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Characterization of fatty acid composition of bull sperm with varied cryotolerance

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Characterization of fatty acid composition of bull sperm with varied cryotolerance

By

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Characterization of fatty acid composition of bull sperm with varied cryotolerance

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The objectives of this study were to determine fatty acid composition and acrosome status from bull sperm with different freezabilities (n = 12). We hypothesized that lipid fractions had differentiated fatty acid compositions and such differences influence sperm freezability and the sperm acrosome. Fatty acids were extracted from fresh frozen sperm and fractionated by solid-phase extraction. Thirty-four fatty acids were quantified. Saturated fatty acids were predominant, accounting for 71 to 80% of fatty acids in both fractions. Differences in composition between fractions existed ($P < 0.001$). Branched chain fatty acid concentrations (15 to 18 µg) were almost twice that of polyunsaturated fatty acid concentrations found in the polar fractions (8 to 9 µg; $P < 0.001$). Sperm with differentiated freezabilities had few differences in 22:0, 18:1 cis 9, and 14:0 13-methyl fatty acids ($P \leq 0.011$). Analyses of acrosome status of sperm revealed that acrosomes were affected differently among bulls.

DEDICATION

I would like to dedicate this thesis to my parents, Robert H. Evans and Ellen S. Pac. Your unconditional support has fueled my passions and has allowed me to purse my dreams. This monumental achievement would not have been attainable without you both. Without my mother's wavering passion for animals, I would have never pursed a career or education in the animal science field. My father has been my number one fan through all of the challenges that life has presented me. Thank you for being by my side.

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CHAPTER I

LITERATURE REVIEW

Cattle production and food security

Food production is a growing concern due to the rapidly increasing world population. There are more than 840 million people world-wide who suffer from undernourishment [1]. Addressing malnourishment begins with agriculture through both plant and animal production. Food animals, however, are unique by providing both food and other products such as leather and fiber (wool). In addition, animals aid in farming practices (plow animals) and pasture management. While these returns from animal production are important to society, it is the dietary impact of meat and dairy product consumption that makes efficient animal production so important when combating malnourishment. In fact, animal-based proteins provided via meat and dairy consumption are more efficiently utilized by the human body as compared to plant products due to the amino acid profiles making it more readily available for utilization in the human body.

While the impact of inadequate food production can be felt worldwide, the United States (US) has fared well compared to other countries due to improvements in agricultural practices and technologies. From a global perspective, however, Brazil is one of the top producers of cattle based on average head of cattle from 1994 to 2017, followed by the US, China, and Argentina [2]. In the US, there are 913,246 total beef cattle and calf operations [3]. Additionally, there has been a

25% increase in the sales of cattle and calves totaling $76.4 billion since 2007 according to the USDA 2012 Census [3]. Furthermore, the US dairy sector had close to a 1% increase in the milk production in pounds just since 2017 in addition to milk production totaling close to 217.6 billion pounds of milk total in 2018 [4].

Nevertheless, the global agricultural sector is changing due to consumer preferences and climate change altering productivity of pastures used for grazing and crop land. In turn, farmers and ranchers are being forced to become more resourceful and efficient in their production so that they can meet consumer demands. Furthermore, the increased need for animal products further pushes the limits when it comes to farmland. Adequate land is becoming difficult to locate due to population growth and farmland urbanization. This leads to overuse of the land, depleting the soil of its nutrients. Since the 1980s, the number of US farms, in addition to farm size, has stayed relatively consistent [3,5] . The amount of farmland decreased from 406.6 million acres in 2007 to 389.7 million acres in 2012, but the number of acres harvested increased from 309.6 to 315 million acres [3]. Although the land is limited, animal production remains in demand.

Successful beef producers must be able to breed their cattle in an efficient and timely manner to satisfy consumer demand. Part of the problem in meeting this need for breeding efficiency goes back to a common practice in the cattle industry called cryopreservation, commonly referred to as sperm banking or freezing. This process, while beneficial to the breeding industry by preserving sperm and making it more readily available, it causes damage to the sperm when it undergoes cryopreservation. Nevertheless, some sperm cells are more resilient than others due in part to the lipid compositions of the sperm cell membrane [6]. Understanding this resiliency is necessary to develop science-based solutions for today's breeding industry.

Spermatogenesis is a continuously recurring process in the male. This helps ensure the replenishment of spermatozoa in the case of injury, trauma, or heat stress to the male, allowing for it to regain normal reproductive efficacy. In bulls, the process of spermatogenesis takes approximately 61 days to complete with new cells being added to the cycle every 13.5 days [7]. Figure 1 depicts the development of spermatogonia as they move through spermatogenesis.

The testicles are the manufacturing sites of spermatozoa, hormones, proteins, and fluids that contribute to the reproductive success in male mammals. They produce the all-important androgen, testosterone, along with other hormones such as inhibin and estrogen. Spermatogenesis is compartmentalized in the seminiferous tubules of the testicles. In the basal compartment, mitosis takes place to allow for the proliferation of spermatogonia to obtain the primary spermatocytes. Primary spermatocytes undergo two cycles of meiosis to further mature to secondary spermatocytes, and then, spermatids. Spermiogenesis, the final stage of spermatogenesis, finishes the cellular transformation of spermatids into mature, motile spermatozoa. Finally, spermiation allows for mature spermatozoa to be released from the Sertoli cells and into the luminal space of the seminiferous tubules. Cells are stored in the caput (head) epididymis. When it is time for the sperm to fully mature, they are pushed into the corpus (body) epididymis. Finally, mature sperm, spermatozoa, are moved into the caudal (tail) epididymis for storage and transport to the vas deferens for ejaculation. Sperm gain their motility from alterations made while traveling through the epididymis and changes are made to the plasma membrane.

Molecular and cellular structures and their functions of bull spermatozoa

Bull sperm cells have several structures that allow for their ability to fertilize the female oocyte. The head is shaped like a kernel of corn. If the head of the sperm cell is not properly shaped, its ability to move with forward, progressive motility to fertilize an oocyte can be compromised. Head shape abnormalities have been associated with immaturity of sperm and reduced fertility [8]. The head is encapsulated by several membrane layers; the plasma membrane, the outer acrosomal membrane, the acrosome, and the inner acrosomal membrane. The cell's nucleus is held within these layers to prevent damage to the genetic material that is held for the fertilization of an oocyte. On the front portion of the sperm cell, the acrosome bends back towards the back of the cell and creates the apical ridge. Towards the center of the sperm head, the acrosome ends and causes midpoint of the head, which is called the equatorial segment. The structures of the head that lie between the apical segment and the equatorial segment are grouped into the principle segment of the sperm head.

The midpiece or neck of the sperm connects to the implantation socket at the caudal end of the sperm head via the capitulum. The midpiece is the home to mitochondrial adenosine triphosphate (ATP) production and fuels the movement of the sperm cell. The midpiece is composed of mitochondrial helices and has inner tubules that are responsible for bending of the sperm tail. There are outer coarse fibers of the midpiece that form a fibrous sheath that surrounds the tail of the sperm. The annulus is the end point of the midpiece and junction point to the tail. The axial filament complex originating from the distal centriole is a matrix of the microtubules. The sliding motion of these tubules allows for the lateral movement of the sperm tail and is driven by ATPases. This is of great importance because ATP production is dependent upon the

environmental temperature, which allows for greater motility. Mitochondria, located in the midpiece, are responsible for ATP production. Mitochondria are biomarkers of sperm cell fertility and are necessary for motility [9]. They have two sets of membranes, the inner and outer mitochondrial membranes, which create an environment for energy-transduction and allows for oxidative phosphorylation to take place [10]. Approximately 60% of ATP produced are used for motion of the sperm and 40% is used for substrate cycling. A very small amount of ATP is used for the maintenance of ionic gradients that are necessary for ATP generation.

Bull fertility

Low fertility is the primary factor limiting efficient production of cattle. The artificial insemination (AI) industry strives to provide the highest quality semen for both dairy and beef industries. However, significant fertility differences still exist among bull studs despite using sufficient numbers of sperm cells with normal morphology, motility, and viability. Production of bull semen and its sale for AI constitutes a major business with an annual economic impact of $260 million to the US. In recent years, about 21 million units of frozen semen were sold annually by AI companies from the US [11]. AI programs are in need of further development due to shortcomings that can make them comparable to utilizing a natural service program in regards to reproductive performance [12]. Decreased reproductive success can be attributed to selective breeding of animals and breeding for production characteristics such as milk yield, instead of selecting based off of fertility.

Male fertility, defined as the ability of spermatozoa to fertilize oocytes and support early embryonic development, is the most important factor controlling animal reproduction. In bull reproductive science, despite considerable amounts of research, cryopreservation of bull sperm

has been poor in addition to other species. In humans, approximately 50% of sperm are lost due to the freezing and thawing processes [13]. Additionally, despite producing high numbers of sperm with normal morphology, motility, and viability, bulls afflicted with non-compensable sperm defects never achieve adequate fecundity [14]. Similar to differences seen between species, differences in freezability exist in sperm from different bulls. However, molecular and cellular mechanisms underlying these puzzles are not well defined. Furthermore, there are no conventional methods to accurately evaluate freezability of semen and predict fertility of bulls. Thus, there is an urgent need to identify sperm biomolecular markers to improve sperm freezability, evaluate semen quality, and predict bull fertility. Along with limitations in evaluation methods, studies concerning the effects of antioxidants on the post-thaw sperm are also limited, and yet, antioxidants are speculated to be important for cryopreservation of bull sperm. Furthermore, lipids in sperm are likely to play vital roles in reproduction, however, their identities and how they are involved in bull fertility remain unclear. These gaps in the knowledge base could translate into financial loss for the cattle industry.

A breeding soundness exam (BSE) is commonly utilized by producers to identify potential areas of weaknesses that could hinder the bull's ability to perform as a breeding animal. A BSE examines the physical attributes and libido of a bull along with its sperm quantity and quality. Examinations, particularly of the sperm motility and morphology, are imperative for producers who may be investing in a new bull so that they know that the bull is of good reproductive health and ability [15,16]. Sperm should be 30 percent motile at a minimum and bulls should produce at least 70 percent normal sperm [17]. Normal spermatozoa will be free of morphological defects and be capable of fertilization. The testes and associated structures such as the scrotum are examined for unevenness, inconsistencies, circumference, and differences in the

left and right testicles. This examination is done as positive associations of scrotal circumference with testosterone production and fertility have been noted [18–21]. Additionally, greater sire scrotal circumference has been correlated with earlier onset of puberty in the sire's daughters. The requirements in a BSE for minimum scrotal circumference are based on the age of the bull. The scrotal circumference should be less than or equal to 30 cm at the age of 15 months, 31 cm or more between 15 to 18 months of age, 32 cm or more between the 18 to 21 months of age, 33 cm or greater between 21 to 24 months of age, and greater than 34 cm after 24 months of age [17]. In addition, the individual's prepuce and penis should be free of damage, swelling, growths, or any other abnormalities. Rectal palpation allows for the examination of other reproductive glands and organs such as the prostate gland and the seminal vesicles. Along with physical examination of the reproductive tract, spermatozoa are collected via artificial vagina, electro-ejaculator, or massaging of the seminal vesicles and ampullae via the rectum to evaluate morphology, motility, and concentration of sperm. Finally, bulls may be introduced to a cow and allowed to mount the cow or a dummy. This will allow for the evaluators to determine if there are structural problems that could hinder the ability to mount and dismount a cow. This portion of an evaluation may not be included in the typical BSE, but these behaviors and the soundness of the bull may be analyzed by owners or potential buyers.

Several other factors play into bull fertility aside from reproductive characteristics. Genetics, nutritional status, and environment all play a key role in bull development and reproductive ability. Characteristics such as scrotal circumference, testicular volume, and width have been demonstrated as having mid to high ranging heritability correlations[22]. It has also been demonstrated that environmental factors such as climate affect testicular size and weight grain of developing bulls with significant deficits occurring in Brahman bulls when moved to northern

locations[23]. Lack of development will in turn reduce productivity of that bull in regards to its breeding ability and the amount that it could be sold for when it is time for processing of that animal. In addition, the age of the bull affects sperm characteristics and reproductive ability of the animal due to several physiological and anatomical changes that occur over time. In a study previously performed, sperm produced by older bulls had impairments of the sperm head and had lower proportions of polyunsaturated fatty acids, docosahexaenoic acid, and other ω-3 fatty acids in the sperm and seminal fluid than in that of younger bulls [24]. Breed plays a role in the average age of puberty. Holstein and Brown Swiss reach maturity at approximately 9 months, Angus at 10 months, Hereford at 11 months, and Brahman at 17 months [25]. A shorter time period to reach puberty is advantageous in that it allows for shorter generation intervals.

Bulls should be free of diseases and physically sound for mounting a female for breeding. In addition, issues such as nutritional stress, heat stress, poor sanitation, and poor overall health can lead to detrimental effects in bull development and fertility. Furthermore, bulls should not be over-conditioned or underconditioned. An overweight bull may have trouble mounting a female and lack the vigor to breed many cows in a given timeframe. An underweight bull may lack the stamina to cover ground if the bull is placed in a large pasture. In fact, libido has been documented as a desirable trait [26,27]. In a bull libido study, mature Friesian, Jersey, Achai, and Friesian x Sahiwal breeds of varying ages were evaluated for libido, and then, collected to assess sperm quality parameters [28]. Jersey sires and mature sires (regardless of breed) demonstrated greater semen quality. Additionally, libido was greater in sires of more mature ages.

Contemporary methods to ascertain sperm fertilizing ability

Fertilization is the cornerstone of mammalian development. The two differentiated cells, a spermatozoon and an oocyte, set the stage for embryogenesis and fetal development for which developmental competency of the gametes is essential. Following fertilization, dramatic reprogramming of gene expression unfolds as embryonic development progresses toward blastocysts. Despite producing vast numbers of spermatozoa with apparent normal motility and morphology, cryopreservation of livestock sperm is poor and differs among males, and more importantly, the causes and mechanisms of this problem are not fully understood. Cryopreservation is an essential tool in animal biotechnology, where sperm are frozen in extenders containing chemicals that prevent cryodamage, and are stored in liquid nitrogen and shipped around the world to inseminate cows using AI. Semen extenders are designed to protect and buffer sperm cells from damaging agents such as reactive oxygen species (ROS) and bacteria. Lipoproteins, commonly milk or egg yolk, are utilized to minimize cold shock to the cells by stabilizing the cellular membrane of the sperm cells [29]. Energy substrates such as glucose provide a lifeline to cells for cellular metabolism. The osmolarity and pH of an extender may also be modified. Nevertheless, significant variations exist in cryopreservability or freezability of sperm among the breeding bulls, however, molecular and cellular markers and mechanisms for these variations are not currently known. Basic extenders for cryopreservation of bull sperm include ingredients such as egg yolk, milk, plant-based constituents, and synthetically generated materials such as lab created liposomes. In a recent study, a liposome-based extender and a soy lecithin based extender was tested for the ability to affect bull sperm post cryopreservation [30]. It was found that chromatin integrity was similar between both treatment groups of extenders and the non-return rate of the extenders were similar when using AI [30]. Silymarin, an extract from a

milk thistle, has also been tested for its efficacy as a natural adaptive to semen extenders. The addition of silymarin significantly improved the percentages of live sperm post-thaw with the control group having approximately 61% and the silymarin treatment samples had approximately 70 to 84% live cells across treatments [31].

There are a number of protocols used to cryopreserve semen. The effects of storage temperature, freezing or cooling technique, and additives have been investigated [32–34]. Improving methods of bull spermatozoa cryopreservation will lead to increased fertility rates and financial benefits as well as preserving high quality genetics in superior bloodlines. More effective cryopreservation methods would ensure that quality sperm could be shipped internationally or preserved long-term with reduced cryodamage. Through the ability to preserve bull spermatozoa long-term and ensure the viability of sperm post-thaw, the genetic material of any bull can be preserved and utilized when needed. This reduces several costs such as standing for a natural service verses the cost of a straw of semen. According to a previously conducted field study , the net cost for a natural service program was $100.49/cow per year whereas the timed AI program was only $67.80/cow per year [12]. AI also eliminates the physical risks of live-cover breeding to both bulls and cows by reducing the chances of contracting diseases and risk of injury [35]. For these reasons, producers are able to maximize use of superior bulls. AI has gained popularity in recent years as a result of the improvement in semen extenders and cryoprotectants.

Sperm cryopreservation has become an important contributor in the progression of livestock industries. This technology increases international availability of bull sperm and allows the genetic preservation of superior sires. However, this method of sperm preservation exposes the spermatozoa to membrane and DNA damage, thus, reducing sperm quality and viability. An important source of damage arises from osmotic stress experienced during freezing and thawing.

During freezing, the spermatozoa are suspended in a hypertonic solution, which causes intracellular water loss and membrane shrinkage. Alternatively, during the thawing process, the cells are exposed to a hypotonic solution, which causes intracellular water uptake and stretching of the membrane. These rapid changes in cell volume result in plasma membrane damage.

Another challenge preventing the successful cryopreservation of bull sperm is the induction of apoptotic pathways as a result of cryodamage. When sperm cells undergo changes in homeostasis initiated by factors such as oxidative stress or DNA fragmentation, programed cell death (apoptosis) pathways can be activated. Furthermore, it has been found that dead or damaged bull spermatozoa have an increased release of ROS, which can lead to oxidative damage of healthy sperm and stimulation of apoptosis. ROS are mainly generated by electron leakage from the mitochondrial electron transport chain. Cryopreservation and thawing increase the production of ROS leading to oxidation, membrane damage, and DNA fragmentation [36–38].

Fertility differences exist among males including the fertility of bulls. Spermatozoon is not able to fertilize an oocyte if they are unable to travel to the oocyte and penetrate the zona pellucida. While it is known that ejaculates are composed of a complex mixture including water, sperm cells, substrates, inorganic salts, and proteins, the concentrations and amounts of these components can vary from bull to bull. Variations in the amounts and forms of key macromolecules impair and diminish sperm viability and cause lower fertility. Certain sperm proteins also affect early embryonic development [39].

To prevent the damaging effects seen in relation to current methods of sperm cryopreservation, many alternative techniques have been studied. Sugar-supplemented extenders have been tested for their ability to provide protection and energy to sperm cells. Sugars can serve as non-penetrating protectants for cells undergoing cryopreservation, which in turn reduces

the possibility of osmotic stress on sperm cells [40,41]. Sugar additives to extenders act as an energy source to the cells through the storage process, which may allow for higher cell vitality [40,42]. Glycerol, a widely used cyroprotectant, has a large molecular weight, making it difficult to permeate the sperm cell membrane, which causes increased osmotic stress. Cryoprotectants with lower molecular weights and greater membrane permeability give way to similar percentages of motile sperm upon post-thaw analysis. However, these smaller cyroprotectants may be causing less osmotic stress than glycerol, thus, making them more effective in improving sperm cell viability [43]. Other studies have aimed at reducing the amount of oxidative damage induced by ROS production during cryopreservation. It has been shown that by supplementing stallion semen extender with the enzyme superoxide dismutase (SOD) quality parameters such as membrane integrity and motility are greatly increased in chilled spermatozoa [44]. Vitamins C and E have been utilized in cattle-based studies and have been found to prevent damage from ROS and cold shock [45]. Catalase, an antioxidant enzyme found in seminal plasma, sperm cells, and other mammalian cells, has been supplemented to stallion semen in an attempt to reduce oxidative damage through sperm processing procedures, but it did not provide significant improvements or hindrance of the cells [46].

Resveratrol is a polyphenol that has been isolated from the skin of red grapes and acts as an antioxidant in addition to having systemic anti-inflammatory, cardioprotective, antimicrobial, and anti-tumorigenic properties in mouse and human models [47,48]. When it was evaluated as a possible supplement in the cryopreservation of human sperm, a decrease in DNA fragmentation [49], decrease in lipid peroxidation, and an increase in SOD and CAT activity were observed [50]. Alternatively, ram sperm cryopreserved with resveratrol supplementation did not show improvement in quality parameters cited as motility, acrosome integrity, or plasma membrane

integrity [51]. Nevertheless, resveratrol was found to reduce ROS in the cryopreservation process with stallion sperm [52]. Furthermore, resveratrol has showed promising results when used in combination with semen extenders in bulls in that it improved mitochondrial activity and lowered DNA damage [53].

Sperm are membrane-rich, specialized cells whose molecular health is critical for successful fertilization and activation of the oocyte to support early embryonic development. The membrane layers surrounding the nucleus, cytoplasm, and the tail all contain critical lipids that play important roles in sperm physiology [39]. Use of flow cytometry techniques and computer-assisted semen analysis (CASA) has allowed researchers to complete observational studies of functional changes in sperm cells [54]. Stallion sperm have been found to have a similar, but lower protein to phospholipid ratio to bulls and a lower cholesterol to phospholipid ratio [6]. There also appears to be an association between the ratio of polyunsaturated and saturated phospholipid bound fatty acids and the overall susceptibility of cold shock [55]. Nevertheless, comprehensive studies on the identities, functions, and associations of these lipids with bull fertility are poorly defined. One notable fatty acid in particular would be cholesterol. It has been found that there are smaller amounts of cholesterol and other polyunsaturated fatty acids in bulls than other species such as humans and rabbits [55], and this lack of cholesterol is associated with poor freezability of the sperm cells.

While sperm can be evaluated in the field via light microscopy, more advanced technologies may be utilized to gain a comprehensive understanding of an ejaculate. For spermatozoa to successfully fertilize an oocyte, the genetic material within the cell must be delivered to the oocyte unharmed. Damaged DNA will not lead to successful breeding and damage can be incurred from natural causes and from external factors such as cryopreservation.

Sperm chromatin decondensation assays (SCDA) can be used to determine the condition of the genetic material held within the cell. When the cells are evaluated, double-stranded DNA will produce a green fluorescence denoting stable DNA, whereas single-stranded DNA will produce a red fluorescence, which denotes damaged DNA. An additional test would be the COMET assay, otherwise known as a single-cell gel electrophoresis or SCGE, which is a method that is used to measure DNA strand breakage in eukaryotic cells such as spermatozoa. Cells are cryopreserved, then, embedded on agarose-coated slides and lysed. Cells will then undergo electrophoresis and fluorescent staining. DNA that is not intact will be observed under a microscope with fragmented DNA giving cells a comet-like tail. DNA integrity may be evaluated using the Terminal Deoxynucleotide Transferase dUTP Nick End Labeling, or TUNEL method for short. Cells are isolated and fixed to a slide where they are then incubated with DNA labeling solution 60 minutes. Samples are rinsed and incubated with antibody staining solution containing a fluorescent dye. DNA fragmentation is then determined based on fluorescence as calculated by flow cytometry. Flow cytometry is another valuable tool that is commonly utilized in sperm analysis in which cells are examined for their physical characteristics. Cells may be treated with probes to allow for specialized evaluation of particular structures or identification of other chemical markers. Flow cytometry can be used for both sperm chromatin structure assay (SCSA) and acrosomal status determination [56].

Determining osmotic activity is of importance because it can provide insight to the membrane structure and integrity of the cells. This is vital to cell survival and protection of the internal structures. A hypoosmotic swelling test (HOST) can be used to determine if the plasma membrane of the cell has been compromised. The process by first isolating cells, and then, re-suspending them in phosphate buffered saline (PBS) [57]. The sperm suspension is then transferred

into preequilibrated hypoosmotic solution for incubation. Spermatozoa are counted such that coiled-tailed spermatozoa will be considered membrane intact, while the straight tailed spermatozoa will be considered membrane damaged.

Finally, an assessment of motility is necessary. Sperm cells are considered to be motile if the flagellum twitches, regardless if the motion is limited or lacks forward motion [58]. Motility can be determined by using CASA technology, a sensitive tool for sperm evaluation. It has been demonstrated that morphological structures of Japanese black bull sperm varied between first and second ejaculate samples by utilizing CASA analyses [59]. The first ejaculate contained greater proportions of rapidly moving and circulating motile sperm than the second ejaculate group. In a previous study, 50 crossbred bull ejaculates were evaluated for their sperm motility dynamics utilizing CASA [60]. Results showed significant differences between motility subpopulations of rapid, medium, and slow speeds. Rapid motile sperm had good forward progressive motility, while medium motile sperm had poor velocity, head movement, and tail movement. While intact, slow cells technically can fertilize an oocyte, it is extremely unlikely that they will successfully encounter an oocyte because they cannot move towards the oocyte in a purposeful manner, which would decrease chances of fertilization.

Lipidomics

Lipidomics is a new frontier in the biomedical and reproductive physiology fields. Through various techniques such as gas chromatography mass spectrometry (GC-MS), tandem mass spectrometry (MS-MS), and liquid chromatography mass spectrometry (LC-MS), scientists are capable of determining how cellular lipids play a role in the biological systems that they are found in. A lipidome can be established, which is simply the complete lipid profile within a

given organism, tissue, cell, or organelle under evaluation. Lipid profiles can include the classes or families of lipids that are present. In depth studies can produce more detailed information such as the quantities of each lipid compound and compound chemical structures. These techniques separate compound based on their molecular weights and are then fragmented to aid in the determination of each compounds based on the location of the breaks in the compound. Isomers of a compound will breakdown in a different manner, allowing for the identification of variations in a given compound.

Lipidomics has already been used to find new and exciting information in regards the fatty acid content of sperm cells from different species. In the stallion, mass spectrometry revealed that (O-acyl)-ω-hydroxy-fatty acids were present specifically in the sperm head and tail which had not been previously found [61]. While the exact purpose of these compounds is unclear, it demonstrated that complex fatty acids, such as (O-acyl)-ω-hydroxy-fatty acids which contain carbon chains of up to 52 carbons, are important to sperm cell membrane functionality [61]. In dogs, changes in the fatty acid composition of sperm cells throughout the process of sperm maturation has been documented with the concentrations of saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids being high in those sperm cells that were collected from the cauda of the epididymis [62]. The cauda sperm had significantly greater amounts of caprylic, stearic, and pentecenoic acid than that of the caput and corpus sperm [62]. Differences were also present in the epididymal fluids of samples with the caput having significantly less stearic, pentadecanoic, and linoleic acids than that of the cauda fluid [62]. In boars, the supplementation of both n-3 and n-6 fatty acids to the diet has shown to alter the composition of sperm cell fatty acids and demonstrated a positive correlation of DHA content with viability and progressive motility of sperm cells [63].

Aside from lipidomic evaluation of sperm cells, lipidomic techniques can be applied to a multitude of cells and tissues. In a past study, fatty acid profiles were determined for both sperm cells and erythrocytes before and after a supplement was given to participants [64]. This study was performed to determine how diet affected fertility and how the Western diet could alter fertility. It was found that several fatty acids including C16:1 cis9, 18:2 (ω-6, LA), 20:5 (ω-3, EPA), and 22:6 (ω-3, DHA) increased significantly in the erythrocytes after dietary supplementation. Additionally, lipidomics has been utilized to identify lipid profile differences between healthy and diseased patients. In a recent study , patients with diseases such as acute lung infections, pulmonary embolism, or acute exacerbation of chronic pulmonary disease showed more than a twofold increase in various lipid elements extracted from patient's blood plasma [65]. Furthermore, liquid chromatography-mass spectrometry could be a powerful tool to determine potential biomarkers for the diagnosis of subclinical coronary artery disease [66]. Patient serum samples were analyzed, and researchers identified a total of 103 lipids. Those patients with severe coronary calcification tended to have greater levels of monounsaturated triacylglycerols and saturated triacylglycerols, which led researchers to suggest that calcification could be associated with cellular autophagy dysfunction.

Fatty acids are stored as triglycerides, which are necessary for normal bodily function and metabolism, but they can collect in tissues such as liver tissue and cause problems (i.e. non-alcoholic fatty liver). Lipidomic evaluation of male and female rats using electrospray ionization mass spectrometry, liquid chromatography with tandem mass spectrometry (LC-MS/MS), gas chromatography with a flame ionization detector revealed significant differences in molecular patterns of lipids within plasma and liver samples [67]. It was reported that males had lower levels of total fat than females. Female rat livers had a greater total monounsaturated fatty acid

(MUFA) percentage than males, but liver samples from males had a greater polyunsaturated fatty acid (PUFA) percentages than that of the female liver samples. They suggested that estrogen was of interest due to its role in lipid synthesis. Obesity markers have also been investigated in rhesus monkeys by GC-MS technologies and ultra-high-performance liquid chromatography/mass spectroscopy (UPLC/MS) to determine the differences in blood plasma profiles of normal and obese monkeys [68]. Notably, concentrations of saturated fatty acid, palmitoleic acid (16:1), and polyunsaturated fatty acid, arachidonic acid (20:4), were greater in obese monkeys than normal monkeys. Findings suggested that these fatty acids could be used as a diagnostic tool for several obesity-related diseases. It is clear that fatty acids serve many purposes in the body but increasing the proportions of fatty acids does not necessarily equate to improvements in bodily function or cellular function.

In conclusion, with the increase in the global population comes the demand for increasing of food production. In the cattle industry, this increase can be achieved through improving of breeding efficiency and this can be done with the applications of new technologies. In particular, the use of lipidomics to identify the lipid profiles of sperm may be the key to the determination of fertility markers and additional avenues for improvement of sperm extenders. This, in turn, can assist in meeting this goal of breeding efficiency, but further research is needed.

CHAPTER II

LIPIDOMIC MARKERS OF SPERM CRYOTOLERANCE

Introduction

Male fertility, ability of the sperm to fertilize and activate the egg and support early embryonic development is essential for propagation of species. Fertilization is the cornerstone of mammalian reproductive development and differences in fertility exist among all male mammals [69]. The union of the terminally differentiated cells, spermatozoon and oocyte, set the stage during fertilization for embryogenic and fetal development, for which developmental competency of the gametes is essential [70]. Although a single bull is capable of producing copious amounts of apparently normal sperm with good motility, post-thaw viability of the sperm and cow pregnancy rates differ significantly. Cryopreservation is the process of cooling and then freezing sperm with the intention of preserving the integrity and function of the cell during the freeze-thaw process so it can be used in the future for artificial insemination (AI). However, cryopreservation always results in significant sperm damage membrane leading to cell death or the inability to function [71,72,38].

Artificial insemination has become increasingly popular among beef and dairy producers because of its several advantages. AI allows for the use of bulls that may be physically incapacitated or unable to perform natural breeding service, eliminates the need for maintaining bulls on the property for natural service thus reducing labor costs, allows for distribution and shipment of valuable genetics, and reduces the instances of disease transmissions by removing

direct animal to animal contacts. Production of bull semen constitutes a major agricultural business in the US and abroad. In 2018, nearly 22 million units of frozen dairy bull semen were sold domestically by American AI companies [11]. Genetic value premiums, semen costs, and bull maintenance costs are some of the top determinants of expected economic differences in natural service and AI breeding systems in regards to herd sizes and cow-to-bull ratios [73].

While there are many advantages to AI, careful insemination requires considerable effort by the producers. They must carefully monitor the time of estrus, ovulation, and then, inseminate the animals within a precise time window to achieve high conception rates [74]. In addition to timing, the sperm must be intact and free of any morphological or functional damage. Cooling and freezing protocols can cause this damage and drastically affect the semen quality upon thawing for use [75]. To prevent this damage, sperm samples are commonly supplemented or enhanced with extenders, which are intended to protect the cells from cellular damage caused by ROS, free radicals, and other agents [76]. Additionally, extenders may contain combinations of nutrients to sustain and prolong cellular life. Extenders can include antibiotics which reduce the possibility of bacterial contamination or damage of which previous studies have demonstrated that antibiotic additives do not adversely affect sperm quality [77]. A serious shortfall to this process is that cryopreservability or freezability of sperm differs greatly among breeding bulls, but in general, about 50% of the sperm will die during the freezing and thawing procedures [71]. Importantly, molecular, cellular, and physiological determinants of sperm freezability are not fully understood.

There are significant differences between whole sperm, its plasma membrane, and the outer acrosomal membrane in regard to certain parameters: the protein to phospholipid components and ratios as well as the cholesterol to phospholipid components ratios [78]. The

protein to phospholipid ratios are largest in the whole sperm, followed by the outer acrosomal membrane, then the plasma membrane [78]. The cholesterol to phospholipid ratio is lower in the whole sperm and the outer acrosomal membrane but the plasma membrane has a greater ratio of the components [78]. Fatty acids have come under increased interest due to their responsibility in providing energy [79] and structure to cells. Docosahexaenoic acid (DHA), for instance, has been documented for its positive correlations with sperm motility [79]. Compositional characteristics of the plasma membrane give way to fluidity and freezability of sperm cells and those with more fluid membranes display improved responses after cryopreservation procedures [80]. Destabilization of the membrane is caused by temperature induced stress in addition to osmotic stressors like water or cryoprotectants which can cause damage or swelling of the membrane [81].

In addition to the motility and morphology, sperm fertility can be impacted by the structural characteristics of the spermatozoon itself. In fact, the membrane layers surrounding the nucleus and cytoplasm, as well as the tail, all contain critical lipids that play important roles in sperm physiology and integrity [39]. Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) make up the composition of lipid membranes in addition to other materials such as sugars and proteins and are vital to successful fertilization [82]. This membrane matrix varies from animal to animal and from cell to cell, which can help or hinder the cell from performing. Sperm with greater proportions of PUFA as compared to SFA tend to demonstrate higher fertility [83]. Lipid peroxidation is the oxidative degradation of lipids caused by reactive oxygen species (ROS) and other influences, being internal or external in their nature. The plasma membrane of the sperm cell can be destroyed by these factors leaving them incapable of delivering genetic material to the oocyte, thus, preventing pregnancy. Species

differences exist in lipid compositions of the sperm plasma membrane, as well as variations among sires within a given species making lipid profiling a vital component to sperm evaluation.

Many technologies are currently utilized to aid in the evaluation of sperm integrity. Computer-assisted sperm analysis (CASA) is commonly used to deliver an objective and detailed analysis of sperm motility and morphology [40,54,57]. Sperm chromatin decondensation assays (SCDA) are also readily performed to determine the amount of DNA fragmentation that occurs after exposing the cells to acidic conditions [84]. Although these techniques help detail pertinent information, they suffer from major shortcomings including inability to provide any information with regards to the sperm membrane composition. Membrane composition could improve or hinder a cell's ability to withstand damage through the cryopreservation process. As such, there is an urgent need for precision breeding tools to better analyze components such as the lipid membranes of the cell and its organelles to assist in predicting the important characteristics of bull's sperm and semen quality.

The acrosome is an essential structural element of sperm and acrosome reaction is a specific set of complex biochemical events which results in changes in membrane phospholipid/cholesterol ratio, membrane fluidity, cAMP concentrations, and net charge on sperm cellular surface for the fusion of sperm and egg for successful fertilization [85]. The lipid composition and metabolism play a significant role in mammalian acrosome reaction [86]. For example, lipids particularly lysophospholipids affect Ca^{2+} channels by disturbing phospholipid biolayers of the membrane thereby influencing acrosome reaction [87]. Also, such catabolites modulate protein phosphorylation cascades during sperm capacitation and acrosome reaction [88]. Arachidonic acid and 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HETE), precursor for signaling lipids, trigger acrosome reaction [89]. Moreover, 1,2-Diacyl-sn-glycerols (DAG) may

play a role in membrane fusion due to its destructive effects on membrane structure during acrosome reaction. Cryopreservation can have detrimental effects on sperm acrosome by inducing modifications that destabilize sperm cellular membranes that contain lipids leading to morphological abnormalities including abnormal and premature acrosome reaction [90,91].

In this study we hypothesized that freezability is altered based on the variety and quantity of fatty acids present in the sperm plasma membrane as revealed by lipidomic evaluation of sperm cells from dairy bulls with different freezability phenotypes. We also hypothesized that cryopreservation affects sperm acrosome status. The rationale for this study is that the quality data generated on molecular underpinnings of lipids involved in cryopreservation will help us better understand and demystify the causes of poor semen quality and its specific effects on fertility. We hypothesized that the bulls of the Poor freezability group will have poor quality and low quantity of the predominate fatty acids that are found in the Good freezability bull group.

Materials and Methods

Sperm freezability determination and experimental design

Bulls (Alta Genetics, Watertown, WI, USA) were selected based on post-thaw viability data that were collected from 2008 to 2016. The semen quality database utilized 860 Holstein bulls and each bull was collected at least 15 times over a two-month period. Post-thaw viability of each bull was computed as an average of all bull samples from which the standard deviation was determined. The overall average post-thaw viability was 54.7% and ranged from 33.02% to 67.2% in individual post-thaw viability. Bulls were arbitrarily placed into Good and Poor freezability phenotypes based on the average post-thaw viability scores as being below or above from the population averages. Bulls selected for this study were outliers of the Poor and Good freezability as post-thaw viability (Table 1 and Figure 2).

Bulls were housed at the same facility and were managed identically. Semen was collected by artificial vagina and a subsample was placed into a separate tube (500×10^6 sperm per bull) and mixed with protease inhibitors. The remaining semen sample was diluted with extender, processed, and frozen according to company standard protocol. The cryopreserved sperm samples were used in the experiments to determine the acrosome status.

The subsample sperm were separated from seminal plasma with 800 × g centrifugation for 15 min. Sperm were washed twice with cold PBS (GE Life Sciences, Logan, Utah, USA) and subsequently centrifuged at 700 × g at 4°C for 15 min after each wash. Following the second wash, the samples were aliquoted into 100 million sperms per tube and the samples were stored at -196°C in liquid nitrogen. The samples were shipped to Mississippi State University in a liquid nitrogen tank for use in the lipidomics studies.

The sperm samples analyzed in this study were obtained from Alta Genetics, Inc., a commercial company, and that experiments conducted in our laboratories did not involve live animals.

Lipid extraction

The cellular fatty acids were extracted using a previously described method [86], with modifications. Frozen aliquots (10^8 sperm/tube) were placed into their own receptible then diluted to 10^7 cells. An aliquot from each bull containing 10^7 sperm cells was pipetted into a reaction vial with 30 μL of 1.5 mg/mL methyl tridecanoate and 30 μL of 1.5 mg/mL tridecanoic acid as internal standards for neutral lipid (NL) and polar lipid (PL) fractions, respectively. Fatty acids were extracted in 500 μL of methanol for 10 min and subsequently in 250 μL of chloroform 2 h. After 15,000 × g centrifugation for 10 min at room temperature, the supernatant was transferred to a new 2 mL polypropylene microcentrifuge tube. The pellet was extracted

again in 500 µL of methanol, 250 µL of chloroform, and 400 µL of water and the supernatant was combined with the previous extract. After similar centrifugation, the extract was allowed to sit overnight in a -20℃ freezer. Following that, the top layer was filtered through a 0.2-µm nylon membrane (Fisherbrand, Fisher Scientific, Pittsburgh, Pennsylvania) and stored at -20℃. The bottom layer containing fatty acids was placed in an amber GC vial (Agilent Technologies, USA) and dried under a gentle nitrogen stream at 40℃ and the residue was stored at -20℃.

Lipid fractionation and fatty acid derivatization

Neutral and polar lipid fractions were separated using a previously described method [92]. Silica gel cartridges (100 mg/1 mL; Agilent Technologies, USA) were conditioned with 500 µL of methanol and 500 µL of chloroform in that order. Vacuum was used to obtain a 0.05 mL/min flow rate. Lipid residues were reconstituted with 200 µL of chloroform. Lipid solution in chloroform was pipetted into the cartridge and collected into a new amber GC vial. The lipids were eluted first with 200 µL of chloroform to obtain neutral lipids. Vials were rinsed with 4 × 200 µL of chloroform a final wash of 300 µL of chloroform and the rinse was combined in the cartridge. The polar lipids were eluted with 6 × 200 µL and a final 300 µL of methanol. Both lipid fractions were dried under a gentle nitrogen stream before being derivatized.

Dried lipid fractions were derivatized as using a previously described method [93] with modifications. Lipids were saponified with 70 µL of 10-N KOH and 630 µL of methanol at 55℃ for 1.5 h. Liberated fatty acids were trans esterified with 58 µL of 24-N H_2SO_4 for 1.5 h in the same conditions. Fatty acid methyl esters (FAME) were extracted in 300 µL hexane, dried, reconstituted with 100 µL of hexane, and stored at -20℃.

Gas chromatography – mass spectrometry analysis

The fatty acid composition was determined by using an Agilent 7890A GC system equipped with a HP-88 capillary column (30 m × 0.25 mm × 0.20 μm), a flame ionization detector, an autosampler, and a split/splitless injector (Agilent Technologies Inc., Santa Clara, CA, USA). The FAME were separated in a 20-min temperature-gradient program with helium as the carrier gas and quantified by an Agilent 5975C inert XL MSD with triple-axis mass detector in a selected ion monitoring mode. Fatty acid methyl esters were identified by comparing their retention times with authentic FAME standards and calculated by an internal standard calibration method.

Calculations and statistical analysis

The gravimetric concentration of each fatty acid was calculated according to methods reported in a previous study [94] with correction by differences in the molecular weights between FAME and their corresponding FA. The normalized percentage of each individual FA (percent of total fatty acid) was used to represent the proportion of a given FA content relative to the total FA content. The saturation index (SI) was calculated as ratio of total SFA to total UFA (including MUFA and PUFA) concentration [94]. A generalized linear mixed model was used to analyze the data with freezability phenotype, lipid fraction, and their interaction as fixed effects. Analysis of variances was performed by the GLIMMIX procedure of SAS 9.4 (SAS Institute Inc, Cary, NC). Means, if different, were separated by the LSMEANS statement of the GLIMMIX procedure. Degree of freedom was estimated by the Kenward-Roger approximation. Actual probability values to determine statistical significance were reported.

Sperm acrosome status

Sperm acrosome reaction in response to lysophosphotidyl choline was assessed by staining of the green-stained acrosomal region of the sperm (a positive acrosome reaction) according to the methods previously described [95]. Briefly, frozen semen in two straws were carefully thawed for 30 s at 37°C, and total sperm was isolated using a percoll gradient to separate the extender. The extender was carefully aspirated using a micropipette and suspended in 500 µL of Bovine Gamete Medium [(BGM-3; 1 M CaCl2, 0.1 M KCl, 2 M NaCl, 0.1 M MgCl2, 0.1 M NaH2PO4, 40 mM HEPES, 21.6 mM sodium lactate, 3 mg/ml bovine serum albumin, and 1 mM sodium pyruvate (pH 7.5)] then centrifuged at 700 g for 5 sec. The supernatant was removed and the pellets in two tubes [one for LPC (5 mg/ml in BGM 3) and one for No LPC)] were resuspended in 250 µL of BGM3 followed by incubation at 39^{o}C in a water bath for 15 min. Sperm were then fixed by adding 10 µL of 0.4% paraformaldehyde and incubating at room temperature for 15 min. Then the sperm were stained with 10 µL of fluorescein isothiocyanate-peanut agglutinin (FITC-PNA; Sigma- Aldrich, St. Louis, MO) and 10 µL of PI (2.4 mM) at 37°C for 20 min.

Acrosome status was evaluated using 10 µL of the samples under epifluorescent microscopy (Life Technologies, EVOS FL Auto) and by counting total of at least 200 sperm/bull with the comparison of LPC challenged and not LPC challenged acrosome reacted percent sperm. The sperm cells in duplicate were evaluated and categorized into three groups namely Acrosome Intact, Acrosome Damaged, and Acrosome Reacted (Figure 3. A-C, A'-C'). We obtained fluorescence patterns of bull sperm stained with FITC-PNA + PI for the evaluation of acrosome patterns and sperm viability. Dead sperm cells with nuclear red PI fluorescence are depicted in Figure 3A-C. The others are depicted in Figure 3A'-C': Acrosome intact cells with

uniform green FITC-PNA fluorescence of acrosome site (A'); acrosome-damaged sperm cell with partial green (B'); acrosome reacted cell with along its outline (C'). For statistical analysis, the percentages of acrosome-reacted sperm were assessed as the quotient of [(number of spermatozoa with reacted or damaged acrosome) / (total number of spermatozoa)] × 100. The data were analyzed using a simple *t-test* to evaluate the statistical significance between sperm from Good and Poor freezability bulls at $P \leq 0.05$.

Results

Compositional characteristics of neutral and polar lipid fractions of sperm

Thirty-four fatty acids were detected and quantified for sperm across the two phenotypes and two lipid fractions. Total fatty acid concentrations were 267 and 238 µg per 10^7 cells for Good and Poor phenotypes, respectively. Fatty acid concentrations of the NL and PL fractions ranged from 55 to 59 µg and 179 to 212 µg, respectively. There were 7 fatty acids in the SFA category, 15 fatty acids in the MUFA category, 8 fatty acids in the PUFA category, and 4 fatty acids in the BCFA category (Table 2 and Table 3).

In the NL, SFA was the predominant FA category ($P < 0.001$), being almost 4 times greater than MUFA and 10 times greater than the PUFA (Table 2). The BCFA category was the smallest ($P < 0.001$), ranging from 0.03 to 0.05 µg per 10^7 cells. Within the SFA category, 18:0 was the most abundant fatty acid (19 to 20 µg), followed by 16:0 (13 to 15 µg). Of the MUFA category, 14:1 cis 9 was the most abundant fatty acid (2 to 2.5 µg; $P < 0.001$), followed by 17:1 cis 10 (2 to 2.3 µg), and 18:1 cis 9 (1.9 to 2.8 µg). In the PUFA category, 18:2 trans 9,12 (0.29 to 0.79 µg), 20:3 cis 8,11,14 (0.16 to 0.32 µg), and 22:4 cis 7,10,13,16 (0.15 to 0.58 µg) were the most abundant fatty acids ($P < 0.001$). The BCFA were not quantifiable in the NL, except for 15:0 14-methyl ranging from 0.03 to 0.05 µg per 10^7 cells (Table 2).

The total fatty acid concentration in the PL fraction was 3 times greater than that in the NL fraction (Table 2; $P < 0.001$). As a result, although predominant fatty acids in the SFA category remained similar, the predominant patterns in other categories were different. Similar to the NL, SFA was the predominant category of FA in the PL, being almost 10 and 20 times greater than the MUFA and PUFA ($P < 0.001$), respectively. However, the BCFA concentration (15 to 18 µg) was almost twice as much as that of PUFA (8 to 9 µg; $P < 0.001$). Similar to the NL, 18:0 (74 to 88 µg) and 16:0 (57 to 67 µg) were the predominant fatty acids of the SFA category ($P < 0.001$). However, in the MUFA category of the PL, 18:1 cis 9 was the predominant fatty acid ($P < 0.001$) with 7 to 10 µg, accounting for more than 50% of the MUFA concentration. Other major MUFA detected in the NL were not found in substantial concentration in the PL, even less than 18:1 trans 11 (1.4 µg; $P < 0.001$). In PUFA category of the PL, the 22:6 cis 4,7,10,13,16,19 (4 to 5 µg) and 22:4 cis 7,10,13,16 (1.8 to 2.0 µg) were the predominant fatty acids ($P < 0.001$). Additionally, these fatty acids were 16 and 3 times greater in the PL than in the NL ($P < 0.001$). However, 18:3 cis 6,9,12 (γ-linolenic acid) was not quantifiable in the PL fraction. The BCFA was the second greatest category (15.7 to 18.5 µg; $P \leq 0.059$) in the PL, much greater than that in the NL ($P < 0.001$).

Despite drastic differences in fatty acid concentrations between the NL and PL, the predominant patterns of fatty acid concentrations were translated into percentages (Table 3). Saturated fatty acids accounted for 71 to 80% of the total fatty acids across NL and PL. The PL had approximately 6 to 7% more SFA than the NL ($P = 0.004$). Within the SFA category, 16:0 accounted for 24 to 25% of total NL fatty acids and 31 to 32% of PL fatty acids. The 18:0 accounted for 34 to 35% of the NL fatty acids and 41% of the PL fatty acids. In the MUFA category, the 18:1 cis 9 accounted for 3 to 5% of fatty acids in the NL and PL; whereas 14:1 cis 9

and 17:1 cis 10 accounted for 3 to 4% of fatty acids in the NL, but only 0.2% in the PL ($P < 0.001$). Total MUFA contributed 23 to 24% of fatty acids to the NL but only 6 to 7% to the PL ($P < 0.001$). The PUFA contributed approximately 4 to 4.5% of fatty acids to both the NL and PL ($P = 0.555$). The 18:2 trans 9,12, 18:3 cis 6,9,12, 20:4 cis 5,8,11,14, and 22:4 cis 7,10,13,16 had greater percentages in the NL than the PL ($P < 0.001$). However, 22:6 cis 4,7,10,13,16,19 had the greatest percentage in the PL ($P < 0.001$), contributing 2.4 to 2.6% of fatty acids to the PL. The BCFA contributed approximately 8.7% to PL fatty acids with 16:0 14-methyl being greatest at 5.5% ($P < 0.001$); whereas BCFA was only found at much lower percentage in the NL (0.04 to 0.09%; $P < 0.001$).

Compositional differences in fatty acids of sperms with Good and Poor cryo-tolerance

There were 2-way freezability × fraction interactions for 22:0 ($P = 0.060$), 18:1 cis9 ($P = 0.058$), and 14:0 13-methyl ($P = 0.472$). For 22:0, there was no freezability difference in the NL ($P = 1.000$) between Good and Poor freezability groups (not quantifiable); whereas in the PL, the Good freezability had 0.08 µg or 0.03% more than the Poor freezability ($P = 0.011$). The 18:1 cis9 concentration did not differ in the NL ($P = 0.464$) but was 2.66 µg greater for the Good freezability in the PL ($P = 0.049$). However, the difference in concentration was not translated into a difference in percentage in the PL (P = 0.468). For 14:0 13-methyl, there was no freezability difference in the NL ($P = 1.000$) because this fatty acid was not quantifiable; however, in the PL, the Good freezability had 0.06 µg or 0.02% more than the Poor freezability ($P = 0.007$). Most of major fatty acids in SFA (16:0 and 18:0), MUFA (14:1, 17:1, and 16:1), and PUFA (18:2 trans 9,12, 18:3 cis 6,9,12, 20:4 cis 5,8,11,14, and 22:4 cis 7,10,13,16) were not different between the Good and Poor freezability ($P \geq 0.067$).

Sperm acrosome status of Good and Poor freezability sperm

The percentages of reacted acrosome status of sperm from Good and Poor freezability bulls are presented in Table 4, and acrosome status of sperm visualized using fluorescent microscopy in Figure 3. Without LPC treatment (No-LPC), percentages of acrosome reacted sperm in Good freezability and Poor freezability were 38.51 and 41.36, respectively. With the LPC treatment (LPC +), the percentages of acrosome reacted sperm in Good freezability and Poor freezability were 71.9 and 78.85%, respectively (Table 4 and Figure 4). Average percent differences in acrosome reacted sperm were 33.4 and 37.4% in Good freezability and Poor freezability, respectively. Percentages of acrosome reacted sperm in No-LPC did not change between Good freezability and Poor freezability ($P > 0.05$). However, percentage of acrosome reacted sperm in Poor freezability was higher than those of Good freezability in LPC (+) ($P = 0.05$).

Discussion

With the recent improvement in technologies, the application of lipidomics has expanded into the livestock industry. This area of research is important because fatty acids play a vital role in the lipid membranes of cells and are essential for signal transduction, metabolism and growth [96], membrane fluidity [80,97], and regulation of gene expression [98]. The fatty acid composition has been characterized in certain cells such as muscle. The evaluation of muscle tissue of horses shows that horses have large percentages of PUFA, ranging from 29 to 38%, across all samplings including mesenteric fat, cardiac fat, and abdominal fat [99]. These findings differ from our present bull sperm study in that PUFA in our samples ranged from approximately 4 to 5%. In a study on fatty acid composition of human spermatozoa, researchers found that pathological samples contained higher levels of PUFA than those from normal samples. Specifically, DHA was almost

at a level of 0.84 ± 0.11 nmol/10^6 spermatozoa (mean ± SEM) in the normal patients and a level of 1.96 nmol/10^6 spermatozoa in the pathological samples[100], an equivalence of 6.4 µg per 10^7 cells. In the present study, DHA ranged from 0.15 to 2.04 µg, which is 3 times less and was located primarily in the PL fraction.

In humans, sperm samples from asthenozoospermic ($P < 0.01$) and oligozoospermic samples ($P < 0.05$) contained high levels of SFA as compared to the normal samples [101]. Samples from men with asthenozoospermia, oligozoospermia, and oligoasthenozoospermia contained lower levels of DHA than that of the normal males [101]. Additionally, DHA was positively correlated with sperm motility ($r = 0.53$) [101]. The fatty acid composition of NL and PL have also been investigated in wild and cultured eel eggs where both fractions shared similar fatty acid composition but interestingly, fertilization levels did not correlate significantly with fatty acid composition [102]. However, there was an obvious negative correlation of total lipid levels found in unfertilized eggs and fertilization rates of those eggs. From these eggs, researchers also determined that 18:1 n-9 (34.73%), 16:0 (17.58%), 16:1 (10.34%), and DHA (6.23%) were the most predominant fatty acid compounds within the NL fraction of the tested eggs. In contrast, DHA (19.69%) was the most predominant fatty acid compound found in the PL fraction, followed by 16:0 (18.24%), 18:1 n-9 (15.46%), and 20:4 (9.99%). In the present study, 16:0 was about 25% in NL and 32% in PL, 16:1 was about 2.5% in the NL and only 0.1% in the PL,18:1 ranged from 3 to 5% in the NL and PL, 20:4 ranged from 0.2 to 0.6% in the NL and PL, and 22:6 was 0.49% in the NL and 2.5% in the PL (Table 2 and 3) which is much less than what was found in the eel eggs. There are vast differences between these two studies but these results indicate the importance of fatty acid difference found in different groups of animals and how management, so to speak, alters cell or egg quality.

In the female reproductive tract, fatty acids such as myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), and linoleic acid (18:2), have been identified as predominant components in the uterovaginal junction mucosa tissues of hens [103]. It was also found, in the same study, that supplementing sperm with 1 mM oleic acid and linoleic acid contributed to higher viability of sperm, although the supplementation did not affect motility [103]. These fatty acids and others including myristic acid (14:0), palmitate (16:0), eicosapentaenoic acid (EPA, 20:5), arachidonic acid (20:4), and DHA (22:6) were predominant in the current study and are similar other studies [104]. The authors reported that these fatty acids were not only predominant, but also varied between summer and winter seasons, possibly due to the heat stress experienced by the animal in warmer seasons or some other contributing factors. These authors used five, cross-bred bulls; whereas we used 12 purebred Holstein bulls, which indicated potential breed effects on sperm fatty acid composition. Our results showed many similarities in SFA, MUFA, and PUFA such as myristic and palmitic acids (Table 2 and 3); however, EPA, arachidonic acid, and DHA were not as predominant in the current study as reported by previous studies [94]. In a recently published study on male bats fed rations with varied fatty acid components, researchers found that as sperm matured and moved from the caput to the cauda of the epididymis, SFA content increased while PUFA remained unchanged and that diet did not alter sperm composition [105]. In a study performed on Shetland pony stallions, 22:5, docosapentaenoic acid, was determined to be the most predominant PUFA and noted that this particular fatty acid increased in addition to decreases in palmitic and stearic acid as breeding season approached [106]. Docosapentaenoic acid was not detected in the present study but it has been identified as a key PUFA in maintaining sperm membrane integrity and cold shock resistance [107].

Large amounts of fatty acids were found in the PL fraction, indicating that sperm lipids primarily reside in the cell membrane. It is important to note that sperm lipids were very saturated with a saturation index of 2.6 to 7.2, compared to 0.5 to 1.0 in most livestock muscle and adipose tissues [92,94,99]. Studies in other species such as rabbits have demonstrated that cholesterol and SFA levels affect membrane structure and function. Cholesterol increases have shown to improve sperm quality and reduce overall abnormalities [108,109]. Cholesterol plays a role in the fluidity of the membrane and gives structural integrity to the membrane, and yet, it has been shown that there are smaller amounts of cholesterol and a higher ratio of polyunsaturated to saturated fatty acids in bull sperm than in humans or rabbits leading to less tightly structured membranes [55]. Lower levels of cholesterol has been associated with poor freezability of sperm cells because cholesterol helps provide structure to plasma membranes [6,110,111]. Sperm from species such as buffalo have been identified as less stable due to the low cholesterol to phospholipid ratio [112]. Researchers found that the cholesterol to phospholipid ratio was double that of the outer acrosomal membrane of the cells but surprisingly, the whole sperm cell ratio did not yield significant difference from the outer acrosomal membrane [78]. It has also been demonstrated that supplementing rooster sperm with cholesterol and desmosterol-loaded cyclodextrin improved post-thaw survival rates and motility while reducing apoptosis activity of the cells that would thus lead to loss of functionality [113]. It was also demonstrated that bulls, based on conception rate percentages, significantly differ when it comes to cholesterol contents of sperm with low conception rate bulls having the lowest cholesterol amount and the high conception rate group having the highest amount of cholesterol [114]. In the same study, it was noted that cholesterol content was strongly correlated with sperm quality parameters [114]. This is an interesting insight to freezability and fertilization because the over supplementing cholesterol

will prevent the acrosome reaction thus inhibiting fertilization. While we did not explore the cholesterol composition of these bulls, this would be an avenue worth investigating further due to the understood importance of cholesterol to the cellular membrane's integrity.

Differences exist in the PL and NL fractions of lipids within sperm membranes due to their specialized physiology which is designed to aid in successful fertilization. Sperm plasma membranes contain a variety of phospholipids, such as phosphatidylserine and phosphatidylcholine, that are biologically active and play roles in maintaining membrane integrity and lipid bilayer systems within the membranes, have been targeted as indicators of sperm cell functionality [111]. Sperm are known to contain a particularly large amount of neutral lipids which varies from other cell types [115,116]. In addition to this, the apparent content of particular fatty acids can alter how the membrane functions. Chains of PUFA are capable of pushing cholesterol to other portions of the membrane which creates domains of weakness within the membranes [117]. However, neutral lipids, phospholipids, and glycolipids are also of importance due to major losses incurred by cryopreservation but interestingly, SFA percentages increased [118]. It has been suggested that fatty acids that are present in the seminal plasma or within the female reproductive tract may interact with sperm cells throughout the fertilization process which could include the biosynthesis of lipids [119]. Much of the mechanisms behind functionality of the sperm membrane fatty acids, their quality, quantity, and role in fertility are still unclear.

Membrane lipids have roles in acrosome reaction that reduced after induction of acrosome reaction [120]; phospholipids are accounted for 67% of membrane lipids, and sterol is another major lipid. The changes in lipid composition in rat sperm may indicate potential lipid marker that linked to acrosome reaction [121]. It was also reported that concentration of total lipids

was decreased in acrosome reacted sperm compared to fresh sperm; classes of lipids namely, phospholipids and neutral lipids lowered in capacitated and acrosome reacted sperm [86]. In this study, our findings that lipid composition changes between freezing groups may explain alterations in the percentages of reacted acrosome in Poor freezing phenotype (Table 3, Table 4, and Figure 4). Although the ratio of cholesterol to phospholipid was not indicated in our study, ratio was reported as double of acrosomal membrane [78]. Also, reduced cholesterol during cryopreservation is related to acrosome reacted sperm [122], and associated with poor freezability of sperm cells [110]. This finding can elucidate our results that poor freezing is likely to have increased acrosome reaction in response to LPC. Fatty acid composition is also critical for fluidity of acrosomal membranes that cryopreservation needs rich levels of PUFA [123]. Therefore, in our study, distinct concentration of PUFA may uncover little increase of acrosome reaction in poor freezing group.

Conclusions

One of the major problems encountered in cryopreservation is the potential for cell membrane damage due to membrane composition, ROS, fatty acid peroxidation, and other harmful agents. Some sperm cells are more resilient than others due in part to the lipid composition of the sperm cell membrane [6]. For example in a previous study, when sperm samples taken from post-mortum bulls were supplemented with DHA, glutathione peroxidase (GPx), and superoxide dismutase (SOD) [124], the samples supplemented with a combination of DHA and SOD displayed higher motility than other treatment groups. DHA is of interest because it is a naturally occurring fatty acids that is found in sperm membranes and SOD aids in the reduction of ROS. Similarly, researchers have supplemented sperm samples from Holstein bulls with extenders with additional n-3 fatty acid and α-tocopherol, an antioxidant agent, and

observed improved cryosurvival of cells in addition to higher ratios of n-3 and n-6 fatty acids pre- and post- freezing as compared to the control group [125]. New technologies such as lipidomic profiling could be integrated into common practices to better understand a bull's ability to perform and provide viable sperm samples that are capable of withstanding harsh cryopreservation processes. Alterations to the sperm membrane through the enhancement of extenders could allow for personalized improvements for bulls in the future, but more research needs to be conducted in order to understand the mechanisms, conditions, and spatial/temporal relationships behind membrane alterations and the incorporation of exogenous fatty acids.

The research performed in this study has allowed us to gain a more comprehensive understanding of the sperm lipidome, and thus, determine lipid associations with freezability. There is a need to uncover the lipid profiles as determinants of cryotolerance. This is key to the sperm cell's resilience and post-thaw viability. Lipidomic methods can be used to identify differences between bull sperm fatty acid composition, but it does not directly indicate how the sperm will perform *in vivo* fertility. Recently, researchers were able to identify the presence of (O-acyl)-u-hydroxy-fatty acids (OAHFA) in stallion sperm for the first time by utilizing MS technologies [61]. These researchers noted that the lipidome was more broad in the sperm cells themselves and more restricted in the seminal plasma. This could be of interest due to the protective qualities of seminal plasma.

Use of the GC-MS equipment is suitable for quantification of FAME study due to the specificity and sensitivity of the machine for small compounds. However, results from this study could be enhanced by the addition of a secondary analytical chemistry tool such as a nuclear magnetic resonance spectroscopy (NMR) which uses magnetic fields to observe local molecular structures and conformations within a solution [126]. The GC-MS and NMR both have been

utilized to identify metabolites of human sperm samples [126,127]. In one study, 69 metabolites were identified in total with certainty by using the technologies, but only four of the metabolites were identified by both the technologies thereby demonstrating how technologies differ in precision [127]. Nevertheless, NMR, in combination with GC-MS analysis, only needs a small portion of a sample and does not destroy the sample [128]. It has the ability to identify low-weight molecules, but it does have low sensitivity so care must be taken when preparing samples and the machinery for analysis [128]. Depending on the complexity of the mixture that is being analyzed, accuracy and sensitivity can be clouded by the predominate compounds in the sample. In a muscle composition study, phosphatidylethanolamine and phosphatidylcholine species predominated the samples, and therefore, were the most accurately quantified [129]. Although NMR was not available for use in this particular study, it is a promising frontier for lipidomic research and should be utilized in future endeavors in this area.

In conclusion, fatty acid profiling will permit comparison of bull sperm against one another or against other species such as stallions or boars to deepen our knowledge of fertility characteristics and markers. The new data and knowledge reported in this study will provide a strong foundation for further studies of cattle reproduction, sperm preservation, and animal and human fertility markers. The present study used a large animal model relevant to human reproductive mechanisms to examine a fundamental lipidomes associated with sperm freezability. The bull is a unique model for the study of male fertility. There are significant similarities between the human and bovine genome well as embryonic development. Contrary to human fertility research relying on anecdotal records of one or few infertility treatment cycles per couple, there is a great deal of field fertility data available in the agricultural sector, focused on improving livestock reproduction. Through our partnership

with the URUS Group (Alta Genetics), a leading animal breeding company, we had sperm samples from bulls with detailed breeding records on thousands of AI services, and well-documented sperm freezability phenotypes that we have used for this project. Furthermore, bulls produce large numbers of spermatozoa so semen can be repeatedly collected from the same donor in large quantities, and IVF and embryo culture are well developed and reliable.

The manuscript describes the results of original research that has exceptional importance because the findings shed lights onto molecular and cellular underpinnings of male fertility, ultimately mammalian reproduction and development. Lipidome of sperm and their roles in sperm cryopreservation, fertilization and early embryonic development in mammals had been mystery for many years; our study has targeted exactly this objective and produced new knowledge. Better understanding the nature of sperm lipidome and how they are associated with sperm freezability and male fertility will lead to improved understanding of mammalian gametogenesis and fertilization, and development of diagnostic/prognostic methods. Sperm lipids identified will serve as potential biomarkers to evaluate semen quality and sperm freezability and predict male fertility. As such, the findings are expected to advance both fundamental gamete science and assisted reproductive technologies (ART).

CHAPTER III

CONCLUSIONS AND FUTURE DIRECTIONS FOR LIPIDOMIC ANALYSIS IN THE AGRICULTURAL INDUSTRY AND OTHER AREAS

Sperm lipidome associations with bull fertility

Demands that are made on the agricultural industry grow daily as the global population multiplies and the desires of consumers continue to change. Products produced by the beef and dairy industry will always be in high demand due to the nutrient value of animal derived products. With industry goals geared towards sustainable, efficient, and profitable production of cattle, producers have the challenge of fine-tuning their operations in order to achieve the highest quality product. Breeding is necessary to the maintenance of the business as well as the production of replacement animals for the operation. Bull fertility is extremely important to the success of both dairy and beef operations. Bulls are expensive to maintain and can impact profitability, depending on the type of breeding program that is utilized and how intensely the bulls are managed. Feed costs alone can make up costs. In one study, bull feed costs made up 38 to 61% of the total bull cost [130]. Bull costs can be controlled by producers that utilize AI programs. Costs per straw can vary greatly depending upon the goals of the producer. Obtaining genetic advantages can benefit production but it will also be at a financial cost to producers due to the net merit of the selected bulls [131].

The use of lipidomic analysis brings animal reproductive sciences and fertility to the forefront of new frontiers in biomedical, biotechnical, and agricultural fields. This area of

research is imperative to the growth and development of new strategies to fortify reproductive technologies and animal production abilities. Lipidomic evaluation is incredibly important to the study of animal fertility and sperm freezability in that it has the capabilities to determine, on a molecular level, what makes a cell resistant to insult such as cryopreservation. As was ascertained in this study, the application of lipidomics can assist in the better understanding of bull fertility.

Administration of dietary fatty acids can alter bull sperm parameters. In a previous study, bulls were given control or a fish oil supplemented diet to determine differences between pre-thaw and post-thaw sperm quality and the lipid content of bull spermatozoa [132]. Semen samples collected from bulls fed a fish oil-supplemented diet had a greater volume of semen, sperm concentrations, and altered fatty acid profiles as compared to the control samples. Upon post-thaw of the samples, the fish oil fed bulls had higher progressive motility (35.62 to 40.62%) than bulls on the control diet (32.05 to 36.88%). The fish oil diet had significantly greater amounts of C18:3 ($P < 0.05$) and significantly greater amounts of C14:0, C18:1, C20:4 (ω-6), C20:5 (EPA) (ω-3), C22:6 (DHA) (ω-3), ∑PUFA, ∑ω-3, ∑ω-3/∑ω-6, and ∑PUFA/∑SFA ($P < 0.01$). In a similar study , bulls were fed encapsulated SFA, flaxseed oil, and fish oil to determine changes in physiological characteristics of both fresh and frozen semen [133]. In both the flaxseed oil and fish oil fed groups, DHA levels increased significantly and docosapentaenoic acid (DPA) significantly decreased. Additionally, the flaxseed oil fed bulls had sperm samples with greater progressive motility and velocity upon post freeze thaw ($P < 0.008$). The authors also discussed the idea of incorporation rates of the dietary fatty acids and how changes occurred as early as approximately 35 days. While dietary incorporation of fatty acids to enhance sperm quality and

seminal plasma (SP) characteristics, lipidomics could help identify weaknesses in samples then incorporate fatty acids *in vitro* in a significantly shorter timeframe.

In another study, bulls were fed diets enriched by alpha-linolenic acid (ALA) or palmitic acid (PA) to examine the effects the fatty acid had on sperm quality and seminal plasma antioxidant capacity [134]. It was determined that there were no differences in antioxidant levels of SP between either treatment. Additionally, both treatments elicited positive effects on cryopreserved sperm sample quality which including plasma membrane intactness and acrosome intactness. Studies in younger bulls have also revealed marked improvements in fatty acid composition when PUFA were supplemented to the diet. The results of these studies suggest that the ratio of fatty acids is vital to the functionality of the sperm and their overall structural integrity. The incorporation of exogenous fatty acids is unclear, but this mechanism is of great importance. It has also been determined that the addition of supplemental dietary PUFA alters both sperm and SP fatty acid composition, but there were not significant differences in fresh semen or SP quality or quantity [135].

Lipids have been investigated for their efficacy as cryoprotectants. Researchers examined sperm samples from six Brown Swiss bulls, pooled the samples, and then, tested the pooled sperm with 4 levels of 3-n fatty acids and 3 levels of α-tocopherol (VE) to evaluate post-thaw characteristics [136]. In a related secondary experiment, researchers compared the pre-freezing and post-thaw qualities of the 0.4 mmol VE and 10 ng ml^{-1} n-3 fatty acids, which was the best combination of fatty acids and VE from the first experiment, and compared it to a control group that lacked the additions of the fatty acids and VE. Results demonstrated that the addition of 3-n fatty acids and VE improved post-thaw characteristics, specifically improved lipid composition with marked improvement in fatty acid composition and ratios of n-3 to n-6

fatty acids. Recently, fatty acid supplementation was tested as a potential avenue for controlling or reducing damage caused by heat stress. The effects of adding vitamin E and PUFA to sperm from *Bos taurus* bulls under heat stress conditions have been previously examined and showed that vitamin E treated samples showed beneficial effects from the added vitamin E in regards to sperm characteristics, but PUFA supplementation decreased quality of samples [124]. Use of lipids in sperm extenders has not been fully investigated as there are still many hurdles to conquer. Previously, ω-3, ω-6, and ω-9 fatty acids were supplemented to sperm extender and emulsified with polyethylene glycol [137]. It was found that the polyethylene glycol significantly decreased sperm motility and sperm parameters were not improved with the addition of any of the fatty acids, regardless as to if the sample was cooled or freeze/thawed.

Cholesterol has been of interest due to its ability to stabilize and reduce the fluidity of the plasma membrane [97]. Cholesterol is particularly interesting due to the role it plays in the sperm capacitation process. The loss of cholesterol from the plasma membrane is necessary for capacitation to take place which destabilizes the cell to allow for fertilization [138]. The addition of cholesterol to semen samples prior to cryopreservation has improved cell membrane integrity post-thaw, but caused a reduction in acrosomal reaction, which is necessary for fertilization, has been noted [139]. Cholesterol, under normal circumstances, will be lost from the sperm membrane to allow for the membrane to take a more fluid state and ultimately deteriorate so that capacitation and the acrosome reaction to take place [140]. Cholesterol has also been shown to improve sperm cell motility and cryosurvival by enhancing the membrane fluidity at low temperatures [141].

One method of determining bull fertility would consist of inseminating multiple fertile cows with sperm from a given bull, however, this is a rather costly and time intensive endeavor

[142]. This alone does not determine the fertility of a bull. Aspects such as sperm morphology and motility must be evaluated. A more cost-effective alternative is a breeding soundness exam (BSE) which is used to evaluate the overall health of a bull, its mating ability, libido, and semen quality parameters, which include sperm volume, motility, morphology, concentration, and viability [143]. Outside of the typical BSE procedures, additional assessments may be carried out to determine sperm quality and fertility. Technologies such as CASA and flow cytometry have been investigated as additional tools for sperm fertility analysis. Post-thaw bull sperm samples have higher DNA fragmentation and oxidation status variability [144]. In another study, ejaculates from Holstein bulls were evaluated by utilizing CASA technologies and flow cytometric tests to determine the relationship between semen quality and field fertility of bulls [144]. They concluded that use of CASA and flow cytometry provided quality predictions of the field fertility of the bulls, but it also suggests that additional research was needed to increase the reliability of these techniques. Basic light microscopy is inadequate and does not provide this type of information which indicates the need for improved biotechnologies and alternative methods. While these methods have proven to be valuable, more potent and sensitive technologies are needed in order to continue improving industry standards. GC-MS technologies are on the forefront of reproductive sciences and the biomedical field due to the quantitative powers that it provides to researchers for a multitude of studies. In our study, we were able to identity and quantify over 30 fatty acid compounds from bull sperm cells. Additionally, these compounds were not only identified but quantified by using a small number of cells. This bolsters the idea that the use of GC-MS technology as a more mainstream tool for identifying weaknesses or strengths in spermatozoa of bulls.

Current challenges and future directions

Since lipidomic techniques are relatively new, several challenges exist. The use of GC-MS to elucidate lipid profiles has proven to be a promising avenue for determination of bull fertility, but this machinery and use of the technology is not widely available and it takes trained personnel to produce reliable data. Prior to evaluating the bull sperm samples for our study, extensive measures were taken to perfect the methods utilized such that the maximum amount of fatty acids would successfully be extracted, fractionated, identified, and quantified. Dry runs of all the procedures were completed, at least three times over, to check for reliability and accuracy of the experiments and their efficacy.

In our study, we did experience some drawbacks from the experimental design. One of the major limitations of our study is that we had a low number of bulls (n = 12) to utilize in addition to low cell counts. Ideally, greater number of bulls would be utilized such that more data could be collected and shared. In addition, the sperm samples analyzed in this study came from selected commercial bulls that all had good fertility and sperm freezability. That is, we did not have samples from the most extreme low freezability bulls. In spite of low sperm cell numbers, the techniques used were still sensitive in identifying differences in lipid profiles. Had a greater number of cells been utilized, more compounds may have been identified because there would have been higher quantities of existing compounds thus making them easier to identify and quantify. Moreover, all of our samples came from Holstein bulls. It would be interesting to compare other breeds and to evaluate potential differences in *Bos taurus* verses *Bos indicus* species due to differences in heat tolerance and other characteristics. *Bos taurus* breeds are known for their positive carcass traits and excellent production abilities when raised in temperate climates. *Bos indicus* breeds do not have the same high-quality meat that *Bos taurus* breeds

produce but they are designed for production in harsher and subtropical environments with shorter hair coats and larger ears, and dewlap which allow for greater heat dissipation and heat tolerance. Reproductive performance of *Bos taurus* and *Bos indicus* species have been evaluated for their response to synchronization protocols, specifically a 7-day CIDR, and has shown that *Bos taurus* had pregnancy rates ranging from 54 to 58% and *Bos indicus* ranging from 33 to 49%, depending on if select synchronization or co-synchronization protocols were used in tandem with the CIDR [145,146]. It would be interesting to compare the differences in the fatty acid content of these species and how they may affect fertility.

In the future, researchers may choose to evaluate the lipid components of seminal plasma as well. While sperm are the male gametes that are needed for fertilization, seminal plasma must be evaluated when assessing bull fertility due to its ability to protect spermatozoa. However, prolonged exposure of sperm cells to seminal plasma can cause damage to the cells. Seminal plasma is produced by the epididymis and other accessory sex glandsand consists of mixture of vitamins, minerals, and proteins [147]. Heparin binding proteins (HBP) have been recognized as important to the capacitation and acrosome reactions, which are necessary for fertilization [148]. HBP can aid in the reduction of cryopreservation injuries that occur during the freezing process of sperm by minimizing ROS [149]. Seminal plasma is advantageous to sperm in that it provides temporary protection from ROS, but also allows for the sperm cells to undergo capacitation and acrosomal reactions that are necessary for successful fertilization. It has been suggested that long-term exposure to seminal plasma could damage sperm cells. In a study, the removal of seminal plasma from bull semen prior to cryopreservation lowered oxidative stress experienced by sperm cells [150]. Filtered samples maintained greater motility characteristics than that of the centrifuged seminal plasma removal group or the control group. Seminal plasma may provide a

source of fatty acids and other phospholipids to the sperm cells that may aid in the temporary protection of sperm cells prior to fertilization. They could maintain a source of fatty acids for membrane alteration throughout the fertilization process. In human subjects, the fatty acid composition of seminal plasma has been evaluated utilizing GC-MS techniques and the results have shown that seminal plasma from asthenozoospermic subjects had higher levels of both oleic and palmitic acid [151]. Authors indicated that this could be a sign of a membrane metabolism disorder. In a similar study performed in Chinese men of varying semen quality, good and poor semen groups differed significantly with regards to sperm motility, sperm morphology, sperm concentration, and sperm count ($P < 0.05$) [152]. Of the observed metabolites, a majority of them related to the metabolism of polyunsaturated fatty acids. These types of studies are vital to the advancement of both human and animal reproductive sciences.

This research is significant because sperm lipidome helps illuminate the molecular and cellular underpinnings of cryopreservation of the male gamete thereby advancing the fundamental science. In addition, lipidomic profiles could be applied as a technique to improve sperm cryopreservation and evaluate bull sperm, thus, providing a better indication of bull fertility. The fatty acid markers can be identified by using GC-MS technologies, and then, supplemented into extenders to enhance sperm cryotolerance and post-thaw viability of bull semen samples. The improvements made could be applicable to bull sperm evaluations and companies that market bulls for breeding purposes. The combined successes will create a paradigm shift in how bulls are accurately evaluated for breeding soundness and will advance preservation biotechnologies. Current methods of bull sperm cryopreservation are limited, tedious, and marginally effective in maintaining viability. With the knowledge gained by future lipidomic research in bulls such as was done in this study, improved methods of sperm

cryopreservation can be attained and applied to increase the success of exporting and importing sperm, conserving the genetics of valuable bulls, and maintaining populations of production animals worldwide.

Bulls, as a model for cryopreservation studies, provide utility to reproductive research. Bull semen is comparable to that of other species due to similarities in the fatty acid profiles found within each species. Cholesterol content of the plasma membrane of bull sperm is comparable to that found in stallion models but differs from that of rooster and boar models [6]. In terms of physical differences between sperm cells, the morphology of sperm cells differs greatly among species. Rat sperm cells have a thin, hook-like sperm head whereas bulls and humans have round, kennel shaped sperm heads [153]. In a past study, 10 mammalian species were utilized to understand the relationships between sperm structures and flagellar length [153]. Researchers found that the outer dense fiber cross-sectional area positively correlated with sperm velocity, midpiece length, principal piece length, and the overall flagellum length of sperm. This comparative study demonstrates relationships of sperm ultrastructure with sperm length and velocity which is inherently important to successful fertilization. Researchers have started to explore the possibility of using sperm as an indicator of health and risk of cancer in male subjects. Post-thaw semen quality of cancer patients are of lower quality than that prior to freezing [154]. Men with testicular cancer have significantly lower sperm cell concentrations but interestingly, patients with other cancer types have been shown to have no differences in normal sperm [155]. These structures and their composition help determine fertility of a given sire but there are still many unknowns that need exploring.

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APPENDIX A

TABLES AND FIGURES

Tables

Table 1 Bull phenotype information.

Bull code	Freezability phenotype	Number of freezing (n)	Average Post-Thaw Viability (%)	StdDev of Post-thaw Viability	% Post-thaw viability as difference from the population average
1	Poor Freezability	79	48.9	10.0	-5.8
2		107	49.2	6.0	-5.5
3		194	52.7	8.9	-2.0
4		264	54.8	9.1	-0.1
5		71	54.9	10.4	0.2
6		229	55.0	8.2	0.3
7	Good freezability	15	58.4	11.5	3.7
8		113	61.9	8.0	7.3
9		153	62.3	8.1	7.7
10		205	64.3	8.8	9.6
11		50	64.4	11.3	9.7
12		116	66.2	5.2	11.5

This table is a detailed list of phenotypic data provided for each individual bull. This data was used to place each bull into the Poor or Good freezability group for evaluation.

Table 2 Fatty acid concentration in neutral (NL) and polar (PL) lipid fractions of 10^7 sperms from bulls classified by Good and Poor freezability phenotypes.

Fatty Acid	Good		Poor		SE	P_p	P_f	P_x
	NL	PL	NL	PL				
Saturated fatty acids (SFA)	40.11	168.45	42.48	143.36	10.66	0.299	<.001	0.212
14:0	2.70	5.26	2.65	4.81	0.22	0.267	<.001	0.374
15:0	0.45	1.90	0.45	1.70	0.08	0.255	<.001	0.225
16:0	13.73	66.89	15.17	57.32	4.10	0.334	<.001	0.194
17:0	2.32	4.16	2.70	3.64	0.33	0.840	<.001	0.187
18:0	19.62	88.08	20.20	73.97	6.06	0.277	<.001	0.239
20:0	1.30	1.91	1.31	1.76	0.05	0.184	<.001	0.139
22:0	0.00	0.25[a]	0.00	0.17[b]	0.02	0.060	<.001	0.060
Monounsaturated fatty acids (MUFA)	13.24	16.48	14.30	12.09	1.93	0.399	0.791	0.174
14:1 cis 9	2.16	0.37	2.55	0.46	0.17	0.180	<.001	0.384
15:1 cis 9	1.03	0.80	1.07	0.47	0.27	0.591	0.135	0.490
16:1 cis 6	1.35	0.21	1.60	0.17	0.22	0.639	<.001	0.514
16:1 cis 9	0.61	0.76	0.38	0.45	0.14	0.067	0.456	0.788
16:1 cis 7	0.74	0.00	0.95	0.00	0.13	0.435	<.001	0.435
17:1 cis 10	2.08	0.56	2.30	0.41	0.38	0.924	0.000	0.626
18:1 trans 11	1.16	1.39	0.59	1.36	0.37	0.440	0.196	0.474
18:1 cis 9	1.90	10.09[a]	2.84	7.43[b]	0.90	0.352	<.001	0.058
18:1 cis 11	0.09	0.66	0.00	0.53	0.10	0.301	<.001	0.854
18:1 cis 12	0.19	0.18	0.21	0.11	0.09	0.768	0.501	0.628
18:1 cis un	0.47	0.07	0.65	0.11	0.30	0.714	0.130	0.807
19:1 cis 10	0.62	0.19	0.54	0.00	0.12	0.264	0.001	0.646
19:1 un	0.41	0.21	0.34	0.00	0.14	0.339	0.071	0.642
20:1 cis 11	0.22	0.00	0.21	0.00	0.05	0.853	0.000	0.853
22:1 cis 11	0.22	1.01	0.07	0.60	0.17	0.114	0.001	0.449
Polyunsaturated fatty acids (PUFA)	2.30	9.34	2.46	8.06	0.77	0.474	<.001	0.358
18:2 trans 9,12	0.29	0.65	0.79	0.32	0.27	0.763	0.84	0.139
18:2 cis 9,12	0.00	0.92	0.00	0.92	0.10	1.000	<.001	1.000
18:3 cis 6,9,12 (γ-linolenic acid)	0.31	0.00	0.46	0.00	0.07	0.316	<.001	0.316
20:2 cis 9,12	0.00	0.00	0.10	0.00	0.05	0.329	0.329	0.329
20:3 cis 8,11,14	0.49	0.14	0.52	0.00	0.13	0.715	0.004	0.526
20:4 cis 5,8,11,14	0.32	0.59	0.16	0.53	0.11	0.326	0.008	0.623
22:4 cis 7,10,13,16	0.58	1.83	0.15	2.04	0.26	0.685	<.001	0.235
22:6 cis 4,7,10,13,16,19	0.31	5.22	0.28	4.25	0.42	0.249	<.001	0.276
Branch-chained fatty acids (BCFA)	0.05	18.49	0.03	15.68	1.00	0.173	<.001	0.180
14:0 13-methyl	0.00	0.10[a]	0.00	0.04[b]	0.01	0.047	<.001	0.047
15:0 14-methyl	0.05	0.34	0.03	0.28	0.02	0.075	<.001	0.444
16:0 15-methyl	0.00	6.22	0.00	5.43	0.33	0.248	<.001	0.248
16:0 14-methyl	0.00	11.83	0.00	9.92	0.65	0.156	<.001	0.156
Total fatty acids	55.69	212.76	59.26	179.18	12.88	0.258	<.001	0.165
Saturation index (SI)	2.83	6.62	2.65	7.20	0.43	0.642	<.001	0.389

P_p, P_f, and P_x: level of significance for freezability phenotype, lipid fraction, and their interaction
SE: pooled standard error
Saturation index = SFA/(MUFA+PUFA)
[a,b] If denoted by superscripts, means without common letters differ.
un: represents an unknown isomer

Table 3 Fatty acid percentage in neutral (NL) and polar (PL) lipid fractions of 10^7 sperms from bulls classified by Good and Poor freezability phenotypes.

Fatty Acid	Good		Poor		SE	P_p	P_f	P_x
	NL	PL	NL	PL				
Saturated fatty acids (SFA)	72.49	78.96	71.38	79.99	1.85	0.983	0.001	0.568
14:0	4.93	2.56	4.48	2.68	0.28	0.572	<.001	0.320
15:0	0.80	0.91	0.76	0.95	0.04	0.965	0.001	0.322
16:0	24.75	31.41	25.49	31.99	0.60	0.287	<.001	0.898
17:0	4.14	1.97	4.51	2.04	0.30	0.485	<.001	0.619
18:0	35.51	41.06	33.91	41.26	1.26	0.584	<.001	0.481
20:0	2.36	0.93	2.23	0.98	0.08	0.634	<.001	0.269
22:0	0.00	0.12[a]	0.00	0.09[b]	0.01	0.065	<.001	0.065
Monounsaturated fatty acids (MUFA)	23.42	7.64	24.18	6.76	1.83	0.974	<.001	0.659
14:1 cis 9	3.84	0.20	4.39	0.26	0.32	0.341	<.001	0.441
15:1 cis 9	1.81	0.33	1.80	0.26	0.29	0.881	<.001	0.927
16:1 cis 6	2.39	0.11	2.67	0.09	0.29	0.667	<.001	0.619
16:1 cis 9	1.07	0.36	0.62	0.25	0.18	0.127	0.007	0.349
16:1 cis 7	1.30	0.00	1.62	0.00	0.20	0.449	<.001	0.449
17:1 cis 10	3.71	0.26	3.83	0.23	0.51	0.927	<.001	0.884
18:1 trans 11	1.98	0.65	0.99	0.76	0.52	0.411	0.155	0.306
18:1 cis 9	3.37[a]	4.67	4.78[b]	4.15	0.49	0.371	0.506	0.064
18:1 cis 11	0.16	0.35	0.00	0.30	0.09	0.276	0.019	0.552
18:1 cis 12	0.32	0.10	0.37	0.06	0.12	0.973	0.040	0.715
18:1 cis un	0.86	0.04	1.26	0.06	0.56	0.712	0.086	0.738
19:1 cis 10	1.11	0.06	0.89	0.00	0.12	0.239	<.001	0.501
19:1 un	0.73	0.07	0.52	0.00	0.16	0.401	0.001	0.661
20:1 cis 11	0.39	0.00	0.34	0.00	0.07	0.739	<.001	0.739
22:1 cis 11	0.38	0.45	0.11	0.34	0.10	0.075	0.157	0.447
Polyunsaturated fatty acids (PUFA)	4.00	4.66	4.40	4.50	1.09	0.914	0.729	0.801
18:2 trans 9,12	0.53	0.30	1.45	0.18	0.46	0.496	0.119	0.273
18:2 cis 9,12	0.00	0.48	0.00	0.51	0.05	0.790	<.001	0.790
18:3 gamma cis 6,9,12	0.53	0.00	0.80	0.00	0.14	0.339	<.001	0.339
20:2 cis 9,12	0.00	0.00	0.15	0.00	0.07	0.329	0.329	0.329
20:3 cis 8,11,14	0.83	0.08	0.94	0.00	0.21	0.940	0.001	0.665
20:4 cis 5,8,11,14	0.60	0.32	0.28	0.30	0.14	0.238	0.346	0.291
22:4 cis 7,10,13,16	1.02	0.86	0.30	1.15	0.29	0.463	0.246	0.093
22:6 cis 4,7,10,13,16,19	0.49	2.64	0.49	2.37	0.34	0.698	<.001	0.702
Branch-chained fatty acids (BCFA)	0.09	8.74	0.04	8.75	0.10	0.861	<.001	0.786
14:0 13-methyl	0.00	0.05[a]	0.00	0.03[b]	0.01	0.063	<.001	0.063
15:0 14-methyl	0.09	0.16	0.04	0.16	0.02	0.218	0.000	0.284
16:0 15-methyl	0.00	2.95	0.00	3.03	0.04	0.330	<.001	0.330
16:0 14-methyl	0.00	5.59	0.00	5.54	0.06	0.675	<.001	0.675

P_p, P_f, and P_x: level of significance for freezability phenotype, lipid fraction, and their interaction
SE: pooled standard error
Saturation index = SFA/(MUFA+PUFA)
[a,b] If denoted by superscripts, means without common letters differ.
un: represents an unknown isomer

Table 4 Acrosome reaction in Good and Poor freezability bulls.

Bull ID	**Bull Number**	**Freezability Status**	**LPC (+)**	**% LPC (-)**	**LPC difference**	**Group average**		
						LPC (+) mean Std. Error Mean	**LPC (-) mean** Std. Error Mean	**LPC difference mean** Std. Error Mean
1	011HO11607	Good Freezability	72.31	43.72	28.59	71.98 ± 1.406	38.51 ± 2.008	33.47 ± 2.500
2	011HO11720		65.63	40.38	25.25			
3	011HO11450		75.85	42.61	33.24			
4	011HO11419		73.95	34.76	39.20			
5	011HO11898		72.22	30.80	41.42			
6	011HO11907		71.92	38.81	33.12			
7	011HO11838	Poor Freezability	76.48	35.19	41.30	78.86 ± 2.805	41.37 ± 1.517	37.49 ± 2.483
8	011HO11870		66.09	40.38	25.70			
9	011HO11389		80.89	43.36	37.53			
10	011HO11256		84.86	43.21	41.65			
11	011HO11506		81.37	40.12	41.25			
12	011HO12002		83.44	45.95	37.49			

Bulls 1 to 6 were defined as high freezability (HF) and bulls 7 to 12 were grouped as poor freezability (PF). Percentage of acrosome reaction in freezability group and difference for each bull represented, and group average was expressed as mean and Std. Error Mean.

Figures

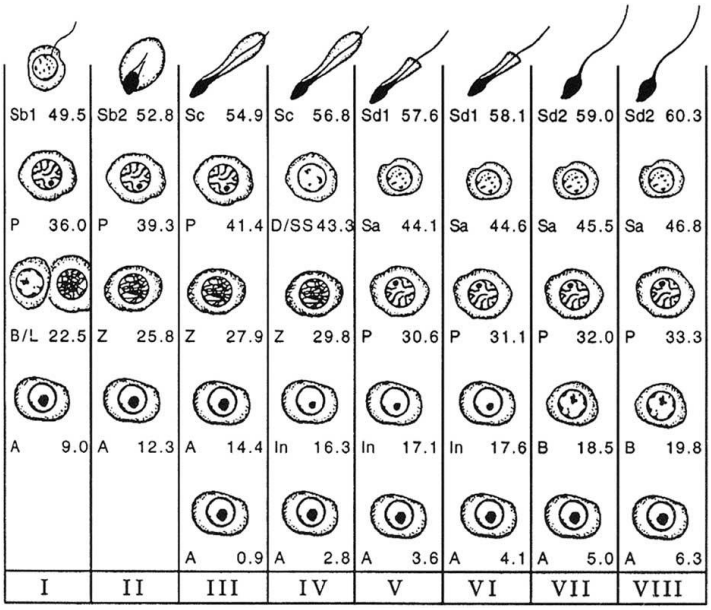

Figure 1 Stages of bovine spermiation in the seminiferous epithelium.

Each stage is denoted by Roman numerals. The spermiation step is utilized as a reference point. Stage VIII is when spermiation takes place. Several germ cells are observed: ‘A’ are type A spermatogonia; ‘In’ are intermediate spermatogonia; ‘B’ are type B spermatogonia; ‘L’ are leptotene primary spermatocytes; ‘Z’ are zygotene primary spermatocytes; ‘P’ are pachytene primary spermatocytes; ‘D’ are diplotene primary spermatocytes; ‘SS’ are secondary spermatocytes; ‘Sa’ are round spermatids; and ‘Sb1’, ‘Sb2’, ‘Sc’, ‘Sd1’, and ‘Sd2’ represents cells in stages of elongation and differentiation. Adapted from [156].

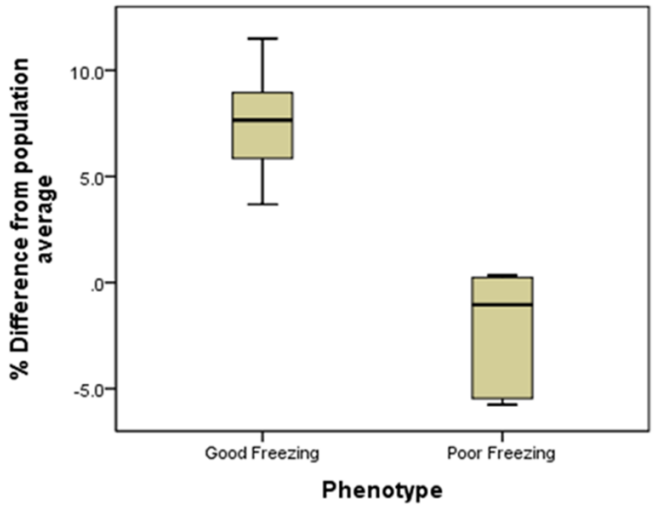

Figure 2 Differences in percentages of Good and Poor freezability bulls.

The differences of each bull were recorded as a percent. The population average was 54.7%. Good freezing bulls showed greater cell viability upon post-thaw whereas the Poor freezing group fell below the population average.

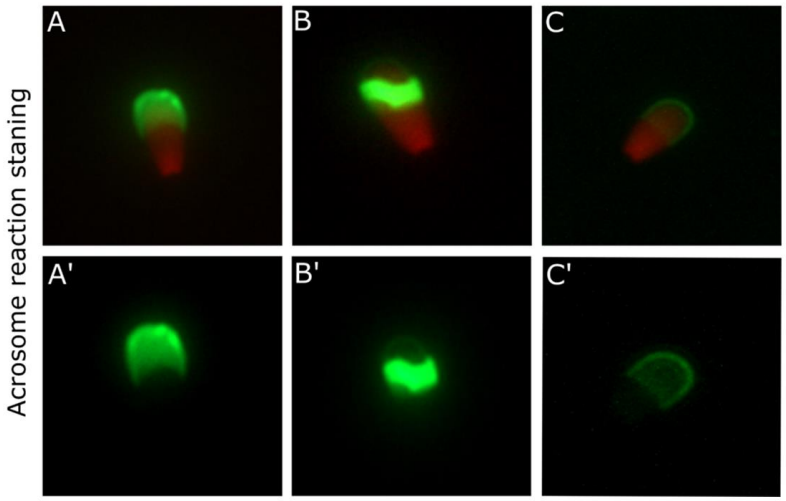

Figure 3 Staining patterns of bull sperm acrosomes.

Dead sperm cells with nuclear red PI fluorescence are depicted in panels A - C. The others are depicted in panel A’ - C’: Acrosome intact cells with uniform green FITC-PNA fluorescence of acrosome site (A’); acrosome-damaged sperm cell with partial green (B’); acrosome reacted cell with along its outline (C’).

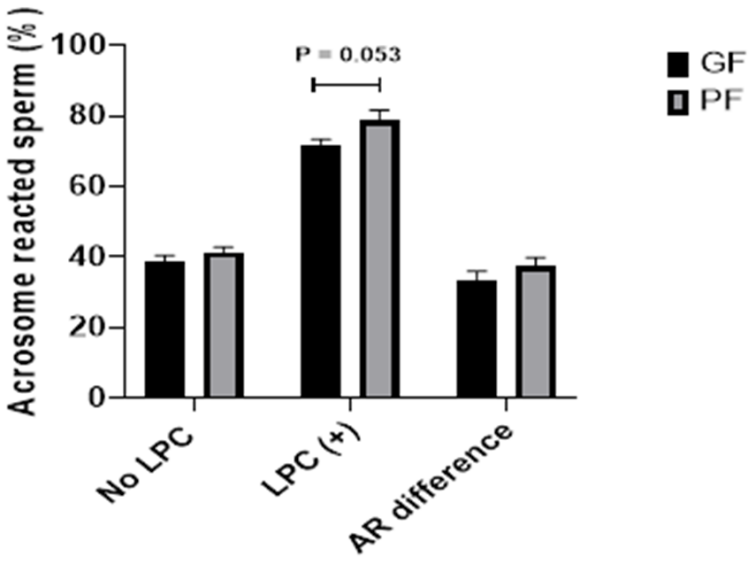

Figure 4 Acrosome reaction percentages in the different freezability groups.

Acrosome reaction (%) in Good (GF) and Poor freezability (PF) groups. Acrosome reaction obtained in absence (No LPC, baseline) and presence (LPC +) of LPC and change in percent of acrosome reaction was compared to No LPC by subtraction LPC (+). The results were presented as mean ± SEM of triplicate from each bull.