglou [96, 155]. Then factors were compared at their high [+] and low [–] extremes [161]. A Technique for Order of Preference by Similarity to Ideal (TOPSIS) analysis was performed as third statistical analysis, to confirm the influence within factors, and rank treatments with respect to sperm parameters [162]. This ranking is a type of multiple attribute decision making method that utilizes normalized and weighted values [162].

4.3. Results

4.3.1. Identification of highly influential factors

The Plackett-Burman design was used to ascertain the most influential factors to post-thaw sperm quality. Table 4.2 provides estimated effects for each factor and sperm quality parameter measured. Factors that influenced three of the four post-thaw sperm parameters were identified as being highly influential. For the present study cooling rate, antioxidant supplementation with GameteGuard®, straw volume, and sodium dodecyl sulfate addition were classified as highly influential factors (P<0.05). Glycerol concentration, cryopreservation extender type and starting osmolality were found to be moderately influential as they impacted some but not all sperm parameters. Equilibration time in the straws prior to cryopreservation and stepwise addition of glycerol were not found be influential.

Table 4.2: Estimated effects of each experimental factor being evaluated in the Plackett-Burman design using *F*-test statistical analysis.

	Effect of Experimental Factors[1]								
Sperm Parameter	A	B	C	D	E	F	G	H	I
Total Motile (%)	-2.4	-4.8	5.4	-1.7	-6.6	-6.6	-3.3	-5.6	1.2
Progressively Motile (%)	-1.3	-3.8	1.9	-0.7	-3.1	-4.5	-3.3	-3.7	0.1
Membrane Impermeable	-4.7	-5.8	9.1	-1.6	0.7	-8.5	-2.0	-6.1	6.2
Acrosome intactness	-25.5	-10.8	6.3	-4.5	2.5	-19.7	-0.9	2.2	1.5

[1]Effects of each experimental factor A-I for the sperm parameters tested. Effect values shaded gray identify significant impacts ($P<0.05$). Experimental factors are assigned as follows: A = Glycerol concentration (%), B = Cooling rate to 4°C, C = GameteGuard® concentration (%), D = equilibration time in the straw before freezing (h), E = cryopreservation extender type (egg yolk TRIS, and lactose yolk), F = straw type/volume (mL), G = Extender starting osmolality, H = sodium dodecyl sulfate (%), and I = stepwise addition of glycerol (1-step vs 2-step extension).

4.3.2. Pairwise comparison of Plackett Burman factors

To detect the difference within a highly influential factor, the influence within each factor was determined using an ANOVA for sperm quality traits (total percent motility, progressive motility, membrane impermeable, and acrosome integrity). Rapid cooling rate and 3% GameteGuard® supplementation were found to improve total percent motility ($P<0.01$). Rapid cooling was also found to have a significant and positive impact on progressive motility ($P<0.01$). Membrane impermeable cells and acrosome intactness were both improved with 2% glycerol, rapid cooling rate, 3% GameteGuard® supplementation, and cryopreservation in 0.5 mL straws ($P<0.01$). A linear regression plot was also generated to visualize the difference within factor but between factor extremes (Fig1).

Table 4.3: Pairwise comparison of factor extremes for the highly influential factors

Sperm Parameter	[1]Factors at their extremes (-1 vs 1)							
	Glycerol concentration		Cooling rate		GameteGuard concentration		straw size	
	+	-	+	-	+	-	+	-
Total Motile (%)	14.3[a]	16.7[a]	13.1[a]	17.9[b]	18.2[a]	12.7[b]	12.1[a]	18.8[a]
Progressively Motile (%)	6.3[a]	7.5[a]	5.0[a]	8.8[b]	7.8[a]	5.9[a]	4.6[a]	9.1[a]
Membrane Impermeable	31.5[a]	35.8[b]	30.9[a]	36.4[b]	38.3[a]	29.0[b]	29.2[a]	38.1[b]
Acrosome intactness	52.9[a]	77.8[b]	60.3[a]	70.5[b]	68.8[a]	61.9[b]	55.2[a]	75.5[b]

[1]Factors at their extremes where -1 and 1 represent low- and high-level extremes respectively. Extreme (+) included 6% glycerol, slow cooling rate, 3% GameteGuard®, and 5 mL straws. Extreme (-) include 2% glycerol, rapid cooling rate, 1% GameteGuard®, and 0.5 mL straws. High and low levels for sperm quality parameters were evaluated for each factor where difference detection was denoted by [a-b] (P<0.01).

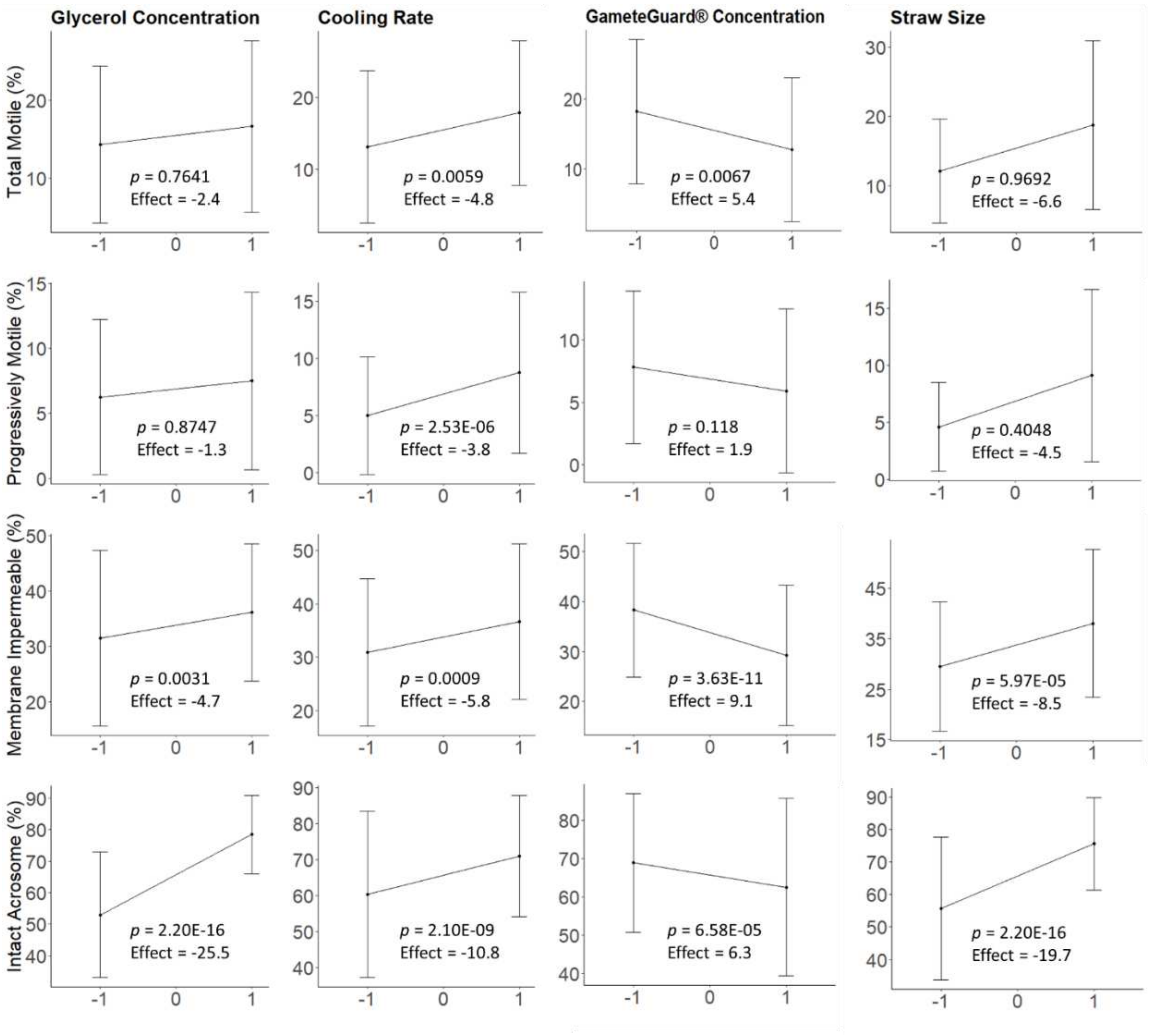

Figure 4.1. Regression plots for each sperm quality parameter and the four influential Plackett-Burman factors (three highly influential), demonstrating the response within the factor extremes (P<0.01). The effects from Table 4.2 are also included with the plots to demonstrate the magnitude the factor effect has on the response within factors. In these analyses, the extremes of the variables are represented as 1 or -1.

4.3.3. Analysis of Plackett-Burman data using TOPSIS

Ranking the impact of the combination of factors between each run and the ability to improve post-thaw sperm quality was performed using a TOPSIS ranking technique (Table 4.4). This helps to further define the influence of high or low factor extremes by ranking of the individual runs and examining the factors within the top runs (combination of factors). When assessing the top three performing runs, 2% glycerol, rapid cooling rate, and 3% GameteGuard®,

were impactful in two out of three runs. Straw size of 0.5 mL is present in all three of the top performing runs. Interestingly, 240 mOsm starting extender is also present in the top three performing runs even though it was influential only for total motility and progressive motility (P<0.01). Similarly, the stepwise addition of glycerol was found to influence membrane impermeability. According to the TOPSIS ranking, one-step extension of sperm and glycerol appears in two of the top three runs. One-step extension was found to significantly improve post-thaw motility and percent membrane impermeable cells (P<0.01).

Table 4.4: Technique for Order of Preference by Similarity to Ideal (TOPSIS) ranking results for individual run and the combination of factors within the run.

		Experimental Factors[3]									Dummy Factors	
TOPSIS Rank[1]	Run number[2]	A	B	C	D	E	F	G	H	I	J	K
1	3	–	+	+	+	–	–	–	+	–	+	+
2	12	–	–	–	–	–	–	–	–	–	–	–
3	2	+	–	+	+	+	–	–	–	+	–	+
4	8	–	–	+	–	+	+	–	+	+	+	–
5	11	–	+	+	–	+	+	+	–	–	–	+
6	6	+	–	–	–	+	–	+	+	–	+	+
7	9	–	+	–	+	+	–	+	+	+	–	–
8	7	–	–	–	+	–	+	+	–	+	+	+
9	4	+	+	+	–	–	–	+	–	+	+	–
10	10	+	–	+	+	–	+	+	+	–	–	–
11	1	+	+	–	+	+	+	–	–	–	+	–
12	5	+	+	–	–	–	+	–	+	+	–	+

[1]TOPSIS ranking of the experimental runs by improved sperm parameters post-thaw.

[2]Run numbers as assigned in the original Plackett-Burman design.

[3]The extremes of the variables are represented as + or – and are as per Table 4.1.

4.4. Discussion

A Plackett-Burman analysis was performed to ascertain the individual effects of nine factors on post-thaw sperm quality. By using experimental evaluation of high- and low-levels or factor extremes, the Plackett-Burman experimental design enables identification of the influential factors that have an impact on post-thaw sperm quality. Such experimental analysis decreases the need for full factorial experimental designs.

The influential factors included cooling rate, antioxidant supplementation with GameteGuard®, straw volume, and sodium dodecyl sulfate addition. This suggests that they are important in the maintenance of both motility and the structural integrity of the cell where other factors may only influence individual aspects of post-thaw sperm quality. It is recognized that the values for total motility in Table 4.3 are lower than current technology, but this experimental method was employed to identify influential factors that warrant further investigating by determining if the factors effect post-thaw sperm quality. This affords the ability to improve upon post-thaw sperm quality by optimizing influential factors for a given cryopreservation extender.

Data from several of the factors in the Plackett-Burman design were congruent with results of previous work such as lower glycerol concentrations [136-138], use of 0.5 mL straws [134, 145-147, 152], and use lactose yolk extender for cryopreservation (310mM in water) [144]. This makes the Plackett-Burman experimental design effective in determining that further investigation of the above factors may be repetitive. As one brief example, increasing glycerol concentration is known to disrupt boar sperm acrosomes [136], however, the Plackett-Burman design must set factors at their extremes to identify the impact of each factor. Thus, this experimental design was effective in confirming the previous work performed on the above three

factors. With that said, some factors demonstrate conflicting results with previous work which will be discussed and may require more detailed investigation.

The Plackett-Burman design focuses on identification of influential factors without the need to be concerned with factor interactions [153, 154]. One such interaction that has been studied is the relationship between glycerol concentration and extender starting osmolality. This study found low glycerol concentration to be ideally paired with hyperosmotic extender of 400 mOsm rather than 300 mOsm extender, likely due to increased cellular dehydration [139]. According to Plackett-Burman and TOPSIS ranking, the top three performing runs all included a starting osmolality of 240 mOsm and the top two performing runs included lower glycerol concentration. Because Plackett-Burman does not focus on factor interactions, these data suggests that glycerol and starting extender osmolality independently influence post-thaw sperm quality parameters.

The use of GameteGuard®, was found to significantly improve post-thaw sperm quality including total percent motile, progressively motile, and membrane impermeability. These results are supported by studies documenting the use of GameteGuard® to improve the post-thaw sperm quality of other species such as bulls [156], bucks [118] and boars [141]. This analysis using Plackett-Burman experimental design supports efforts toward optimizing GameteGuard® concentration as an antioxidant supplement in a full factorial design.

Antioxidant supplementation is useful because the variability observed in the post-thaw boar sperm quality is believed to be due to the imbalance of reactive oxygen species (ROS) generation and antioxidant mediated reduction [2, 51]. It is well understood that the processing of boar sperm for cryopreservation increases the prevalence of ROS which in turn causes reduced motility and premature acrosome reactions [1, 2]. This is believed to be due to the low

cholesterol content of the cell membrane and high concentration of polyunsaturated fatty acids leading to increased susceptibility to cold shock and oxidative stress [112, 134].

A fresh boar ejaculate is rich in antioxidants necessary to create a natural redox balance of the generation and reduction of ROS [4, 25], but processing of ejaculates necessarily results in removal of the seminal plasma derived antioxidants by centrifugation or at a minimum, dilution [134]; therefore, the natural defense from ROS is removed and often not replaced when extended into a cryopreservation extender. Note that the present study worked with the raw ejaculates and did not centrifuge and resuspend sperm into the treatments commonly seen in boar sperm cryopreservation protocols. Because the study required splitting the ejaculate across 12 treatments, seminal plasma was diluted to a similar concentration found in cooled stored boar sperm. Thus, GameteGuard® supplementation demonstrated an impact in post-thaw sperm quality. Additionally future work will focus on optimizing an extender that incorporates a centrifugation step.

As the swine industry traditionally maintains liquid boar semen between 15° to 17°C, boar studs do not have adequate facilities for cooling and cryopreservation. Therefore, ejaculates are often extended, cooled to 15° to 17°C, and shipped overnight to a cryopreservation lab. As a result, these partially cooled samples take less time to reach the point of lipid phase transition. Cooling rate appears to be a matter of debate with respect to the successful cryopreservation of boar sperm. The cooling rate for boar sperm is commonly used at -0.1°C/min which yields acceptable results [2]. Other findings have demonstrated that a much faster cooling rates of -0.4°C/min and -1.5°C/min may be beneficial [140]. This made cooling rate an ideal factor for assessment in a Plackett-Burman design to determine its influence on cryosurvival within the present system. Not surprisingly, cooling rate was an influential factor where the rapid cooling of

sperm (-0.4°C/min) positively influenced total motility, progressive motility, membrane impermeability, and acrosome integrity.

The use of TOPSIS ranking confirmed individual influence of factors by ranking the runs and validated further factorial experiment focus. However, one factor that was not found to be influential, the stepwise addition of glycerol, is commonly used in boar cryopreservation protocols [151, 152]; yet, the TOPSIS ranking suggest that boar sperm may tolerate the immediate addition of glycerol in a 1-step full extension prior to cooling the sperm to 5°C.

Another factor, sodium dodecyl sulfate, was found in the results of Plackett-Burman design to be spermicidal at the concentration used (0.5%). Sodium dodecyl sulfate, the active ingredient in Equex STM paste Orvus (Minitube; Verona, WI), is an anionic detergent known to solubilize proteins [163, 164] but has also been hypothesized to actively solubilize and alter the proteins of sperm membranes [150]; Because the present study used the extremes of 0% and 0.5% (v/v%) sodium dodecyl sulfate, perhaps a lower concentration may yield more desirable results.

4.5. Conclusion

The Plackett-Burman design is more commonly used in the fields of chemistry, biochemistry, and engineering to quickly identify factors that should receive more focus in full factorial designs [153]. While not routinely used in animal and reproductive science, this method has previously demonstrated efficacy with respect to identification of influential human sperm extender additives [165]. The successful use of Plackett-Burman in the present study suggests that advances in animal and reproduction sciences can be achieved by improving the quality rather than the quantity of screening variables of interest.

The present study made significant progress towards development of a freezing-system for boars by utilizing the Plackett-Burman experimental design to assess the impact of 9 individual factors that influence post-thaw sperm quality. Cooling rate, GameteGuard® supplementation, straw size, and sodium dodecyl sulfate were determined to have a high impact on post-thaw sperm quality as it relates to both motility and the structural integrity of the sperm cells. Thus, these factors will receive prioritized focus in future full factorial designs.

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