EFFECTS OF KREBS CYCLE ACIDS AND ERYTHRITOL IN CLARIFIED EGG YOLK MEDIA ON DAIRY BULL SPERM MOTILITY

A Thesis

by

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ABSTRACT

Motility rates of sperm cells affect conception rates, which affect overall dairy production rates. Cryopreservation of cells can damage the cell's integrity and biological functions, including motility. Fresh and thawed motility rates of bovine spermatozoa are important to consider for assisted reproductive technologies (ART) used within the dairy industry. This study was designed to develop a clarified egg yolk cryoprotective bovine semen extender using acids other than citric acid, to increase bovine spermatozoa motility for ART and reduce media debris for less interference with motility. This experiment consisted of two phases. Two basis media were created for Phase 1, Basis A and B, were comprised of industry standard components, and Basis B included 125 mM of added erythritol to act as a cryoprotectant. Treatment acid media were created from Basis A and B by using nonbiological or biological acids, with emphasis on biological acids within the Krebs cycle. Phase 1 screened 16 fresh clarified extenders with 33% homogenized egg yolk (HEY) across 5 dairy bull ejaculates to determine acids studied in Phase 2. Motility and progressive motility percentages were recorded for each treatment up to 13 days and statistically analyzed. Seven treatments advanced to Phase 2 for temperature stress tests. Phase 2 utilized fresh and cryopreserved clarified extenders with 50% raw egg yolk (REY), due to better clarification than HEY, across 11 dairy bull ejaculates. There were significant differences among the Phase 2 fresh treatments for motile ($p=.004$) and progressive motile percentages ($p<.0001$), with α-ketoglutaric acid (AKG) and citric acid, the control (CON), being similar and greater than all other

treatments, except malic acid (MAL) and fumaric acid plus pyruvate (PYR). Phase 2 postfreeze data also illustrated that there were significant differences in treatment for motile (<.0001) and progressive motile percentages (<.0001), with AKG and CON being similar and possessing the highest motility than all other treatments, except MAL. CON and AKG were similar and retained the highest progressive motility percentages compared to all other treatments except for PYR. Evidence showed that the hypothesized most advantageous treatment, citric acid plus erythritol (CAMERY), was out performed by the previously mentioned acids.

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NOMENCLATURE

AI	Artificial Insemination
AKG	α-ketoglutaric Acid
AKGERY	α-ketoglutaric Acid Plus Erythritol
ART	Assisted Reproductive Technologies
AV	Artificial Vagina
BETH	Proprietary Washing Media for Sperm Staining
CAM	Citric Acid Monohydrate
CAMERY	Citric Acid Monohydrate Plus Erythritol
CON	Control
CRE12G	Production Media With 12% Glycerol Without Egg
DEN15	Densimeter used to collect optical density of 15 µl media
EDTA	Ethylenediaminetetraacetic Acid
EY	Egg Yolk
ERY	Erythritol
FUM	Fumaric Acid
FUMERY	Fumaric Acid Plus Erythritol
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
HEY	Homogenized Egg Yolk
HR	Hour
HT CASA	Hamilton Thorne Computer Aided Sperm Analysis

IQR	Interquartile Range
LDL	Low-Density Lipoprotein
MAL	Malic Acid
MALERY	Malic Acid Plus Erythritol
OS TALP	Tyrode's albumin lactic acid pyruvate (as used in sperm staining at STgenetics®)
pH_i	Initial pH
pH_f	Final pH
PYR	Fumaric Acid Plus Pyruvate
QC	Quality Control
REY	Raw Egg Yolk
SE	Standard Error
SUCC	Succinic Acid
SUCCERY	Succinic Acid Plus Erythritol
TP	Time Point
TRIS	Trisaminomethane
TRIS-CAM	Trisaminomethane-Citric Acid Monohydrate
TRIS-CAM-EY	TRIS-Citrate-Egg Yolk
TRIS WS 300	STgenetics® Current Medium
TRT	Treatment

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION

Artificial insemination (AI) is a highly utilized, worldwide assisted reproductive technology (ART), especially within the dairy cattle industry. The sperm cells utilized for this ART are stored in semen straws and either cooled by refrigeration or cryopreserved with liquid nitrogen. AI using fresh, cooled semen in the cattle industry has been around since the 1900s, and cryopreservation of diary bull spermatozoa began in the 1950s for long-term storage of genetic material (Webb, 1992; Pacey and Tomlinson, 2009). Advancement towards higher conception rates, herd genetic improvement, and straw unit quality are efficient and economical goals for the use of this technology. It is known that freezing any germplasm decreases its overall function once it is thawed for an ART (Khalil et al., 2018). Keeping cryopreservation methods consistent and improving semen extender media to increase the survival and overall mobility, due to decreased media debris, would allow for higher quality sperm, with a longer life span, to reach the oocyte more efficiently with a lower cell count per straw. Discovering another beneficial cryoprotectant for extender media could also increase motility of bovine spermatozoa when volume fluctuation during cryopreservation occurs. Minimal research has been conducted over adding erythritol to extender media as a cryoprotectant for bovine germ cells. Additional low-cost acids that aid in the overall function of bovine spermatozoa should continue to be researched. This study will explore if nonbiological acids and biological acids, particularly those in the citric acid

cycle, could beneficially be used to clarify the egg yolk (EY) in extender media for cryopreservation of bovine spermatozoa. This would allow the ART industry to determine if one or more acids aid in enhanced cellular respiration, metabolism, and motility in sperm cells after thawing. Easily accessible, affordable, and advantageous components for cryopreservation media are a priority for semen straw production.

CHAPTER II

LITERATURE REVIEW

Overview of Artificial Insemination

The primary goal of AI is to increase the sperm cell density where fertilization occurs at the ampullary-isthmic junction (Ombelet and Van Robays, 2015). This technology provides the dairy cattle industry with the benefits of bypassing common obstacles within the female tract and the ability to implement elite genetics into herds for favorable production traits, such as higher milk yields (McGetrick et al., 2014; Loomis, 2017). AI also decreases the distance and time that spermatozoa must travel to reach and fertilize a mature oocyte for conventional or sex-sorted semen. With high quality semen from a leading genomic bull, this facilitates the obtainment of select replacement females or bulls for a herd. As of 2016, 66% of all dairy females in the United States were artificially inseminated (Cothren and Gryder, 2016). The percentage of dairy females that are bred through the AI process is continuing to grow due to influential drivers within and outside the industry. AI has not only gained popularity for improving herd genetics and achieving excellent production traits in offspring, but the expanding use of AI technology stems from the increased demand for dairy products from the continuously growing consumer population (Vishwanath, 2003). It is important that the efficiency of dairy industry meets consumer demands in livestock production to sustain the human population with fewer animals that can produce higher yields. Conception rates for AI are lower than natural service conception rates, and the next challenge for

the industry is to increase AI conception rates. A study conducted in 2007 found that 60.1% of the US diary female pregnancies were achieved through AI (USDA, 2009). Improving cryopreservation media that assists in increasing the cell's motility and travel efficiency could support the production of more genetically superior offspring and the production demands of consumers.

Cryopreservation of Mammalian Spermatozoa

Cryopreservation is an integral part of the AI process because hundreds to thousands of doses of diluted bovine semen are not all used at once, so they must be stored in straws for later use. Conventional and sexed semen can be frozen which permits for long-term storage for AI and other assisted reproductive procedures. Cryopreserving sperm cells is extremely beneficial to the ART industry, especially when a highly sought breeding male is deceased because his genetics can be utilized for many years after he is no longer producing semen. Cryopreservation can harm the general function of the spermatozoa, or it can injure them to the point that the cells are no longer viable; this can result in a total loss of a batch (Richardson et al., 2017). With the desire to cause diminutive damage to spermatozoa, this makes cryopreservation an intricate and delicate process for higher sperm cell function after thawing. The main objective of cryopreservation is to protect the integrity of the cells while eliciting minimal stress on the cells for a higher chance of conception. Ice crystal development within the membrane from rapid freezing and the cryopreservation media used can harm cells from extended exposure to a hyperosmotic environment when spermatozoa are cooled too

slowly are the two main factors that could render an ejaculate as waste (Loomis, 2017). Cryopreservation causes the cell to undergo osmotic stress and lose water; therefore, this procedure must be completed with consistency and balance when lowering temperatures to keep the majority of the cells intact. After ejaculation, the cells are held at ambient temperature before cryopreservation; this allows the industry more time to handle and freeze collected sperm cells. Studies have shown that sperm cells age which can affect their membrane integrity and overall motility (Krzyzosiak et al., 2001). Krzyzosiak et al. (2001) found that sperm samples held at ambient temperatures, 18-20°C, had the same motility for the first two days of storage while the motility of sperm without an intact plasma membrane decreased faster than those with a healthy plasma membrane. This indicates that timing during the cryopreservation process is crucial for sperm function because the cells are stressed from the time of ejaculation through the time of freezing. Cryopreservation media components, handling and storing methods, and timing are of the utmost importance when protecting sperm cells during the cryopreservation process.

Egg Yolk for Cryopreservation Media

EY from chickens was found to protect spermatozoa from cold shock during the cryopreservation process (Lasley et al., 1942; Lasley and Mayer, 1944; Bogart and Mayer, 1950). EY acts as a cryoprotectant in extender media for sperm cells to prevent perforation of the plasma membrane during freezing and maintain their form and performance when thawed. The industry standard for EY for cryopreservation media is 20% (v/v), and the low-density lipoprotein (LDL) fraction of EY coats the

spermatozoa to protect their cell membrane from cold shock and cold storage. The lipid portion of LDL is more vital to the cells than the protein fraction; however, the protein portion's function is to solubilize the lipid and bind it to the sperm cell membrane (Watson, 1981). This allows for protecting the cells during temperature fluctuations during freezing and thawing processes. It is known that EY present in semen extenders increases fertilization ability for sperm cells for semen stored at ambient temperatures (Anand et al. 2014). However, the fertility of sperm declines as storage time in EY increases (Krzyzosiak et al. 2001). This must be taken into consideration when handling and preparing ejaculates for freezing since the industry wants to preserve sperm cells at their highest motility percentages. Trisaminomethane-citrate-egg yolk (TRIS-CAM-EY) continues to be the most commonly applied basis medium for semen straw production, and it has been widely accepted since research on most bovine semen extenders concluded in the 1960's on media components for bovine semen extenders. The industry has been looking for an alternate means for a cryoprotectant because EY can carry bacteria that are harmful to spermatozoa, but antibiotics such as strep-penicillin are added to the extender to kill any bacteria that could interfere with the spermatozoa. Thun et al. (2002) discovered that commercially used TRIS-CAM-EY extenders resulted in higher sperm motility, morphology, osmotic resistance, and survival rates after thawing than Biociphos-Plus®, which is completely sterile and produced from soybean extract. Nevertheless, EY can cause agglutination of the acrosomes of the sperm cells which renders them unusable, and soybean lecithin does not cause aggregates of sperm cells (Aires et al., 2003). Watson (1981) proved that EY is superior to soybean lecithin during

cold storage through the study he performed. Therefore, EY continues to be one of the most commonly used bases for cryopreservation media in the industry at this point in time.

Clarification of Egg Yolk for Bovine Semen Extender

EY clarification is the process where the yolk that is used in cryopreservation media is centrifuged and the supernatant of the centrifuged fluid is decanted and utilized as the media and the pellet is discarded. For successful preservation of sperm, EY clarification aids in the removal of any solids or unsupportive lipids that would interfere with the mobility of the sperm cells once thawed and inseminated into a female tract (Wall and Foote, 1999). Clarification of EY would reduce media debris for the increase in sperm mobility to reach the oocyte more efficiently. The type of EY is known to affect the clarity of the centrifuged media. Previous research conducted by STgenetics® in 2014 found that there is a significant difference in the pellet size after centrifugation of media between pasteurized homogenized egg yolk (HEY) and raw egg yolk (REY). HEY is utilized within the ART industry for its time saving efficiency because companies no longer have to physically separate yolks daily to make an amount of REY based media for extender each day, but HEY is usually not clarified by the cryopreservation companies. The research showed that even though HEY saves time, REY yielded a larger, more defined pellet, indicating that more solids or unsupportive lipids are removed from the media when utilizing REY. Clarification of REY aided in visual analyzation of sperm cells. When using the industry standard of 20% (v/v) EY for extender media, clarification does not compromise the metabolic function of sperm cells

and is considered a safe process for the cells to undergo. An osmolarity of about 300 mOsm is the desired measurement for the clarified EY fluid for appropriate freezing of the media and for the protection of the sperm cells, so they are not perforated by ice crystal formation. One study discovered that EY particles possess extremely similar scatter properties to spermatozoa which are assessed as viable sperm with intact acrosomes. However, with appropriate staining, the EY particles will not absorb the stain since they do not contain DNA and will not be considered a sperm cell by any computer program (Nagy, 2003).

Erythritol as a Cryoprotectant

Insufficient research has been reported regarding erythritol being added to extender media as a cryosupportive chemical for bovine spermatozoa. Glycerol is a permeating solute that aids in the degree of dehydration of spermatozoa during the freezing process, and it is the industry standard cryoprotectant (Sieme et al., 2016). Previous investigations have indicated that glycerol is not vital for cryopreservation of spermatozoa (Gibson and Graham, 1969; Nagase et al., 1964; Nagase and Niwa, 1964; Berndtson and Foote, 1972). Non-permeating cryoprotectants such as sorbitol and xylitol are polyols, sugar alcohols, that have been researched more in-depth as cryoprotectants for extender media for several species' spermatozoa thus far (Garcia and Graham, 1989; Yang et al. 2013). Setyawan et al. (2009) studied D-sorbitol, that is found in epidydimal fluid, and they found that glycerol was more supportive than D-sorbitol for volume regulation of bovine spermatozoa. These treatments have been used because they reduce

osmotic stress and are cheaply made through hydrogenation of sugars. Erythritol is created in industry by fermenting sugars with yeasts, such as *Moniliella* sp., and its use is relatively small due to its higher price when compared to other sugar alcohols (Rzechonek et al., 2018). Erythritol has become more readily available over the years as a non-caloric sweetener, which has lowered the cost to $3.50/kg, and it is the first polyol to be manufactured on a commercial scale by fermentation (Cargill, 2019). Garcia and Graham (1989) were among the first researchers to work with erythritol and found that the percent motility of bovine sperm with erythritol, sorbitol, and xylitol performed better than glycerol, with sorbitol being the highest performing cryoprotectant. Further research over erythritol should be conducted to discover and confirm if erythritol is as effective or better than other cryoprotectants.

Biological Acids for Spermatozoal Function

The Krebs Cycle, or the citric acid cycle, is important to consider for the respiration and function of bovine spermatozoa. There are five acids within the cycle that contain 4 or 5 carbons per molecule, are available at a reasonably low cost, and are found inside bovine sperm cells for metabolic and overall function. These acids include: citrate, α-ketoglutarate, succinate, fumarate, and malate. One of the other acids, pyruvate, is derived from glucose through glycolysis for later conversion into acetyl-CoA so that it may enter the cycle for cellular respiration. EY-citrate media has become the most commonly used cryopreservation media for sperm cells in the ART industry (Wall and Foote, 1999). In the last century, all acids within the Krebs cycle have been

studied in spermatozoa across multiple species, and citrate continues to be most commonly used due to its low cost and acceptable function in fresh and frozen extender media for many species. AKG has been studied in rat spermatozoa, and it was found that it can be used as an important energy source, ATP, for the motility of sperm (Li et al., 2010). In previous studies, citrate, fumarate, succinate, and malate were found to be recovered easily when the citric acid cycle was studied in bovine sperm metabolism and rate-limiting reactions (Howe and Flispe, 1959; Flispe, 1964). These acids are already found within spermatozoa for cellular respiration; therefore, adding one or more of these acids exogenously to sperm could allow for prolonged metabolic function. An earlier study discovered that washed sperm, without seminal fluid, utilized added succinate, pyruvate, and egg phospholipids to maintain and support cellular respiration and flagellar motility (Lardy and Phillips, 1945). Revisiting these acids' effects could assist in uncovering a more efficient extender media for bovine spermatozoa function and motility.

Preface of Study

The data for this study was collected from the semen of *Bos taurus* dairy cattle that include individual Holstein, Jersey, and Milking Shorthorn bulls from the livestock reproductive facility, STgenetics®. In this study, sperm cells were screened for motility differences when using different acids, especially acids within the Krebs cycle, and added erythritol for cryoprotection of the cells.

The benefits of discovering an acid and/or cryoprotectant that will improve sperm cell motility rates during cooling, cryopreservation, and thawing, while decreasing the amount of debris from EY in extenders through the clarification process will create an opportunity for more live cells to be utilized for ART fertilizations. With more effective and more motile spermatozoa, this could lead to higher conception rates and desired genetic advancement within dairy herds. It also can be applied to other livestock species industries with continued research.

Objectives and Hypothesis

The objectives of this experiment were to develop a clarified egg yolk cryoprotective bovine semen extender using acids, other than citric acid, that increase the bovine spermatozoa motility for ART and to reduce media debris for more efficient sperm cell travel. This project has the following hypothesis:

Citric Acid Monohydrate (CAM) with added erythritol (ERY) in the extender media will show the highest motility and progressive motility percentages with higher clarity within the media for more efficient sperm cell travel.

CHAPTER III

MATERIALS AND METHODS

No AUP Required

All procedures were approved by Texas A&M Institutional Animal Care and Use Committee and STgenetics Research and Development Department and CEO (No Approval Number. Please See "No AUP Needed" memorandum in Appendix A, after the references pages). The experimental period was approximately 270 days in length with two phases within the study.

Bulls & Collection Procedure

Predominately Holstein and Jersey bulls (n=16) were collected using a Missouri artificial vagina (AV) at STgenetics® in Navasota, Texas. Bulls were collected twice on a given morning by STgenetics handlers for regular scheduled straw production. Bulls collected for this experiment were housed onsite and cared for equally, watered *ad libitum*, and fed a diet based on each animal's specific needs with a 26% crude protein content and a maintenance NE of 0.7 Mcal/lb. After two ejaculates are collected, each ejaculate was examined in the lab by the same researcher for volume, concentration, the total number of cells, percent motility, and morphology proportions. Each ejaculate needed to meet the passing requirements for motility equal to or greater than 70% and primary and secondary morphologies equal to or less than 30% to be used for this research study.

Phase 1

Media Preparation for Phase 1

Two basis cryopreservation media were made for the collected cells. Two liters of Basis A or trisaminomethane-citric acid monohydrate (TRIS-CAM) served as the control extender medium with 2.5 mM of CAM, 10 mM of sodium pyruvate salt, 10 mM of fumaric acid, 3 mM of Ethylenediaminetetraacetic acid (EDTA), 10 mM of D-(+)-Glucose, 3.5 mM of potassium phosphate dibasic (anhydrous), 1 bottle of GTLS, which is a semen extender antibiotic for bovine semen, and 40 mM of TRIS . Two liters of Basis B was comprised of TRIS-CAM with 125 mM of added ERY. Tests mentioned in this section may be omitted or reduced. Under the direction of the Research and Development Department some acids were ruled out preliminarily to Phase 1.

Preliminary tests to Phase 1 included nonbiological acids, such as adipic, that were ruled out as they caused immobility of the cells and rendered high static percentages. This preliminary portion allowed for the determination of the concentrations of the treatment media that would be used. This method development segment also aided in training the researcher on how to use and read Hamilton Thorne Computer Assisted Sperm Analysis (HT CASA) observations by using test ejaculates from 3 bulls that were not used in the study. Most acids selected for Phase 1, the screening phase, to test were chosen based off previous research.

Phase 1 served as a screening experiment to determine acids that would be used in Phase 2 of the experiment. Research and Development chose to focus on acids that fall within the Krebs cycle or are isomers of acids in the Krebs cycle, such as maleic

acid, because they are biological and are most likely to be exogenous supportive acids to bovine spermatozoa. The types of acids included: Krebs cycle acids, other biological acids, and nonbiological acids. The list of acids that were tested in Phase 1 include: CAM, α-ketoglutaric acid (AKG), fumaric acid (FUM), succinic acid (SUCC), Malic (MAL), Maleic, TRIS WS 300, which is STgenetics' current medium, Trehalose Dihydrate, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Each test acid was added to one of the two basis media to create each Test Acid Medium for Phase 1. Acids that were found to be toxic or unsupportive of the cells were ruled out, and Phase 2 utilized the supportive acids that were determined in Phase 1.

To prepare the test acid medium, 250 mL media bottles were tared for each test acid bottle. 90g of Basis A was combined with 1200 µl Sigma's 100xPenStrep Solution, Lot# P0781-Penicillin-Streptomycin, along with the specified amount of test acid and 45g of warmed HEY. The HEY was warmed in the lab at room temperature for 15 minutes before adding it to 250 mL bottles. The HEY, "Easy Eggs", was stored in cardboard cartons at 4°C that were acquired from Michael Foods™ (No AUP needed). HEY was added to each treatment medium for a 33% (v/v). The estimated amount of TRIS was added to each test acid medium. Then the initial pH (pH_i) of each test acid medium with added TRIS was recorded. Then pH was adjusted to 6.8-7.0 pH by using added amounts of TRIS if the medium was too acidic or CAM if it was too basic. Each bottle of test acid was placed in a cooler at 4°C for winterization for 12 hours. The calculated amounts of each test acid added to its respective bottle, calculated TRIS, pH_i, added TRIS or CAM, and final pH (pH_f) after winterization can be found in Table 1.

These steps were repeated with 5 of the 9 acids and added to Basis B (125 mM erythritol). Table 2 gives the calculated amounts of each test acid added to its respective 250 mL bottle, calculated TRIS, pH_i, added TRIS or CAM, and pH_f after winterization.

After winterization, each finalized treatment medium was transferred from the respective 250 mL bottle into a 500 mL conical centrifuge tube to clarify the finalized treatment media. The treatment media were then centrifuged at settings of 1200G for 30 minutes with an acceleration of 9 and a break of 2. Then 10 mL of supernatant was removed with a 5 mL pipette from each centrifuge tube and aliquoted into a 15 mL falcon tube, and the pellets in the centrifuge tubes were discarded. DEN15, mOsm, and pH_f measurements were recorded from the 15 mL Falcon Tubes that were filled with 10 mL of each treatment medium supernatant. A DEN15 measurement determined the optical clarity of the media, and each measurement was recorded from a 590B Densimeter. Table 4 shows the DEN15, mOsm, and pH_f measurements. A Model 3250 Single-Sample Osmometer was used to test the osmolarity of each treatment media, with a goal of around 300 mOsm, and the pH_f was collected with a laboratory grade pH meter with a goal of 6.8-7.0 pH since bull sperm require a slightly acidic to neutral environment. Each test acid medium had a total of 6, 15 mL conical Falcon Tubes of supernatant that are frozen upright and stored in a freezer until they were needed for spermatozoa analysis. Figure 1 depicts the experimental design of Phase 1 and shows that there were 8 treatments per ejaculate.

Test Acid (250 mL bottle)	Estimated Acid (g)	Actual Acid (g)	Estimated TRIS (g)	Actual TRIS (g) for 6.8-7.0 pH	pH_i	Added CAM (g) for 6.8-7.0 pH	pH_f
CAM	1.153	1.152	2.18	2.3387	7.13	0.1662	6.8
Trehalose dihydrate	9.0800	9.0847	--	--	6.44	--	6.45
AKG	1.169	1.1691	1.938	2.0246	6.53	--	6.78
SUCC	0.9447	0.9443	1.938	2.0012	6.6	--	6.8
FUM	0.9286	0.9283	1.938	1.9381	7.47	0.1203	5.85
MAL	1.6091	1.6091	1.45368	3.0075	4.43	--	6.75
Maleic	1.3928	1.3928	1.45368	2.9489	5.14	--	6.85
TRIS WS 300	---	---	--	0.0703	6.67	--	6.79
HEPES	2.8600	2.8616	1.454	1.4561	7.75	0.6788	6.74

Table 1 Basis A (No added erythritol) Test Acid Media Preparation Phase 1.

Test Acid	Estimated Acid (g)	Actual Acid (g)	Estimated TRIS (g)	Actual TRIS (g) for 6.8-7.0 pH	pH_i	Added CAM for 6.8-7.0 pH	pH_f
CAMERY	0.577	0.5771	1.090	1.2243	6.95	0.976	6.8
AKGERY	0.585	0.585	0.969	1.0458	6.45	--	6.76
SUCCERY	0.472	0.4721	0.969	1.0358	6.54	--	6.79
FUMERY	0.464	0.464	0.969	0.969	6.8	--	6.43
MALERY	0.805	0.805	0.727	1.5243	4.83	--	6.78
Maleic+ERY	0.696	0.696	0.727	1.4868	5.47	--	6.8

Table 2 Basis B (125 mM of added ERY) Test Acid Media Preparation Phase 1.

Figure 1 Phase 1 Experimental Design

Total of 8 Treatments/Ejaculate

Spermatozoa Analysis of Phase 1

The preparation day (Day 0) for bull ejaculates (n=5, lettered A-E) were collected from one Holstein, one Jersey, and two Milking Shorthorn bulls. All ejaculates were checked in by the same lab researcher for acceptability. Then the ejaculates were washed and re-concentrated by the proprietary stabilization method of STgenetics® used prior to staining for sorting. A calculated amount of Tyrode's albumin lactic acid pyruvate (OS TALP) was added to each sample ejaculate based on the concentration of each stabilized ejaculate. OS TALP is a proprietary staining method similar to TALP used as a supportive gamete and embryonic media for IVF. The stabilized ejaculates were stained at 6.0 x 10e8 cells/mL and 98 µM Hoechst 33342 for 70 minutes at 34°C. The stained cells then held for ~60-65 minutes at room temperature before aliquoting the sperm into Treatment Acid Media holding samples. Next, the stabilized, stained sperm samples (100 µl) were placed into 4 mL capped polypropylene tubes which had been aliquoted with 900 µl of the appropriate Treatment Acid Media that were developed with Basis A or Basis B. Accordingly, the stored sample volume was 1.0 mL at a 9:1 ratio media to sperm. The concentration of the sperm during storage was ~55 million sperm/mL. The Treatment Acid Media holding samples were held overnight at 18°C, and analysis started on Day 3 for all bulls. The holding tubes containing 1 mL of extended sperm were held overnight at 18°C over the entire course of the 13-day test. These holding samples were then agitated and aliquoted from on analysis days to make observation

sample tubes to collect data, which will be described subsequently. This created a unique sample storage tube for each bull × treatment × day.

HT CASA was the instrument that determined the motility of the cells for each treatment. HT CASA data was collected on Day 3, 6, 8 and 13. Data points that were collected on the analysis days included Count Numbers and Observation Percentages. Count Numbers include: Total, Motile, Progressive, Static and Slow cell number counts. Observation Percentages were Motile, Progressive Motility, Static and Slow cell percentages from the initial Count Numbers.

For each observation day, a 33 µl sample of extended semen from the 4 mL tubes that were held overnight, each night, were aliquoted into a new, labeled, 4 mL observation tube with 300 µl of room temperature OS TALP, for a 10-fold dilution with ~5.5-6.0 million sperm/mL. The diluted sperm were warmed on a specimen warmer block set at 38°C for 30 minutes. Two 10 µl samples of each treatment with sperm were pipetted onto a warm, clean microscope slide and covered with a warm, clean cover slip. Each slide was measured using HT CASA and counted three separate times for triplicate data sets. Then each measurement was recorded for statistical results that determined which acids would proceed to Phase 2.

Phase 2

Media Preparation for Phase 2

Basis Medium Preparation

To conduct Phase 2 of the experiment, 2 L of one basis medium was created using 3.51 g of EDTA, 11.9 g of HEPES, 6.74 g of TRIS, 40 mL PenStrep (Lot #SLBJ2495V), a bottle of semen extender antibiotic supplement for bovine semen, and 2 bottles of GTLS. pH_f of the Basis Medium was 7.0, and the final mOsm of the Basis Medium was 76. Then treatment media was created using the acids that advanced from Phase 1, which included: CAM (CON), AKG, SUCC, FUM, Fumaric plus Pyruvate (PYR), CAMERY, and MAL.

Great Value™ White, Grade A Eggs were cracked and separated by hand to obtain raw egg yolk (REY) to be used as the cryoprotectant. The uncracked egg shell was washed with mild soap and water, dried, sprayed with alcohol, and the shell was air dried. Once the shell was dry, it was cracked, and the white was separated from the yolk by using a steel egg yolk separator. While each yolk was still inside the albumin, it was rolled on filter paper and squeezed with pressure from the researcher's hand for the contents to drain out of the membrane and into a beaker for later distribution into the 250 mL treatment media bottles.

Sterile, 250 mL media bottles were tared for each test acid. 150 g of the basis medium was combined with the specified amount of test acid, and 155 g of warmed REY for 50% (v/v). The estimated amount of TRIS was added to each test acid media. Next, the pH was adjusted to 6.8-7.0 using added amounts of TRIS if the medium was

too acidic or CAM if the it was too basic. Each bottle of test acid was placed in a cooler at 4°C for winterization for 12 hours. The calculated amounts of each test acid added to its respective bottle, calculated TRIS, pH_i, added TRIS or CAM, and pH_f after winterization can be found in Table 3.

After winterization, each test acid medium was transferred from the 250 mL bottle into a 500 mL conical centrifuge tube. The treatment media were then centrifuged at settings of 1200G for 30 minutes with an acceleration of 9 and a break of 2. Then 10 mL of supernatant was removed with a 5 mL pipette from each centrifuge tube and aliquoted into a 15 mL falcon tube, and the pellets in the centrifuge tubes were discarded. DEN15, mOsm, and pH_f measurements were recorded from the 15 mL Falcon Tubes that were filled with 10 mL of each treatment medium supernatant. Each DEN15 measurement was taken from a 590B Densimeter to measure the optical density of each test acid media. Table 5 shows the DEN15, mOsm, and pH_f measurements. A Model 3250 Single-Sample Osmometer was used to test the osmolarity of each treatment media, with a goal of around 300 mOsm, and the pH_f was collected with a laboratory grade pH meter with a goal of 6.8-7.0 pH. Each test acid medium had a total of 6, 15 mL conical Falcon Tubes of supernatant that were frozen upright and stored in a freezer until they were needed. Figure 2 illustrates the experimental design of Phase 2 and that there was a total of 14 treatments per bull.

Experimental Media Preparation

A second level of media preparation occurred in Phase 2. These special media are not standard to the industry and were used for semen straw production. All these

media contain Hoechst 33342 so that the sperm will be stained during processing in a manner which makes them fluorescently similar to sorted sperm in CASA analysis.

TRIS A Analog Preparation

The individually clarified test media samples described above. Also called "TRIS A" Analogs, since they are used like TRIS A media (without glycerol) is used in standard bovine cryopreservation. TRIS A was created by combining 80 volume parts TRIS WS 300 with 20 volume parts Raw EY and clarification.

BETH/Hoechst Preparation

A sperm staining media labeled BETH/Hoechst mixture consisted of 2 µl of Hoechst 33342 standard stain (5 mg/mL stock solution) in each 1 mL of BETH (proprietary washing media for sperm staining). The BETH/Hoechst mixture was used at room temperature for washing and at 34°C for staining. The entire sperm sample from each bull was prepared in the BETH/Hoechst mixture.

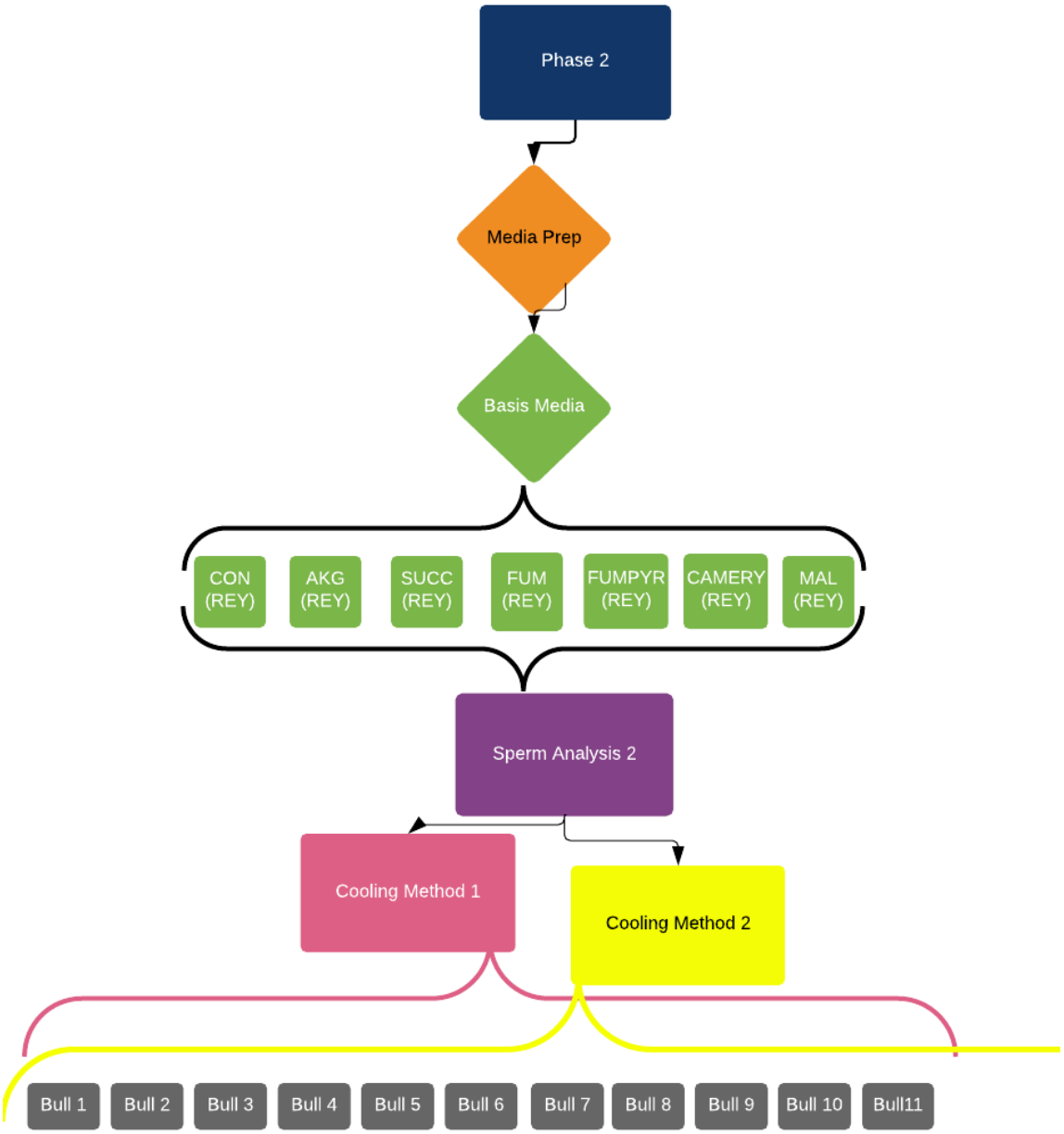
CRE12G/Hoechst Preparation

A CRE12G/Hoechst mixture was made and comprised of 2 µl of Hoechst 33342 for every 1 mL of TRIS WS 300. CRE12G is an egg free TRIS A to which 12% (v/v) glycerol in added to TRIS WS 300 that contains double the amount of glycerol required for cryopreservation. This media was added cold (4°C), prior to cryopreservation, at a ratio of 1:1, to provide 6% (v/v) glycerol to all samples.

Test Acid	Estimated Acid (g)	Actual Acid (g)	Estimated TRIS (g)	Actual TRIS	pH_i	Added CAM	pH_f
CAM	3.5450	4.1009	--	7.1271	2.99	--	6.55
AKG	3.2870	3.2871	--	5.6267	2.32	--	6.4
SUCC	2.6570	2.6571	--	5.5811	3.62	--	6.47
FUM	2.6120	2.6122	--	5.5828	3.12	--	6.31
PYR (FUM+ Pyruvate)	**FUM** =1.724 **Pyruvate** = 1.634	**FUM** =1.7241 **Pyruvate** =1.6341	--	3.8206	3.19	0.0611	6.29
CAMERY	**CAM** =1.181 **ERY** =5.495	**CAM** =1.1811 **ERY** =5.4953	--	2.0285	3.66	--	6.29
MAL	2.0100	2.01	3.72	1.3046	3.07	--	6.42

Table 3 Test Acid Media Preparation Phase 2. It was decided to add TRIS until pH reached 6.8-7.0; therefore, Estimated Tris is not applied to this table.

Figure 2 _Phase 2 Experimental Design



Total of 14 Treatments/ Ejaculate

Spermatozoa Analysis of Phase 2

11 Bulls were used in a cooling-holding test, commercial freezing, and commercial thawing processes for this portion of the experiment.

Quality Control

Ejaculates collected from one Jersey, and ten Holstein bulls, n=11 and numbered 1-11, that pass incoming quality control (QC) was used for this experiment. QC was conducted by the same lab researcher for an estimate of Visual Motility, Volume of Ejaculate, Concentration of Ejaculate, and Percent Primary and Percent Secondary morphology defects. The date, time, and jump (ejaculate) number ejaculate was recorded, along with the volume, motility, primary and secondary morphology, Nucleocounter concentration, and total cells in billions using a Nucleocounter. All 11 bulls were jumped twice in the event that their first jump did not meet a concentration of at least 3 billion sperm, regardless of volume. If the first jump did not have a 3 billion sperm count, then the bull's second ejaculate was combined to his first for the study.

Spermatozoa Preparation

Using a collected volume of raw semen that contained close to 3 billion sperm, that volume of ejaculate was combined with 2x that volume of BETH/Hoechst, held at room temperature, for a sperm:BETH of 1:2. Then the sperm concentration was determined by Nucleocounter. The cells were washed in the BETH in a 15 mL Falcon Tube and were centrifuged at industry settings of 2400 RPM for 15 minutes. The supernatant of BETH and seminal fluids were discarded. A final sperm cell concentration of ~1600 million sperm/mL was determined by Nucleocounter after the

supernatant was removed. Then 1 µl of Hoechst 33342 for every 40 million sperm, ~40µl, was added to the washed cells directly and mixed in quickly with aspiration by pipette, and the tubes with added stain were then incubated at 34°C for 30 minutes. During the incubation period, one empty 4 mL sample tube was labeled for each bull, cooling method, and treatment medium. The TRIS A Analog for each tube was made during the incubation period by combining 2 mL of each treatment medium that had 50% (v/v) REY with 2.5 mL of TRIS WS 300/Hoechst at room temperature.

After staining, sperm cells were transferred into 14, 4 mL sample tubes, per ejaculate, for a concentration of 120 million sperm/mL, which is somewhere between ~80-100 µl of washed, stained sperm. Then a volume of the TRIS A Analog (2.5 mL TRIS WS 300/Hoechst mixture + 2 mL 50% REY treatment media) to make a total volume of 2 mL was added to 2 of the 4 mL sample tubes that had 120 million sperm. The difference between the 2 sample tubes was the cooling method that was labeled on each tube; this will be discussed in the next section. After adding TRIS A Analog, it made the sperm concentration around 60 million sperm/mL and the EY concentration about 28.75%.

Cooling

Cooling Method 1

To imitate cooling in a catch tube, half (7) of the 4 mL treatment tubes were placed in a 50 mL Falcon tube with 20 mL of room temperature water and then placed in the Cooling Castle for 90 minutes before glycerolization. Glycerolization was a 2-step addition of 1 mL + 1 mL of CRE12G/Hoechst, which created the TRIS AB Analog. The

final concentration of sperm in the 4 mL tube was 30 million sperm/mL at this point. Fresh motility was checked within 2 hours of glycerolization; observations collected on fresh motility after glycerolization was deemed as Time Point 0. Then the samples were held overnight in the Cooling Castle before filling 0.25 cc straws the following day. The EY concentration was 14.4% and the sperm concentration was ~6-7 million sperm/straw at the time of freezing.

Cooling Method 2

The second set of (7) treatments in 4 mL tubes with a total volume of 2 mL of sperm (120 million sperm/mL) and TRIS A Analog were held overnight at 16-18°C. These 7 samples were cooled, glycerolized to make TRIS AB Analog, and frozen on the following day. In this way, both sets of straws were filled, sealed and frozen at the same time on the following day. Fresh motility was checked within 2hrs of glycerolization in each case.

Both methods were held in the Cooling Castle during the fresh data collection day 5, or Time Point 5.

Cryopreservation

Four straws per treatment for each cooling method were hand filled with 230 µl of extended sperm, sealed, and frozen in a timely manner or as soon as possible with a production freeze so that cells stay suspended in the media within the straw and did not collect on one side of the straw. Cryopreserved straws were thawed in an industry standard thaw bath at 35°C for 45 seconds and tested for visual motility through HT

CASA. Frozen straws were stored at industry standards in their own Research Nitrogen Tank and retrieved only when they were needed for analysis.

Data Collection

The motility of both freshly held and cryopreserved thawed sperm from the 4 holding methods for each of the 7 treatments was measured in triplicate using HT CASA. Data points that were collected included Count Numbers and Observation Percentages. Count Numbers included: Total, Motile, Progressive, Static and Slow cell number counts. Observation Percentages were Motile, Progressive Motility, Static and Slow cell percentages from the Count Numbers.

Fresh outcome data was measured at two time points, Time Point 0 and Time Point 5. Time Point (TP) 0 was taken after two hours of glycerolization of each cooling method, and TP 5 was taken on Day 5 for both cooling methods. For TP 0 and TP 5 observations, a sample of the cooled and glycerolized 4 mL tubes were transferred into a new 4 mL tube and warmed on a specimen warmer block set at 38°C for 30 mintues, and then two, 10 µl, samples of each treatment with sperm were pipetted onto a warm, clean microscope slide and covered with a warm, clean slide cover. Then each slide was analyzed in HT CASA.

Post-thaw motility was observed and recorded from 2 of the 4 straws for each treatment. Thawed outcome data in post-thaw motility included the industry standard thawing process of placing a treatment semen straw in a thaw bath at 35°C for 45 seconds, drying the straw with a paper towel, cutting the crimped end of the straw, and plunging the cotton end to deposit the thawed sperm into a warmed, sterile, labeled 4 mL

tube that had been in a specimen warming block at 38°C for at least 15 minutes. HT CASA was collected in triplicate observations at Hour 0 and at Hour 3 post-thaw and those measurements were used for statistical analysis. After triplicate data was collected at Hour 0, the 4mL tubes that contained each treatment were placed in a 34°C water incubator until the 3rd Hour for a final triplicate set of data collection before discarding. This is the standard protocol for industry QC straw analysis.

CHAPTER IV

SUMMARY AND DISCUSSION

Egg Yolk Discussion

DEN15 Measurements

The clarity of each treatment media was determined by utilizing a densimeter, which is normally used to measure sperm cell concentration. It was determined that the amount of EY required to obtain the clarity of each media was 15 µl. DEN15 is an arbitrary standard that uses a linear range to create a relative measurement of the optical density.

Based on data found in Table 4 for Phase 1, the DEN15 measurements suggested that CON and CAMERY were more efficient than other acids at discarding unsupportive solids that would interfere with sperm cell motility within the media containing 33% (v/v) HEY. This indicated that adding ERY could be just as effective if not more effective in sperm motility in the sperm analysis phase of Phase 1. Table 5 for Phase 2 contradicts the DEN15 measurements collected in Phase 1. Table 5 implied that CON and CAMERY were not as effective at precipitating out obstructive solids due to both treatments having some of the highest DEN15 measurements, which signified low clarity, that suggested lower sperm motility that would be stored within treatment media including 50% (v/v) REY.

Test Acid Media	DEN15 B/mL	mOsm	pH_f
Citric Acid Monohydrate	**0.86**	**277**	**6.8**
α-ketoglutaric acid	**1.35**	**307**	**6.78**
Fumaric acid	**1.66**	**309**	**5.85**
Malic acid	**1.53**	**372**	**6.75**
Succinic acid	**1.18**	**304**	**6.8**
CAMERY	**1.17**	**285**	**6.8**
AKGERY	**1.36**	**294**	**6.76**
FUMERY	**1.38**	**320**	**6.43**
MALERY	**1.73**	**358**	**6.78**
SUCCERY	**1.30**	**320**	**6.79**

Table 4 DEN15 HEY Clarification of Treatments Phase 1. According to the DEN15 measurements that were taken, CAM, the control (CON), had the most clarified or lowest optical density, and CAMERY possessed the next lowest DEN15 measurement. The osmolarity range was at or between 277-358, which was acceptable for Phase 1 to proceed to the next step of adding spermatozoa for analysis. The pH_f was taken after bringing each treatment to an initial pH of 6.8-7.0 using TRIS (Basic) or CAM (Acidic) and winterization at 4°C overnight. It is important to note that both fumaric acid and FUMERY treatments' pH were lower than all others because it takes longer for fumaric to equilibrate with added TRIS than the other acids; therefore, an unexpected additional drop in pH occurred during the winterization period, which was not corrected.

Test Acid Media	DEN15 B/mL	mOsm	Final pH
Citric Acid Monohydrate (CON)	**2.16**	**304**	**6.55**
α-ketoglutaric acid (AKG)	**0.41**	**320**	**6.40**
Fumaric acid (FUM)	**0.11**	**327**	**6.31**
Malic acid (MAL)	**2.28**	**288**	**6.42**
Succinic acid (SUCC)	**0.13**	**322**	**6.47**
CAMERY	**2.95**	**328**	**6.29**
Fumaric + Pyruvate (PYR)	**0.09**	**334**	**6.29**

Table 5 DEN15 REY Clarification of Treatments Phase 2. The osmolarity range for the treatment medias was 288-334, which was acceptable for Phase 2 to proceed to the next portion of sperm analysis. The final pH was taken after bringing each treatment to an initial pH of 6.8-7.0 using TRIS (Basic) or CAM (Acidic) and winterization at 4°C overnight. These treatments with REY show a lower average pH than HEY did in Phase 1.

Type of EY

In Phase 1, HEY was added to each treatment medium for a 33% (v/v), which is more than the industry standard of 20% (v/v). This concentration of HEY was implemented to act as a buffer for the maintenance of the spermatozoa over the 13-day holding period for the fresh data observations to be recorded. Phase 2 utilized 50% (v/v)

REY instead of 33% (v/v) HEY for the treatment media; this was due to REY allowing for better clarification of the EY according to previous, private research conducted by STgenetics®. REY began at a concentration of 50% in the extender media in Phase 2 to create a safeguard for when the REY was diluted to about 14% for fresh data and postfreeze data collection

.

Phase 1 Results

The differences among bulls are accepted as such in the statistical analysis portion of this experiment since the goal of this research was to find significant differences among treatments, not differences between bulls. There are bull effects in both Phases that are significant, but the bulls were considered random effects for the treatment comparisons.

Over the observation period of 13 days, specifically Days: 3, 6, 8, 13, there was an expected decrease in motile and progressive motile percentages in all treatments shared across all 5 bulls that can be seen in Figure 11 and Figure 12.

The ART industry concentrates on Motility and Progressive Motility quality measurements for semen straw production; therefore, the following tables and figures will concentrate on these two sperm characteristics.

Effects→ Responses	TRT	Day	TRT*Day
Motility%	0.0235	<.0001	0.9963
Progressive Motility%	0.0002	<.0001	0.6655

Table 6 Interactions Between Effects and Influences of Effects on Responses Phase 1. The table shows that no interactions existed between Treatment (TRT) and Day effects based off their p-values in Table 6. Treatment has a significant effect on motility and progressive motility percentages (P<0.05). Day also influences motility and progressive motility percentage responses.

Effect-> Response	Day 3	Day 6	Day 8	Day 13	SE	p-value
Motility	87.67%	83.16%	68.88%	37.07%	1.852%	<.0001
Progressive Motility	69.22%	62.86%	47.88%	16.81%	2.09%	<.0001

Table 7 Effect of Day on Sperm Quality Percent Measures Phase 1. The table shows that the day the sperm were observed over a 13-day period had significant effects on motility and progressive motility with Day 3 producing the highest percentages for both responses and then depleting as time continued, which is expected. Motility and progressive motility stayed above 50% at a week's time; this holding time could be utilized in industry for fresh shipped ejaculates. Progressive motility was always ~20% lower than overall motility of cells.

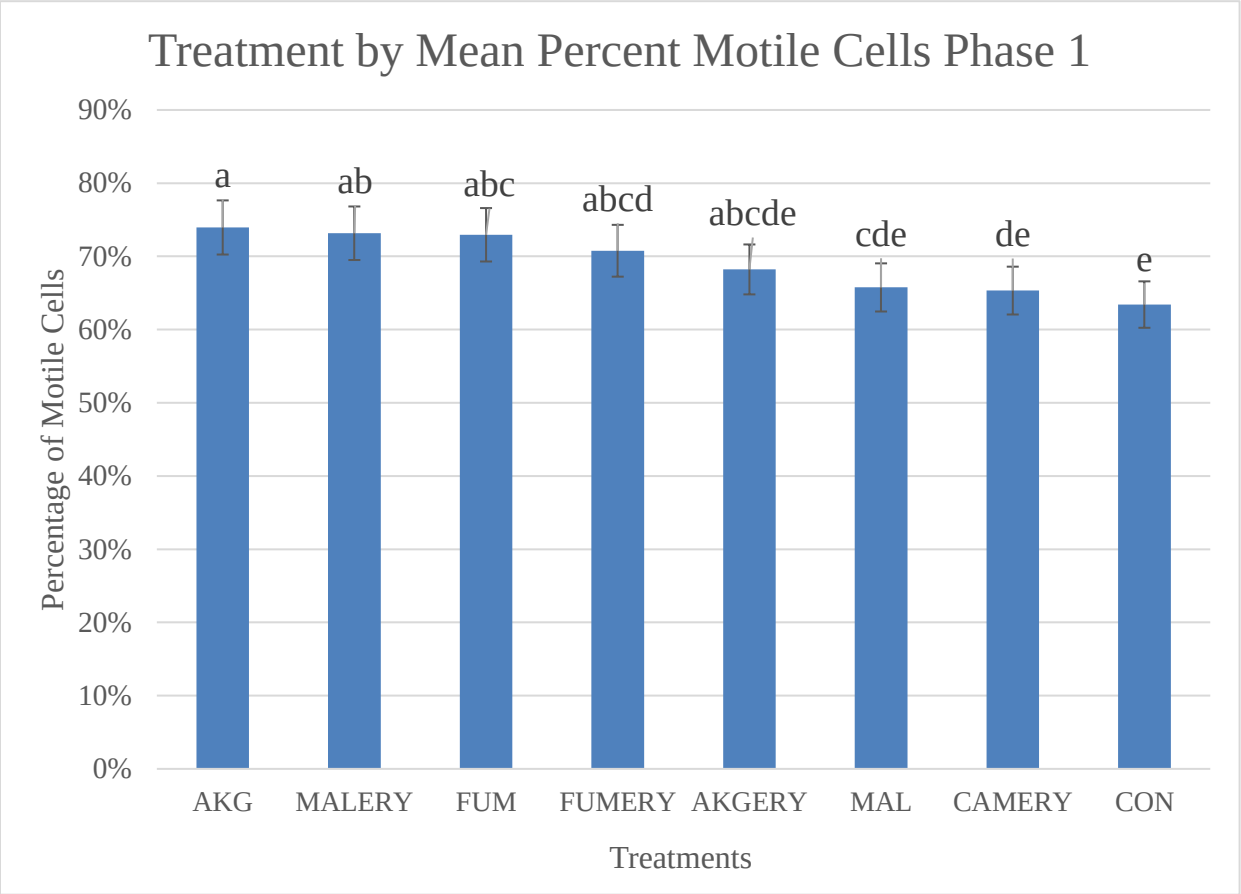


Figure 3 Treatment by Mean Percent Motile Cells Phase 1. Each bar depicts the mean percentage of motile cells across all observation days. There were differences in motility among treatments (p=.0235). Noncommon superscript letters differ (P < 0.05). Figure 3 shows that CON was significantly different and had lower motile cells than FUMERY, while CAMERY, MAL, and AKGERY were intermediates and did not differ from the other. AKG through FUMERY were statistically similar and had greater motility than all others except AKGERY. These treatment differences helped determine which treatments would advance to Phase 2 of the experiment.

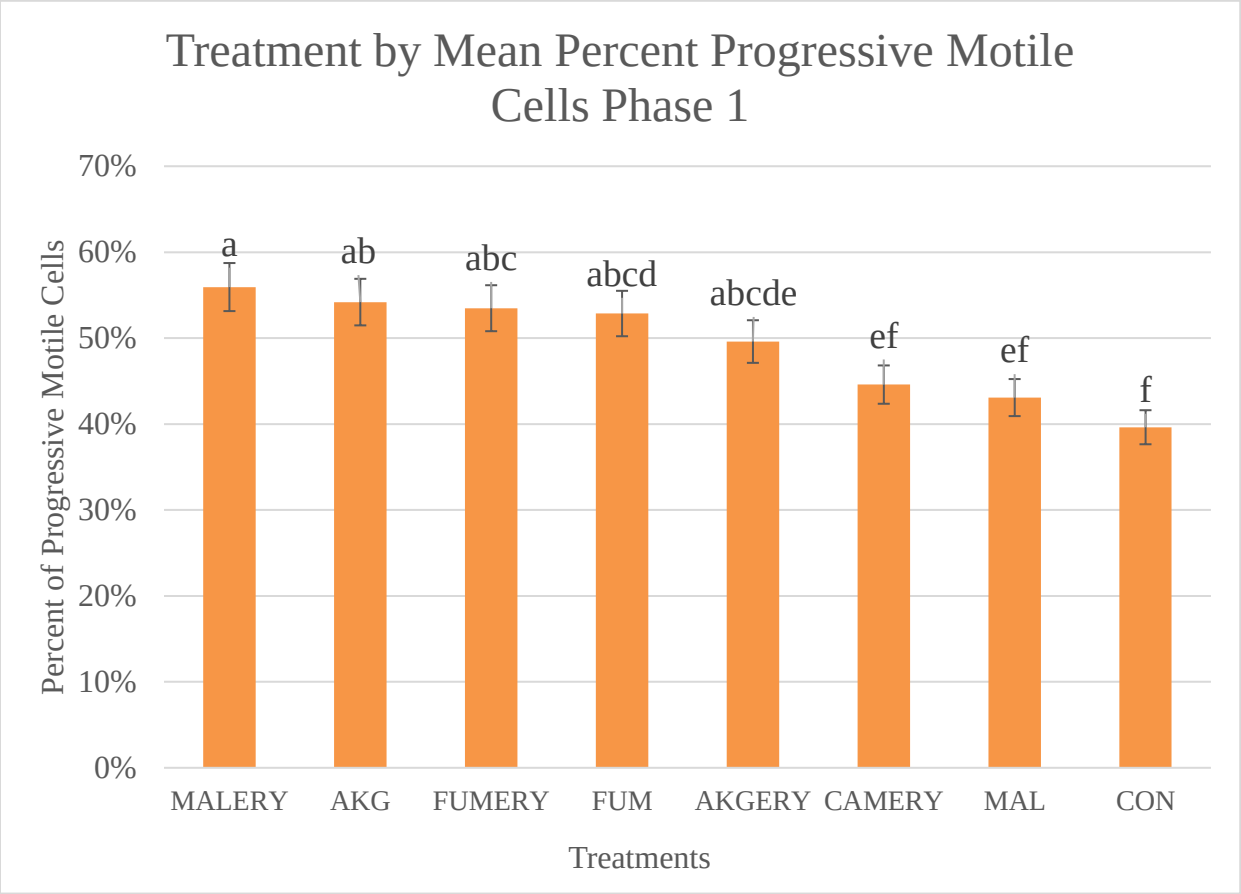


Figure 4 Treatment by Mean Percent Progressive Motile Cells Phase 1. Each bar depicts the mean percentage of progressive motile cells across all observation days. Noncommon superscript letters differ (P < 0.05). There were differences in progressive motility among treatments (p=.0002). Figure 4 shows that CON was significantly different had lower progressive motile cells than AKGERY, while CAMERY and MAL were intermediates and did not differ from the other. MALERY through FUM were statistically similar and had greater motility than all others except AKGERY.

Phase 2 Results

The differences among bulls are accepted as such since the goal of this experiment was to find significant differences among treatments, not differences between bulls.

Acids that showed to be the most supportive in Phase 1 that advanced to Phase 2 included: CON, AKG, FUM, SUCC, MAL, and CAMERY. Fumaric acid plus pyruvate (PYR) was not analyzed in Phase 1, but it was investigated in Phase 2 based off previous research conducted by STgenetics®. Fresh and thawed data was observed and collected among all 7 treatments. Fresh data shows that AKG and CON are statistically very similar and further research is warranted to confirm these discoveries.

As expected, Cooling Method 1 did better overall when compared to Cooling Method 2 because Cooling Method 1 slowed down the metabolic rate of the cells quickly before freezing, and Cooling Method 2 allowed for the cells to metabolize at normal rates for an extended time in ambient temperature before glycerolization.

The storage type of the cells also rendered results as expected. Fresh stored spermatozoa had higher overall motility and progressive motility rates than frozen stored and later thawed semen straws. This is due to cryopreservation, which is known to damage any cell or tissue that undergoes the process.

The following tables and figures show a treatment name that has been condensed. CON, AKG, FUM, PYR, SUCC, and MAL will remain the same in this section. CAMERY will now be shortened to “ERY” since it is the only treatment with added erythritol in Phase 2.

Effects→ Responses	TRT	Cooling Method	Storage	TRT*Meth	TRT*Stor	Meth*Stor	TRT*Meth*Stor
Motility%	0.0005	0.0016	<.0001	0.8419	0.9452	0.7076	0.9893
Progressive Motility%	<.0001	0.0155	<.0001	0.9549	0.7846	0.8269	0.9842

Table 8 Interactions Between Effects and Influences of Effects on Responses Phase 2 – Total Data. According to the interactions table above, there are no interactions between effects for the entire data set collected in Phase 2. Table 8 does demonstrate that TRT influenced both percentage responses. Cooling Method and Storage type effects also had an effect on Motility and Progressive Motility. Time, or Day, is not an effect in this data set since all data does not share specific Days or Time Points to compare. Time will be discussed in following tables and figures.

Effects→ Responses	TRT	Cooling Method	Time Point	TRT*Meth	TRT*TP	Meth*TP	TRT*Meth*TP
Motility%	0.0044	0.0071	<.0001	0.8719	0.7382	0.6122	0.9791
Progressive Motility%	<.0001	0.0622	<.0001	0.9270	0.9689	0.3356	0.9355

Table 9 Interactions Between Effects and Influences of Effects on Responses Phase 2 – Fresh Data. The table shows that there are no interactions among effects based off the p-values of the fresh data collected and analyzed at each time point (TP). TRT and TP influenced Motility and Progressive Motility percentages; however Cooling Method only had an effect on the response of Motility percentage. There were two TP in which data was collected on the fresh extended spermatozoa. TP 0 was fresh data recorded at 2 hours after glycerolization for each Cooling Method, and TP 5 was data collected at Day 5 for both cooling methods.

Effect Response	TP 0	TP 5	SE	p-value
Motility	54.58%	38.90%	1.836%	<.0001
Progressive Motility	33.10%	23.10%	1.44%	<.0001

Table 10 Effect of Time Point on Sperm Quality Percent Measures for Phase 2 – Fresh Data. The table displays that Time Point (TP) had a significant effect on motility and progressive motility. Time Point 0 (2 Hours after glycerolization of TRTs, depending on their Cooling Method) shows the highest percentages for Motility and Progressive Motility. The percentages decrease when these measurements were checked at the later TP, which is expected. Progressive Motility started out ~20% lower than Motility at TP 0, and the range tightened between motility and progressive motility by TP 5 because Motility decreased at a faster rate than Progressive Motility.

Effect Response	Cooling Method 1	Cooling Method 2	SE	p-value
Motility	50.26%	43.22%	1.836%	<.0001
Progressive Motility	30.01%	26.19%	1.44%	<.0001

Table 11 Effect of Cooling Method on Sperm Quality Percent Measures for Phase 2 – Fresh Data. The table displays that Cooling Method had a significant effect on motility, but not progressive motility. Cooling Method 1 shows the highest percentages for Motility and Progressive Motility, which is expected because the ejaculate was cooled and glycerolized immediately. Progressive Motility always stayed about ~20% lower than Motility in both cooling methods.

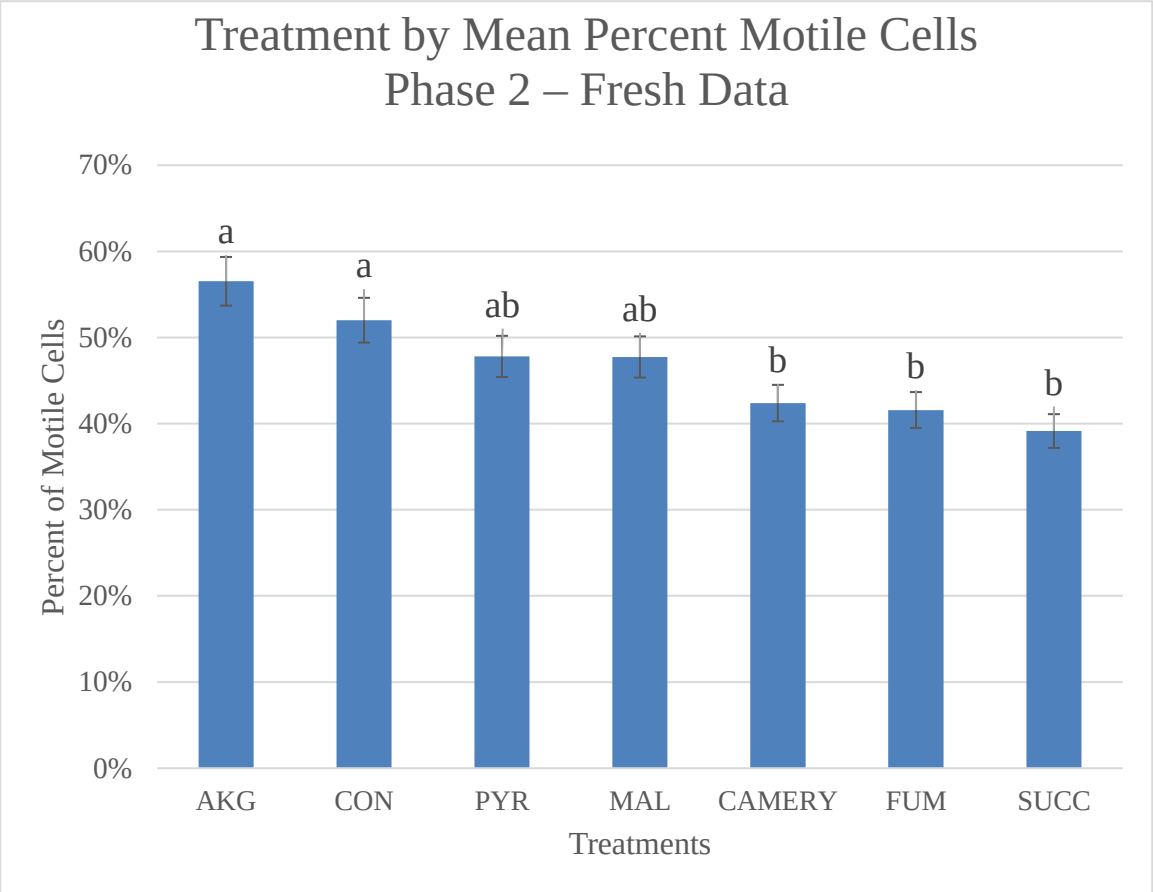


Figure 5 Treatment by Mean Percent Motile Cells Phase 2 – Fresh Data. Each bar depicts the mean percentage of motile cells across all TP. There were differences in motility among treatments (p=0.0044). Noncommon superscript letters differ (P < 0.05). Figure 5 shows AKG and CON had higher motile cells than and were statistically different from CAMERY, FUM, and SUCC, while PYR and MAL were intermediates that were similar to both subgroupings of treatments.

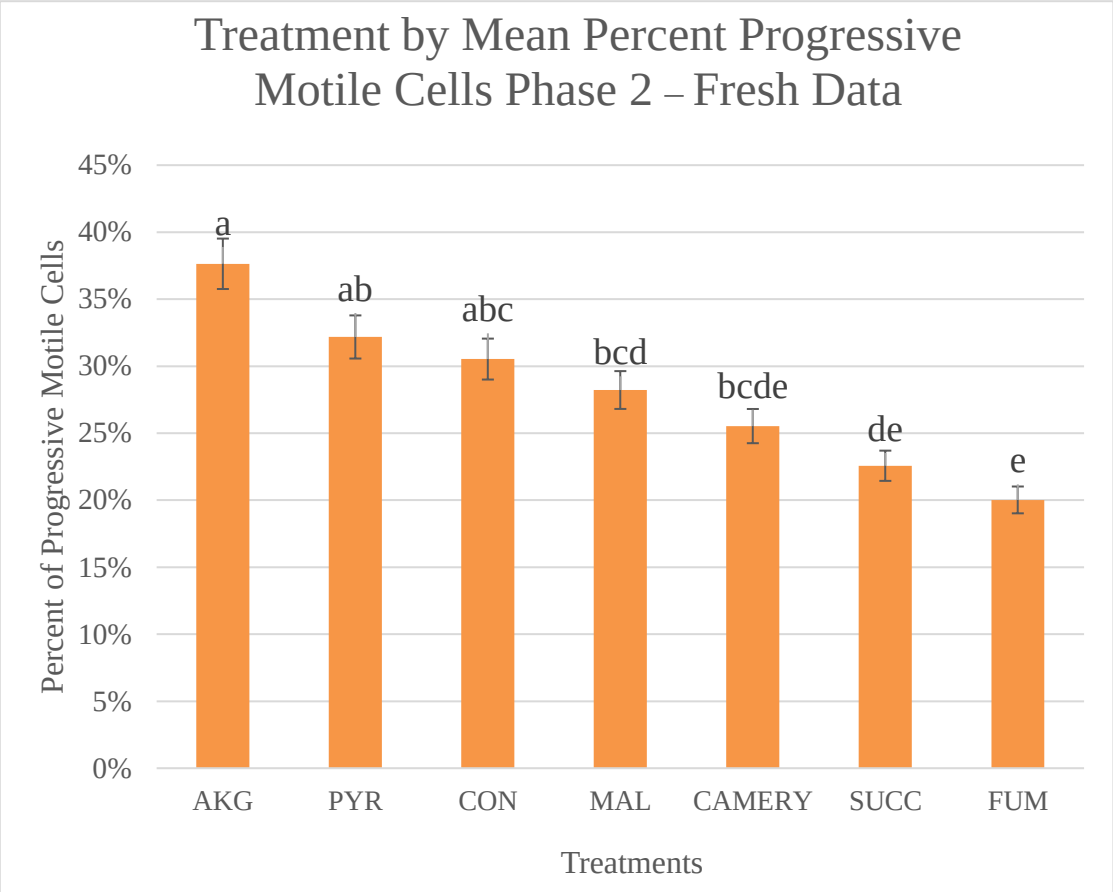


Figure 6 Treatment by Mean Percent Progressive Motile Cells Phase 2 – Fresh Data. Each bar depicts the mean percentage of progressive motile cells across both TP for fresh extended sperm. Noncommon superscript letters differ (P < 0.05). There were differences in progressive motility among treatments (p<.0001). This figure shows that AKG through CON were statistically similar, and they were significantly different than and had higher progressive motile cells than SUCC and FUM. MAL and CAMERY were intermediates that did not differ from the other, and shared similarities among subgroups.

Effects→ Responses	TRT	Method	Hours	TRT*Meth	TRT*HR	METH*HR	TRT*METH*HR
Motility%	<.0001	0.0016	<.0001	0.6335	0.9183	0.8109	0.9953
Progressive Motility%	<.0001	0.0083	<.0001	0.7956	0.5567	0.5596	0.9668

Table 12 Interactions Between Effects and Influences of Effects on Responses Phase 2 – Thaw Data. This table shows that there are no interactions among effects based off the p-values. TRT had a significant effect on both responses (P<0.05). Cooling Method and Hours at which data was collected on thawed cells influenced motility and progressive motility percentages.

Effect Response	HR 0	HR 3	SE	p-value
Motility	39.58%	31.33%	1.335%	<.0001
Progressive Motility	25.98%	15.68%	0.9536%	<.0001

Table 13 Effect of Hour on Sperm Quality Percent Measures for Phase 2 – Thaw Data. The table provides information on how the Hour after thawing frozen semen straws had a significant effect on motility and progressive motility. HR 0 shows the highest percentages for Motility and Progressive Motility, and then they decrease as time continues, which is expected. Motility and Progressive Motility did not decrease more than 10% within the 3-hour time span. Progressive Motility was always ~15% lower than Motility.

Effect Response	Cooling Method 1	Cooling Method 2	SE	p-value
Motility	38.46%	32.45%	1.335%	<.0001
Progressive Motility	22.62%	19.04%	0.9536%	<.0001

Table 14 Effect of Cooling Method on Sperm Quality Percent Measures for Phase 2 – Thaw Data. This table displays that Cooling Method had a significant effect on motility and progressive motility. Cooling Method 1 shows the highest percentages for Motility and Progressive Motility, which is expected because the ejaculate was cooled and glycerolized immediately. Progressive Motility stayed about ~15% lower than Motility in both freeze methods. Cooling Method 1 and 2-Thaw had lower than Table 8 (Cooling Method 1 sand 2-Fresh) percentages, which is expected due to cryopreservation.

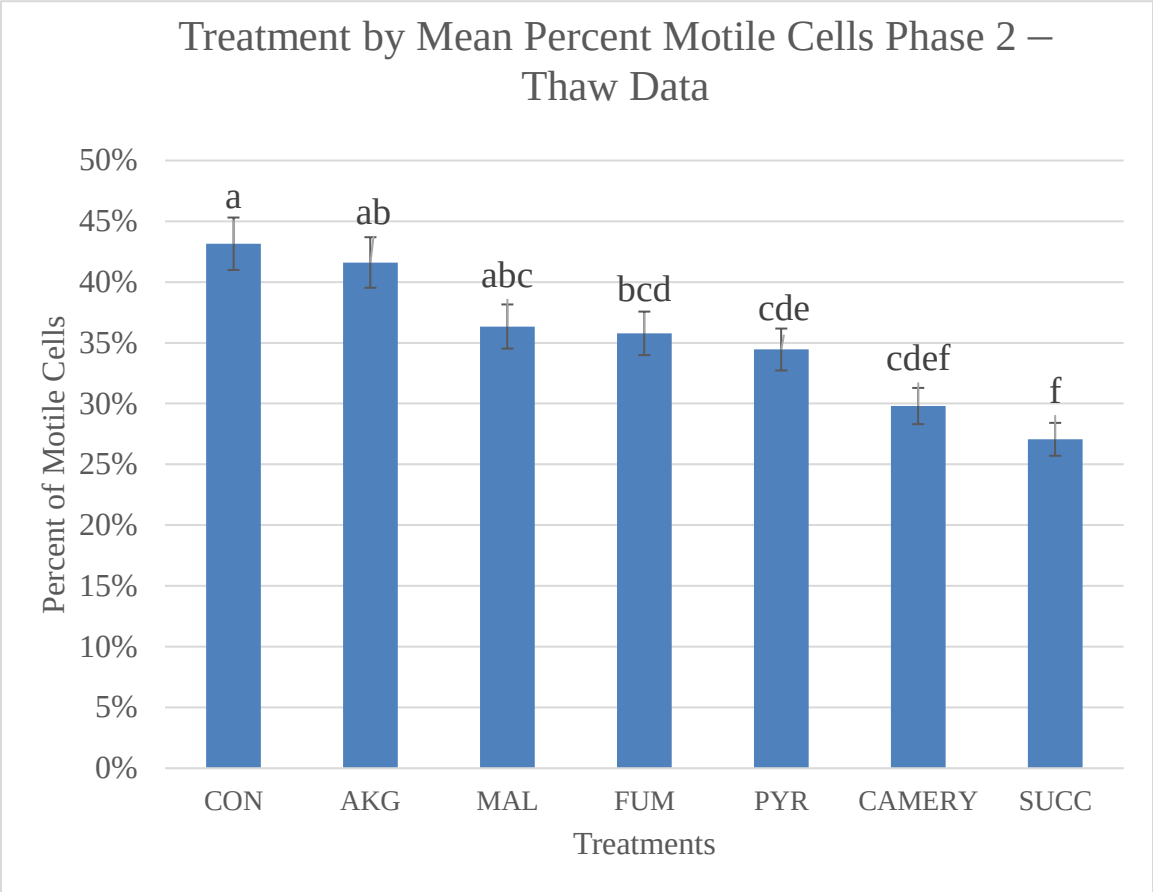


Figure 7 Treatment by Mean Percent Motile Cells Phase 2 – Thaw Data. Each bar depicts the mean percentage of motile cells across all hours of thawed data. There were differences in motility among treatments (p<.0001). Noncommon superscript letters differ (P < 0.05). Figure 7 creates a representation of how CON through MAL are statistically similar and that CON through PYR are all significantly different from CAMERY and SUCC TRTs as they all had higher motile cell percentages than CAMERY and SUCC.

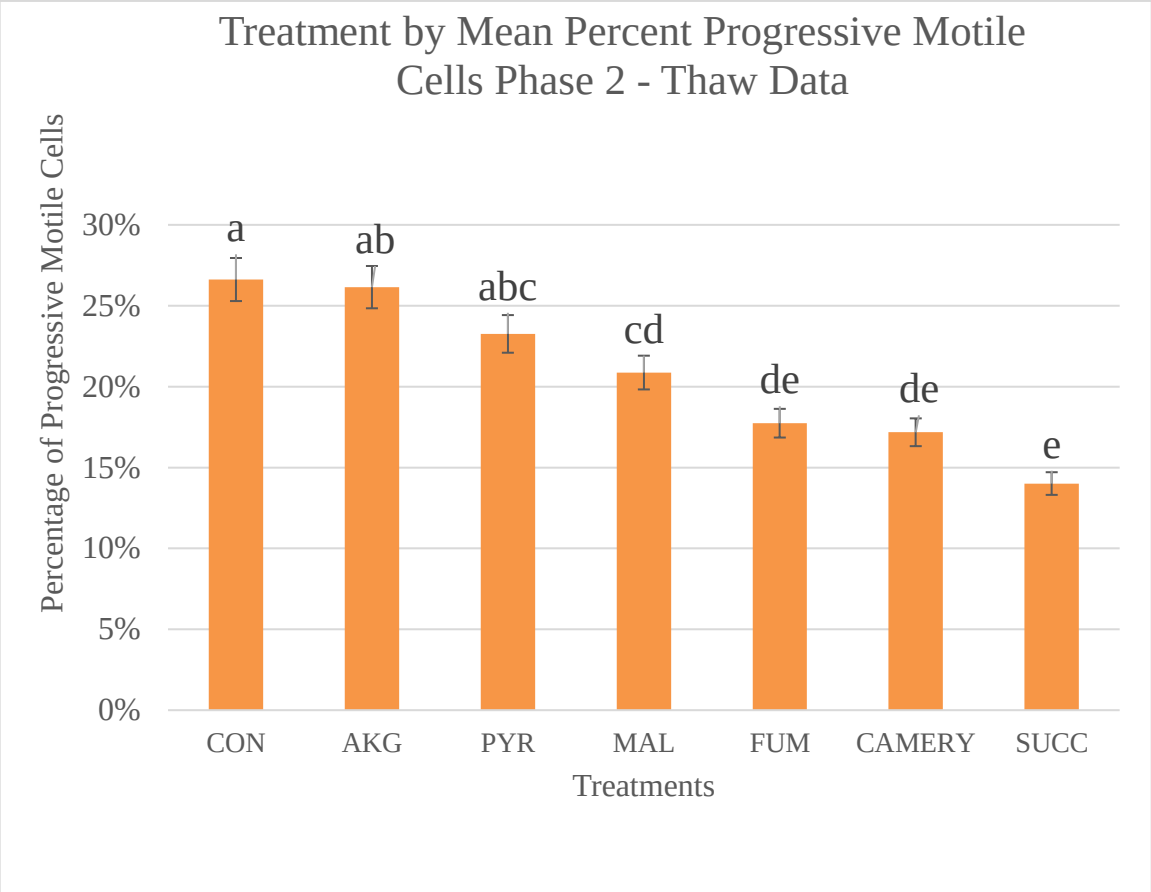


Figure 8 Treatment by Mean Percent Progressive Motile Cells Phase 2 – Thaw Data. Each bar depicts the mean percentage of progressive motile cells across both hours for thawed sperm. Noncommon superscript letters differ (P < 0.05). There were differences in progressive motility among treatments (p<.0001). This figure shows that CON through PYR were statistically similar, and they were significantly different than and had higher progressive motile cells than SUCC, CAMERY, and FUM. MAL was an intermediate that did not differ from FUM or CAMERY.

Effects→ Responses	Difference TRT	Difference Cooling Method	DiffTRT*DiffMeth
Motility%	0.7091	0.6845	0.3839
Progressive Motility%	0.2506	0.5508	0.3147

Table 15 Interactions Between Effects and Influences of Effects on Responses Phase 2 – Difference Between Fresh and Thaw Data. This table presents p-values that show that there were no interactions between TRT and Method effects when the difference between Fresh and Thaw data from Phase 2 was analyzed. The Difference between Fresh and Thawed TRT did not show any significant influence on Motility and Progressive Motility responses (P>0.05). The Difference between Fresh and Thawed Cooling Method also did not influence either response. This indicates that the difference value that was derived from thaw data that was subtracted from fresh data shows that the difference remains the same across all TRTs. Due to p-values being above 0.05 and effects having no significant influence on the two responses, there will be no comparative figures following this table.

Conclusion

Hypothesis

In conclusion, the data collected in this experiment rejected the null hypothesis that CAMERY would prove as the soundest additive for bovine extender media to achieve higher motility and progressive motility percentages of spermatozoa and possess the greatest clarity as an extender.

The highest motility and progressive motility percentages belonged to AKG, MALERY, FUMERY, FUM, and AKGERY in Phase 1. In Phase 2, the data indicated that AKG, CON, and sometimes PYR and MAL had the highest motility and progressive motility percentages in both Fresh and Thawed observations.

The greatest clarity among extender media in Phase 1 was the CON media, which used HEY as most companies in the industry would for the EY portion of the extender. CAMERY had the second highest clarity in Phase 1 with 33% (v/v) HEY. CAMERY had the lowest DEN15 measurement in Phase 2 with PYR rising to the top with the highest level of clarity.

Phase 1 Fresh

Data shows that AKG, MALERY, FUMERY, and FUM are significantly different from CON as they had higher motility and progressive motility rates. CON shows to be the most variable in fresh, HEY extended media. It would be warranted for future studies to investigate why CON had the highest clarity (DEN15 measurement) but having the least motile and progressive motile percentages. It could be due to CON "salting out" or the acid precipitating into the pellet that is discarded.

Biological acids, especially citric and malic, look to do well with 125 mM of added erythritol to fresh media, and it could be useful for shipping fresh/cooled semen as it has become very affordable at commercial volumes at $0.43/mol. This is compared to glycerol $0.10/mol and other polyols, such as sorbitol at $.30-.60/mol (Cargill, 2019). Therefore, further exploration of erythritol is necessary to potentially discover motility and progressive motility percentage benefits. While erythritol appears to aid in increased motility in certain acids, there is at least one variable that needs to be addressed. The variable in question is if erythritol is supporting the cells' motility, or if the sperm have an increased motility due to a lesser amount of test acid in the media. Additional research of Malic + Erythritol (MALERY) should be warranted as well since it performed with some of the highest motility and progressive motility percentages and was not studied further in Phase 2.

Phase 2 Fresh

Data reveals that AKG, and CON were always statistically different than SUCC and FUM with higher motility and progressive motility percentages across the 13-day test perios. Also, Phase 2 fresh data exposes that there are some qualities about PYR and MAL that are occasionally statistically similar to AKG and CON. It would be advantageous to continue research on the four Krebs cycle acids mentioned that are added exogenously.

Phase 2 Thaw

The thawed extender data shows that CON, AKG, and MAL are similar from a statistical standpoint in their motility percentages, while CON, AKG, and PYR are similar from a statistical standpoint in their progressive motility percentages. AKG, and CON were always statistically different from CAMERY and SUCC as they possessed higher motility and progressive motility percentages. CAMERY treated sperm did not do well during the Thawed analysis; therefore, conducting additional research on added erythritol to treatments for cryopreservation may confirm this because it could be due to the variables mentioned in Phase 1 Fresh.

Egg Yolk Clarification Investigation

Beginning an experiment that concentrates on the difference in sperm cell motility in HEY versus REY would prove beneficial to see what is causing CON to have poor motility percentages in HEY infused extender. CON and CAMERY treated with REY had the highest DEN15 measurements, the lowest clarity, which could indicate that their structures are keeping supportive lipids and solids in the emulsified egg yolk intact for the sperm cells to utilize and that the acid is not salting out into the pellet.

Final Inference

AKG is largest candidate for further research since it possessed some of the highest motile and progressive motile percentages throughout each phase, and it should be continued to be compared to the industry standard acid, citric acid. STgenetics® has potential and opportunity to patent a semen extender with AKG in recipe after prolonged research on the subject matter.

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APPENDIX A

SUPPLEMENTAL DATA

Treatment Box Plots

Phase 1 Box Plots

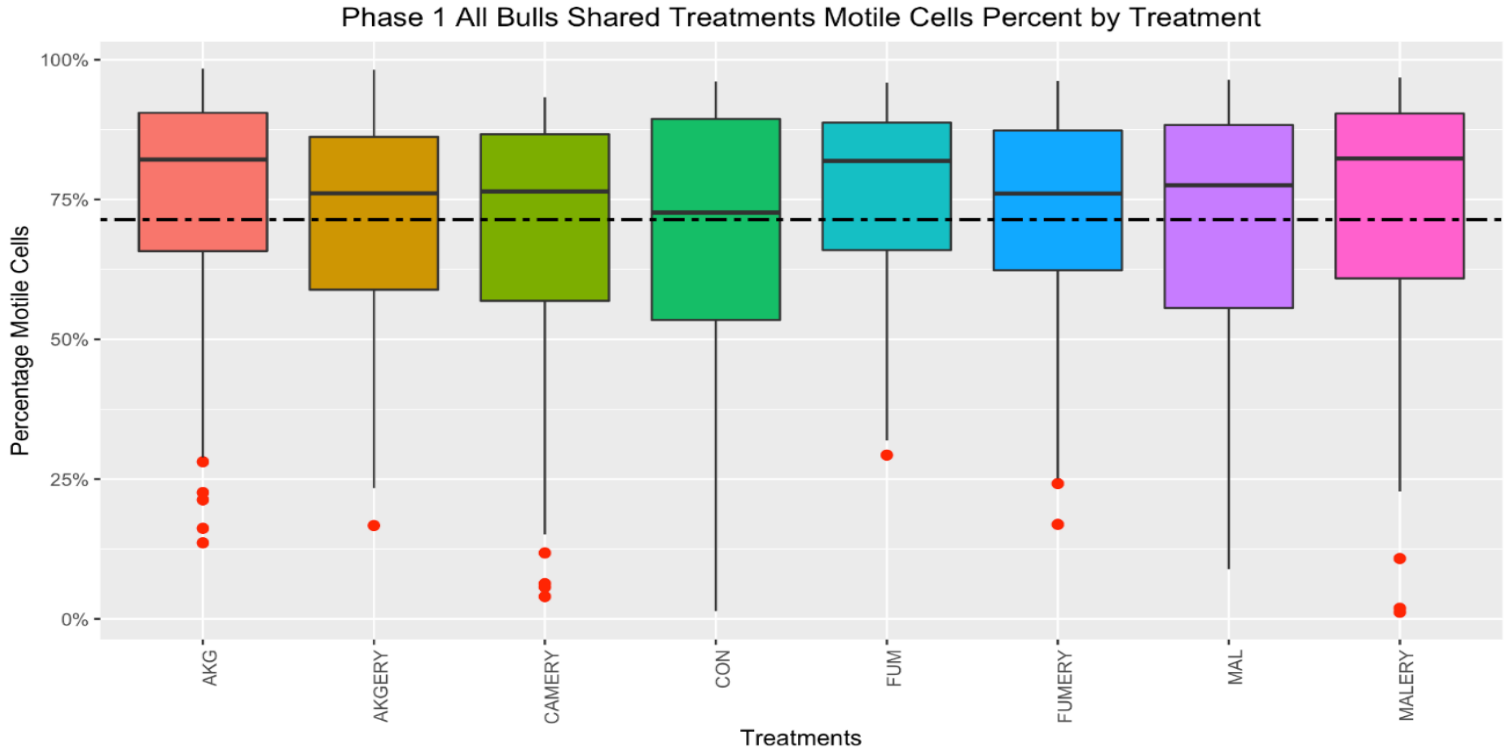

Figure 9 Phase 1 All Bulls Shared Treatments Motile Cells Percent by Treatment. The box plot shows the treatments along the X axis, and Percent Motile Cells along the Y axis. The dotted line represents the mean for all cells across all TRTs and all bulls. The red dots illustrate outliers. Since all data points are percentages, data points cannot be below zero. Also, this data was not trimmed for outliers, that is why the outliers appear in the box plots. If the box plot was trimmed for outliers, the 6 treatments with outliers might be higher on the motile percentage axis and had a smaller range. Furthermore, this box plot does not include time, or Day, as an effect. The plot only shows TRT as an effect across the entire data collection period. Figure 9 depicts that CON is the most variable in its motile cell percentages and that CAMERY had a tighter range with a higher median than CON. AKG, FUM, and MALERY had the highest medians above the mean.

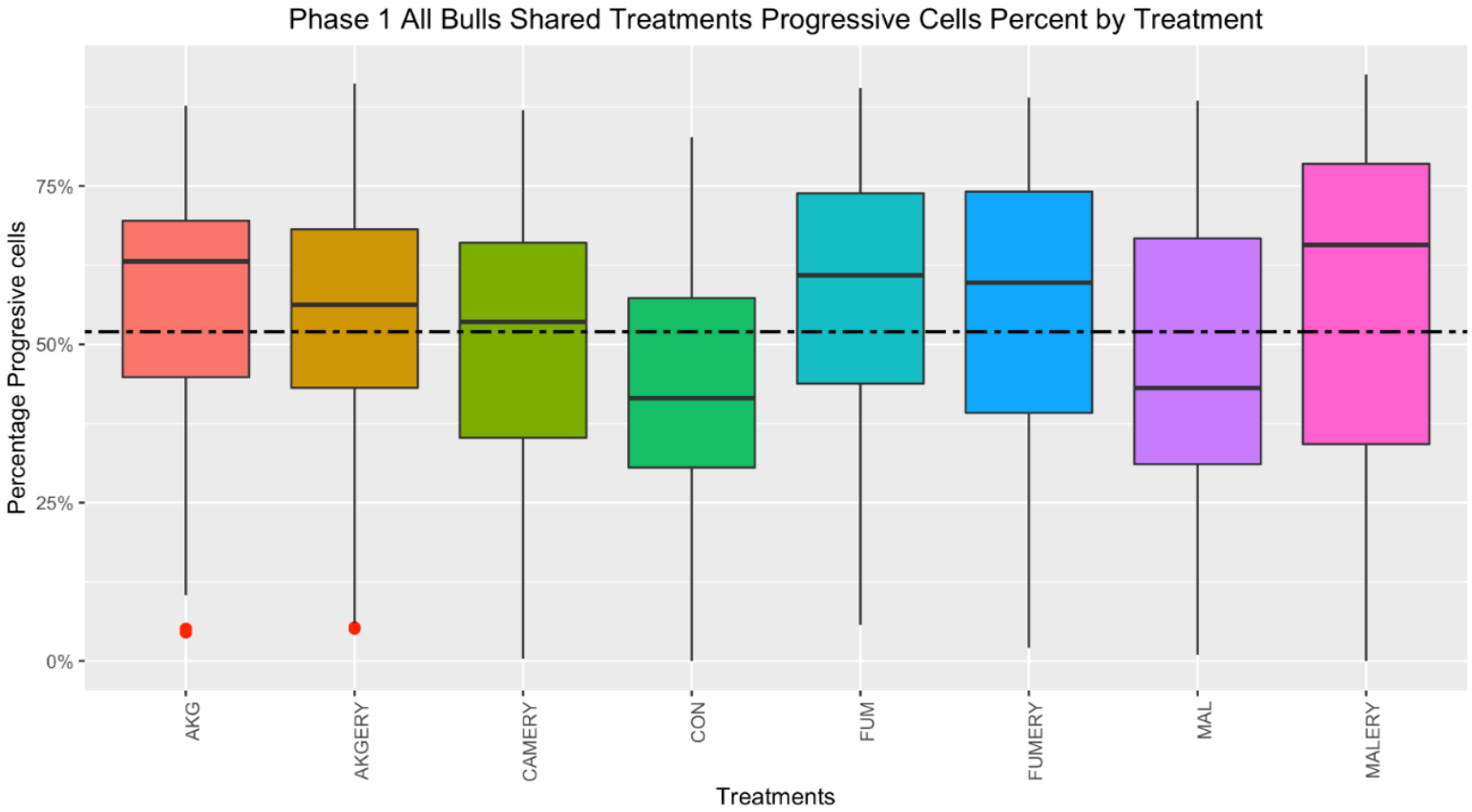

Figure 10 Phase 1 All Bulls Shared Treatments Progressive Cells Percent by Treatment. This box plot explains the percent of progressive cells by TRT. This plot does not include time, or Day, as an effect; it only shows TRT as an effect across the entire data collection period. Figure 10 depicts that CON and MAL are the only TRTs that had medians below the progressive cell mean. However, their added erythritol counterparts, CAMERY and MALERY, had higher medians above mean. Lastly, it is shown that AKG and MALERY had the highest means above the median with MALERY having the largest range.

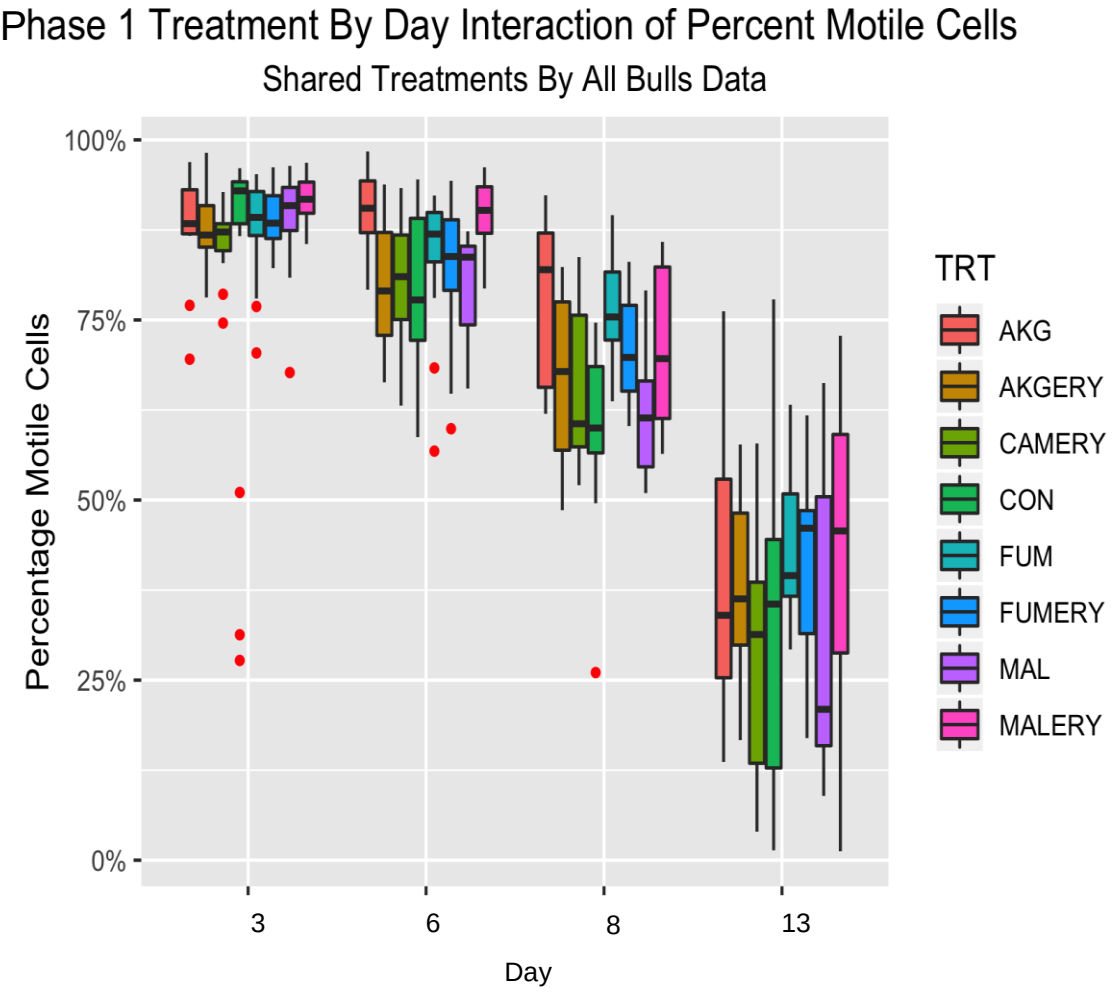

Figure 11 Phase 1 Treatment by Day Interaction of Percent Motile Cells. This figure is set up with Day along the X axis, Percent Motile Cells along the Y axis, and a TRT legend to the right of the box plots. The box plots give a graphical representation of motile percentage trends over time. It proves the visible trend over the 13-day data collection period that percent motility of all TRTs declined, as expected. This is due to the spermatozoa aging and metabolizing at normal rates outside of the body, which is congruent with Krzyzosiak et al. (2001) findings; as storage time for the cells in EY increased, fertility and motility decreased. The box plots illustrate that the percent of motile cells across treatments stay relatively the same and are tightly grouped initially. Day 6 shows that AKG and MAL had the highest medians above 85% at a week's time; this holding time could be utilized in industry for fresh shipped ejaculates. Overall, the percent of motile cells decreased over time and the range for each treatment became larger and more variable, especially by Day 13.

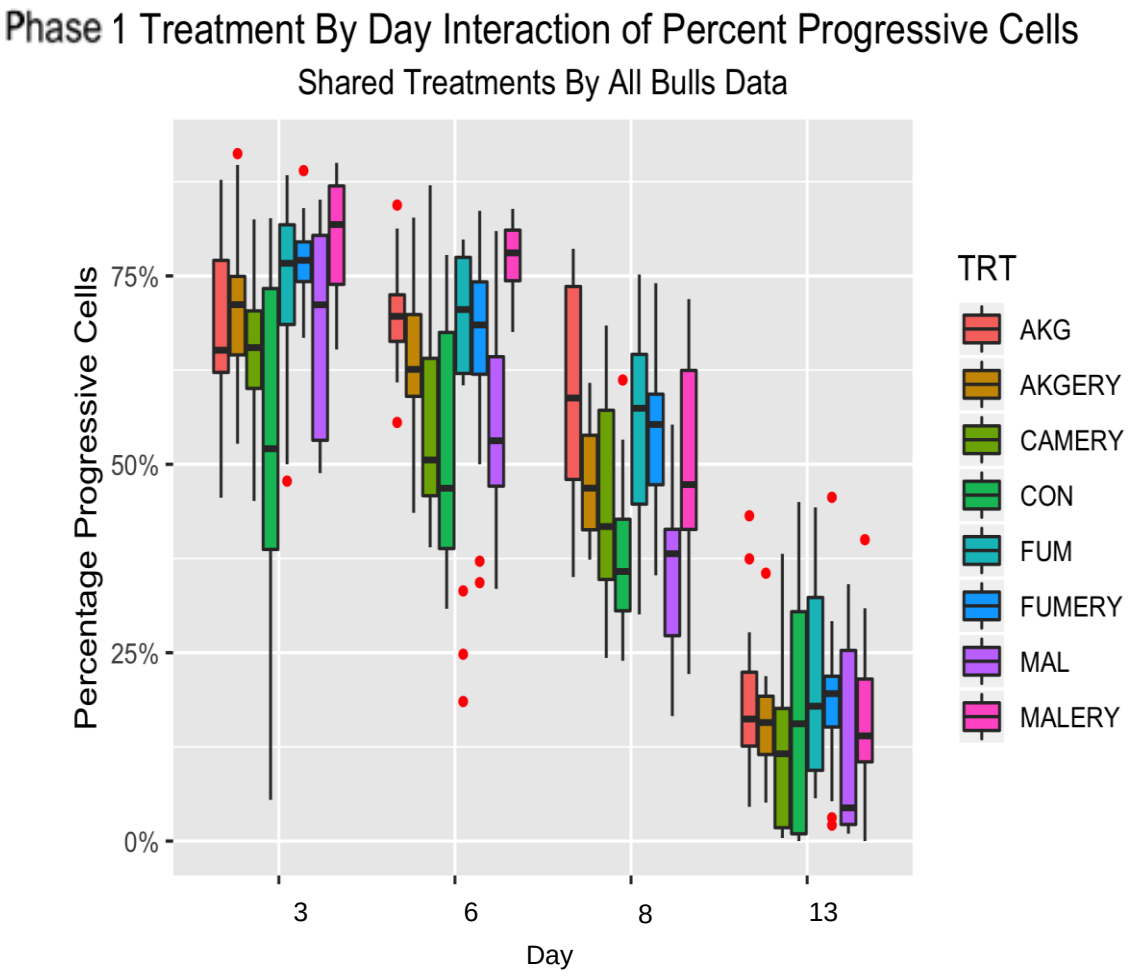

Figure 12 Phase 1 Treatment by Day Interaction of Percent Progressive Cells. The box plots give a graphical representation of progressive motile percentage trends over time. This figure shows the progressive motility percentage over the 13-day test period across all TRTs. There is an apparent trend over the data collection period that the percent progressive motility of all TRTs declined, as expected, because of the extended time that the cells were held in EY. CON is the most variable, especially on Day 3. These box plots also show that an added 125 mM of ERY may improve citric acid and malic acid. ERY near 125 mM in fresh held media is worth further exploration based on this figure. Progressive motility percentage tends to always be lower than motility percentages when comparing Figure 11 and Figure 12. Table 7 also shows this when comparing the average percentages between motile and progressive motile across all days.

Phase 2 Box Plots

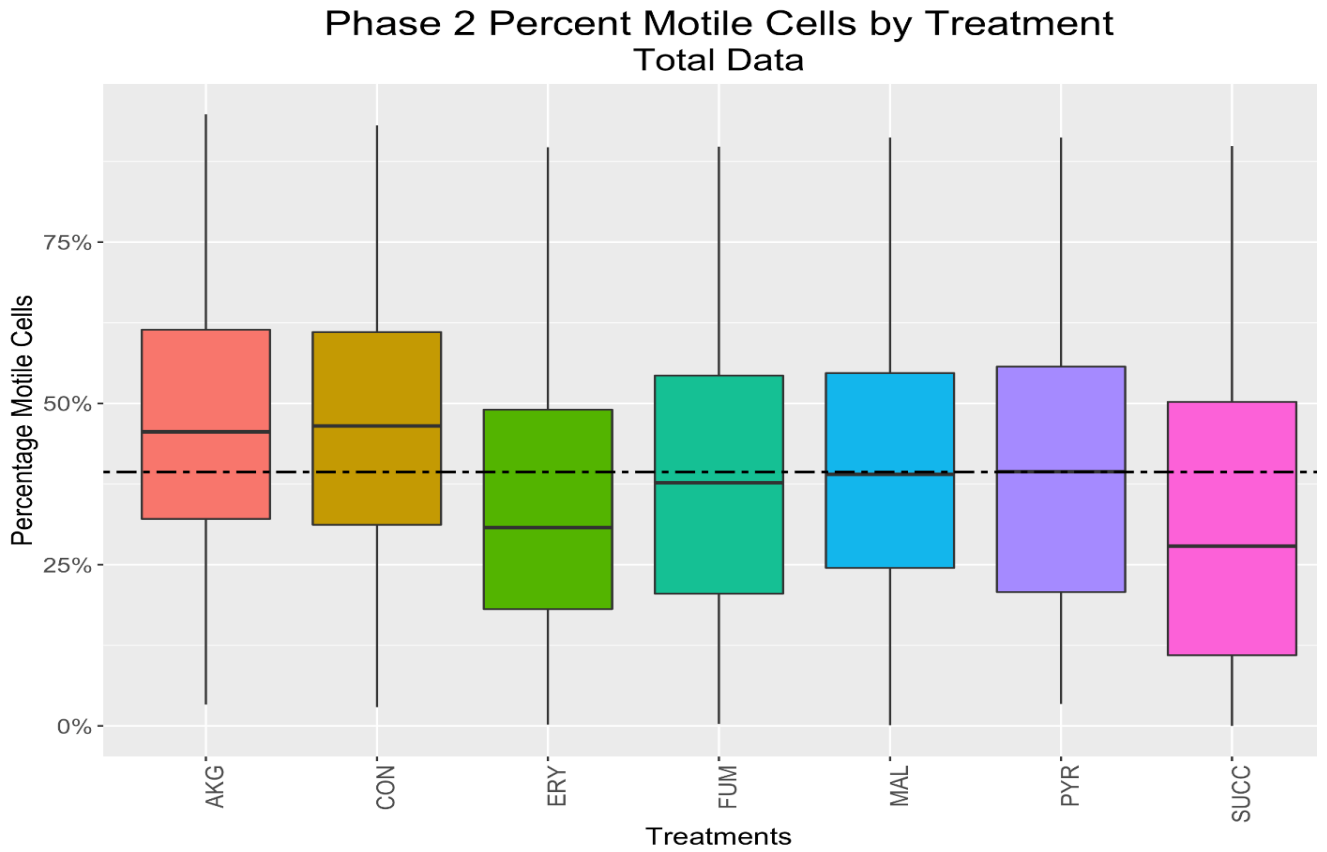

Figure 13 Phase 2 Percent Motile Cells by Treatment – Total Data. This box plot does not include time, or Day, as an effect. The plot only shows TRT as an effect across the entire data collection period. Figure 9 displays that AKG and CON are the only treatments across the total data set of fresh and thawed cells, both TRTs had the highest motility and they are the only TRTs with medians above the overall mean. SUCC had the largest range among treatments, and SUCC and CAMERY were the treatments with medians that are the most noticeable below the total data mean.

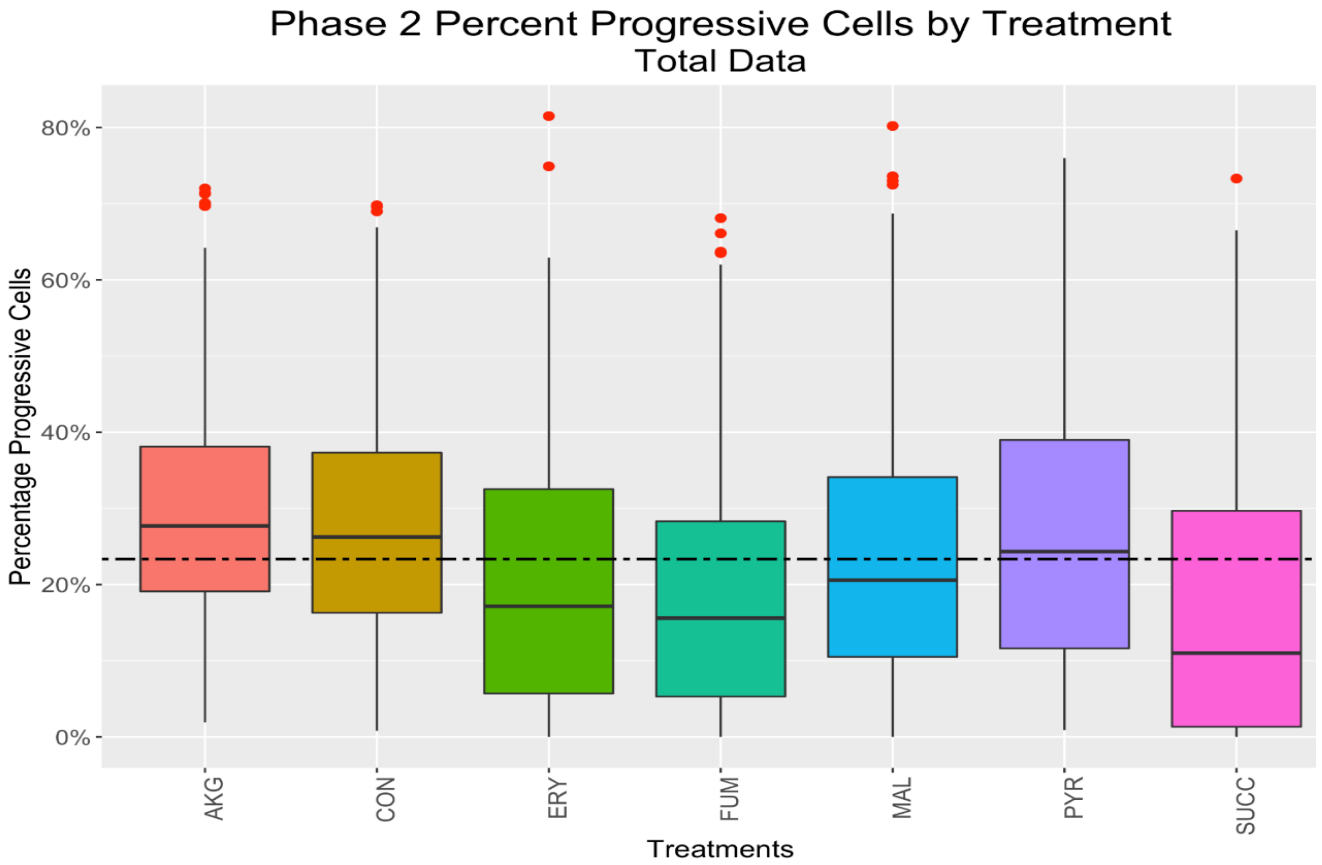

Figure 14 Phase 2 Percent Progressive Cells by Treatment – Total Data. This box plot does not include time, or Day, as an effect. The figure only shows TRT as an effect across the entire data collection period. Figure 14 depicts that AKG and CON also had the highest medians for progressive motility. PYR's median is just above the mean, following AKG and CON. All other TRT medians fell below the mean on progressive motility percentages. There are a few high outliers in this figure that are most likely lifting the medians for most of the TRTs up.

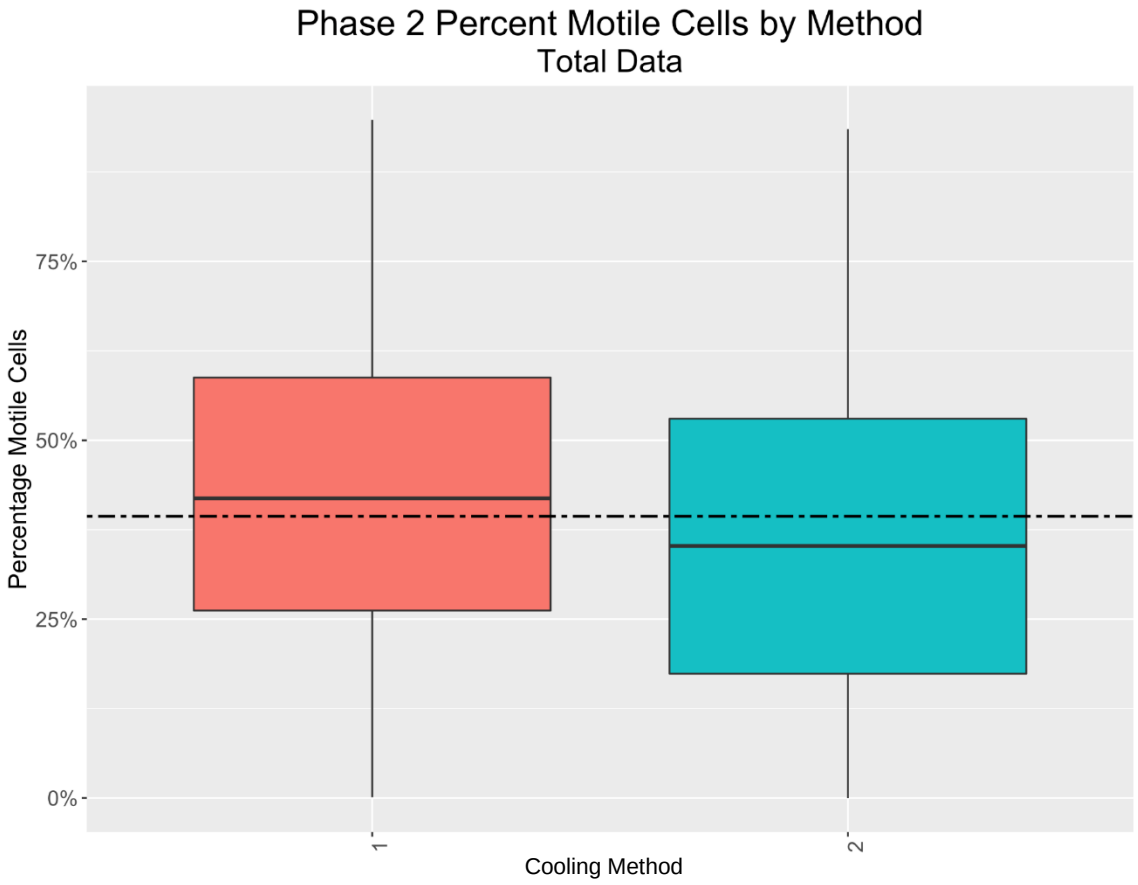

Figure 15 Phase 2 Percent Motile Cells by Method – Total Data. This box plot does not include time, or Day, as an effect. The figure only depicts Cooling Method as an effect over the entire data collection period. Figure 15 proves that Cooling Method 1 rendered higher percent motile cells than Cooling Method 2. Cooling Method 1 slowed down the metabolic rate of the cells quickly by cooling and adding glycerol immediately after the cells were extended. Cooling Method 2 allowed the cells to metabolize at normal rates for an extended time in EY at ambient temperature before cooling and glycerolization. This is the most probable cause for Cooling Method 2's median being the only one below the mean.

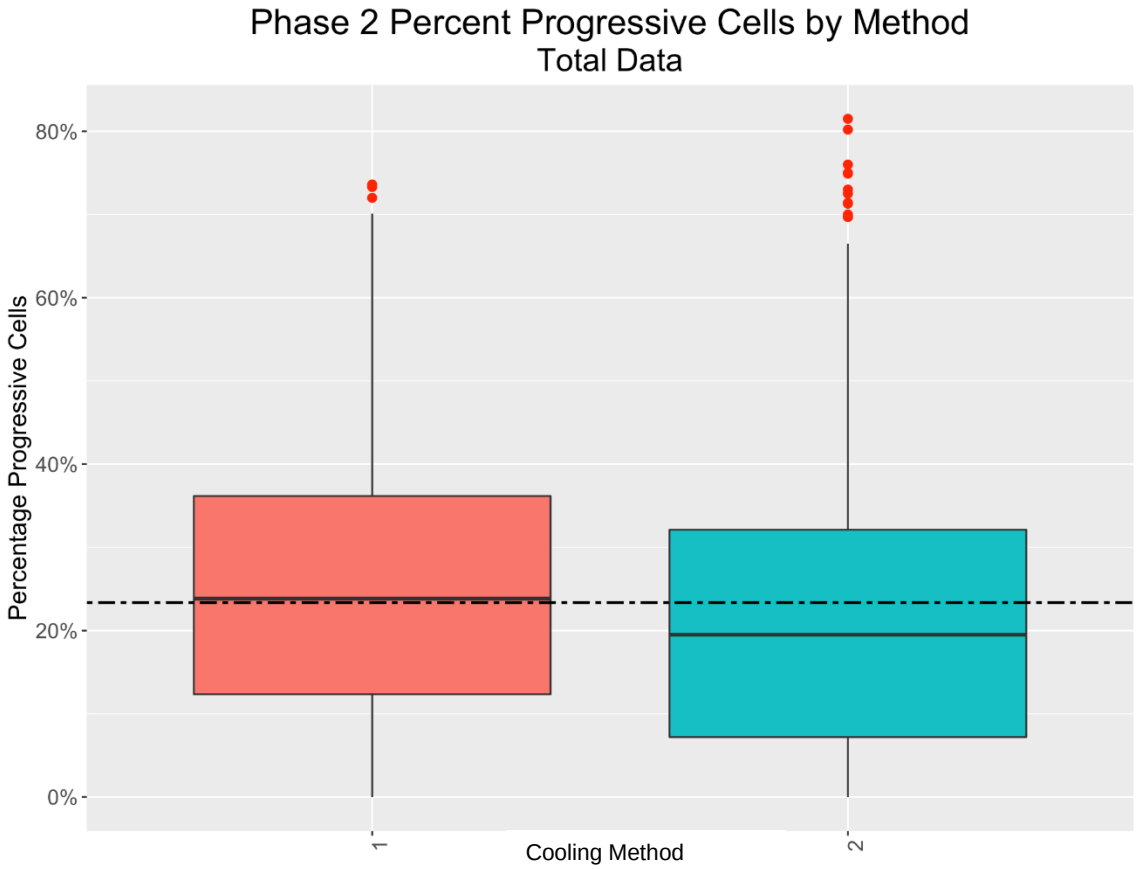

Figure 16 Phase 2 Percent Progressive Cells by Method – Total Data. This box plot does not include time, or Day, as an effect. The figure only depicts Cooling Method as an effect over the entire data collection period. Figure 16 also shows that Cooling Method 1 had higher progressive motile cells than Cooling Method 2, and Cooling Method 1's median is on the overall progressive motile cell mean. Cooling Method 2's median is visibly below the mean. Method 2 had a substantial number of high outliers, but Method 2 was significantly lower than Cooling Method 2 due majority of the cells being were extremely low in progressive motility. Cooling Method 2's outliers are most likely what caused the mean to rise to Cooling Method 1's median.

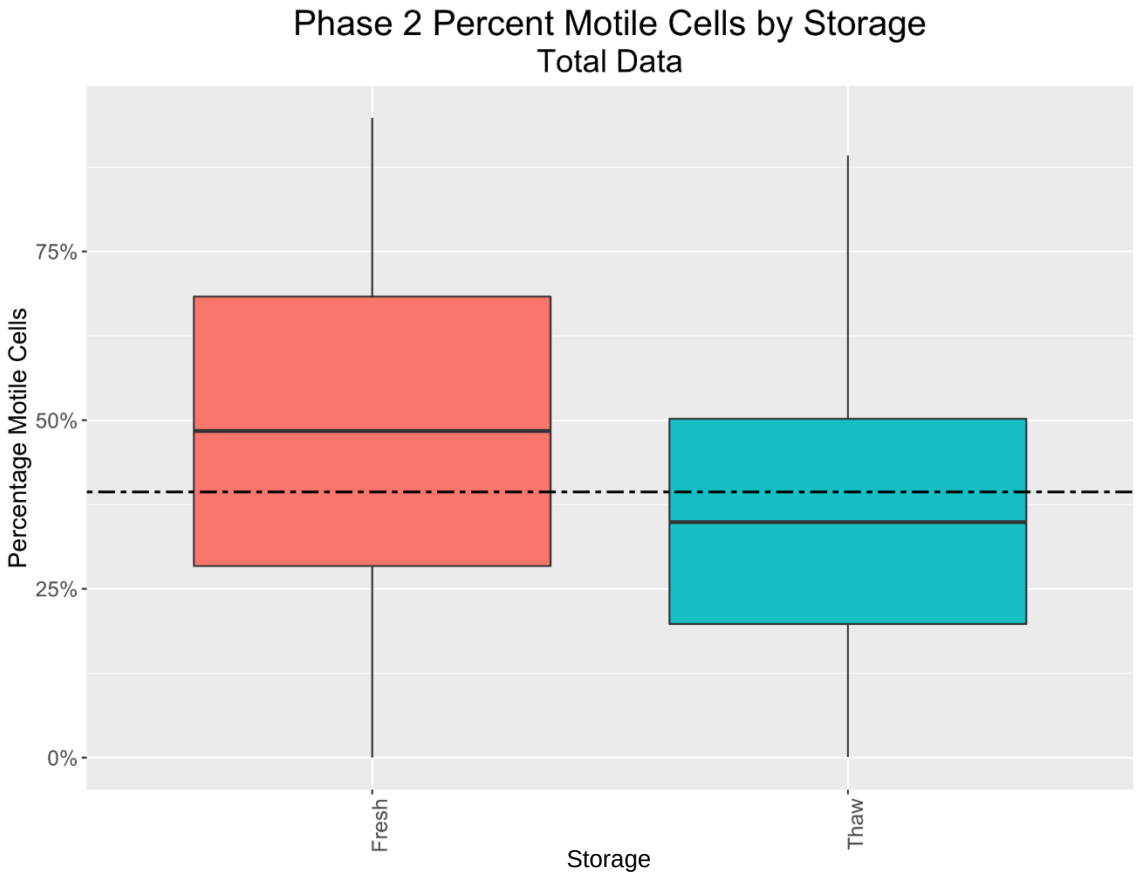

Figure 17 Phase 2 Percent Motile Cells by Storage – Total Data. This box plot does not include time, or Day, as an effect. The figure only shows Storage type as an effect over the entire data collection period. Figure 17 verifies what researchers, such as Richardson et al. (2017), have proved to be true. This figure confirms that cryopreservation damages the overall motility of spermatozoa. Fresh stored cells had higher motility percentages than frozen and then thawed cells because the fresh stored cells never underwent the cryopreservation process and osmotic stress; therefore, most of the cells' membranes are still intact unlike the cells that were frozen and then thawed.

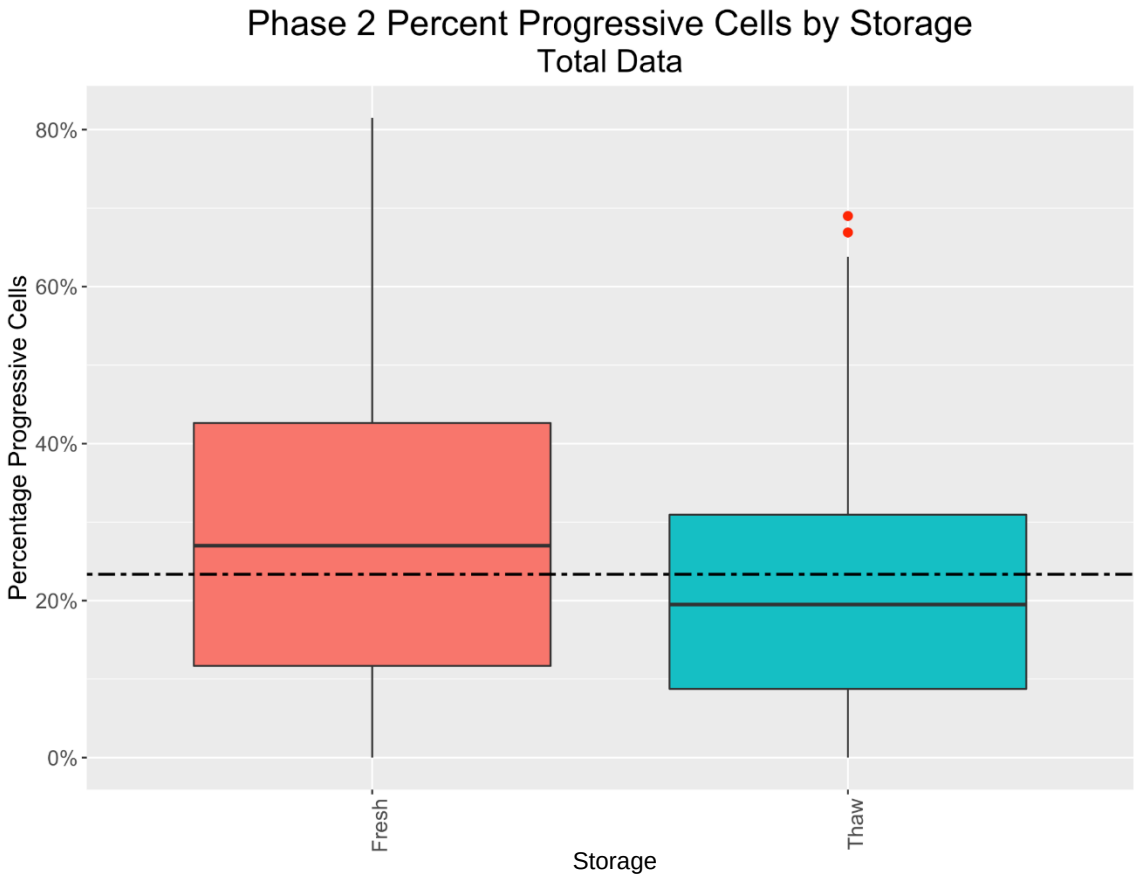

Figure 18 Phase 2 Percent Progressive Cells by Storage – Total Data. This box plot does not include time, or Day, as an effect. The figure only shows Storage type as an effect over the entire data collection period. Figure 18 confirms what was discussed in Figure 17. Fresh extended sperm cells had higher progressive motility percentages, and fresh stored cells had a median higher than the overall mean. Again, frozen and then thawed spermatozoa had the lowest progressive motility and the median for this storage type is below the mean.

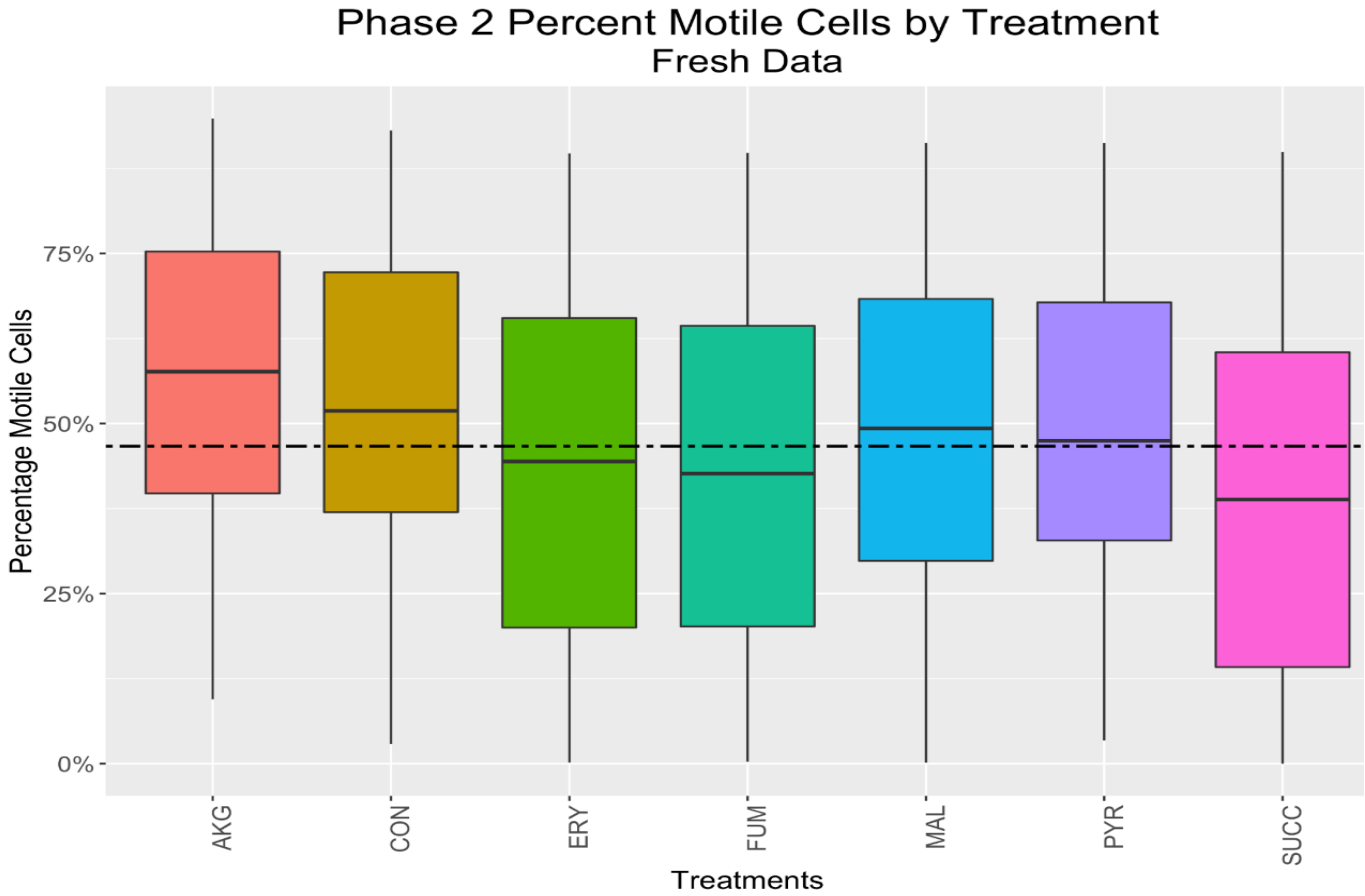

Figure 19 Phase 2 Percent Motile Cells by Treatment – Fresh Data. This figure shows percent motile cells by TRT across both TP that fresh data was collected. Figure 19 illustrates that AKG and CON had the highest percent motile cells in fresh media across the two TP. Both TRT also had the highest medians above the mean. MAL and PYR medians are right above and right at the mean, while the other 3 TRT had the largest ranges and their medians are below the mean.

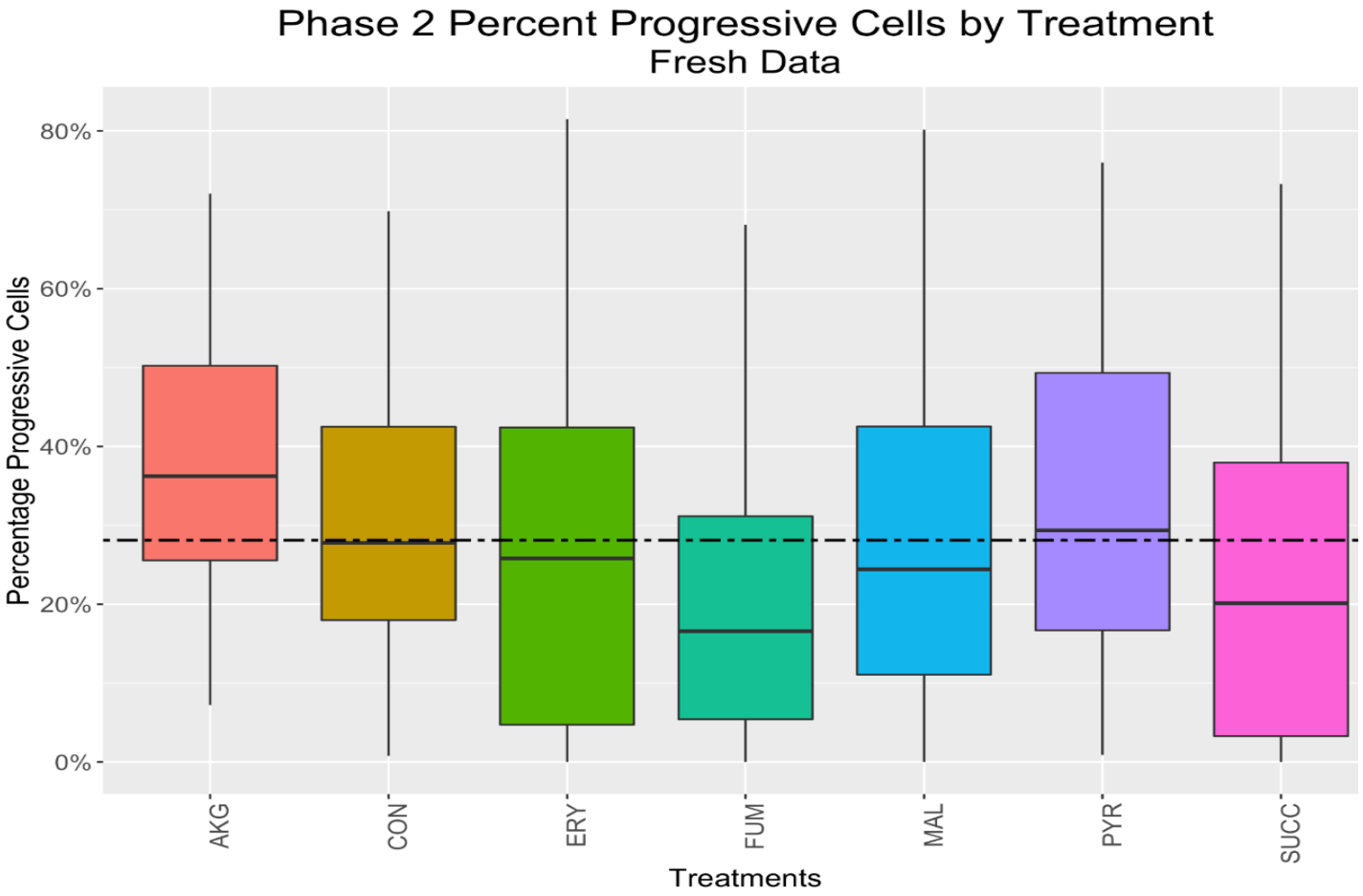

Figure 20 Phase 2 Percent Progressive Cells by Treatment – Fresh Data. This figure shows the percent of progressive motile cells by TRT across both TP that fresh data was collected. Figure 20 shows that AKG is the most distinct TRT with a median above the mean. PYR's median is just above the mean, and CON's median is on the mean. All other TRT medians are below the mean, and CAMERY and SUCC had the largest range of progressive motile cells.

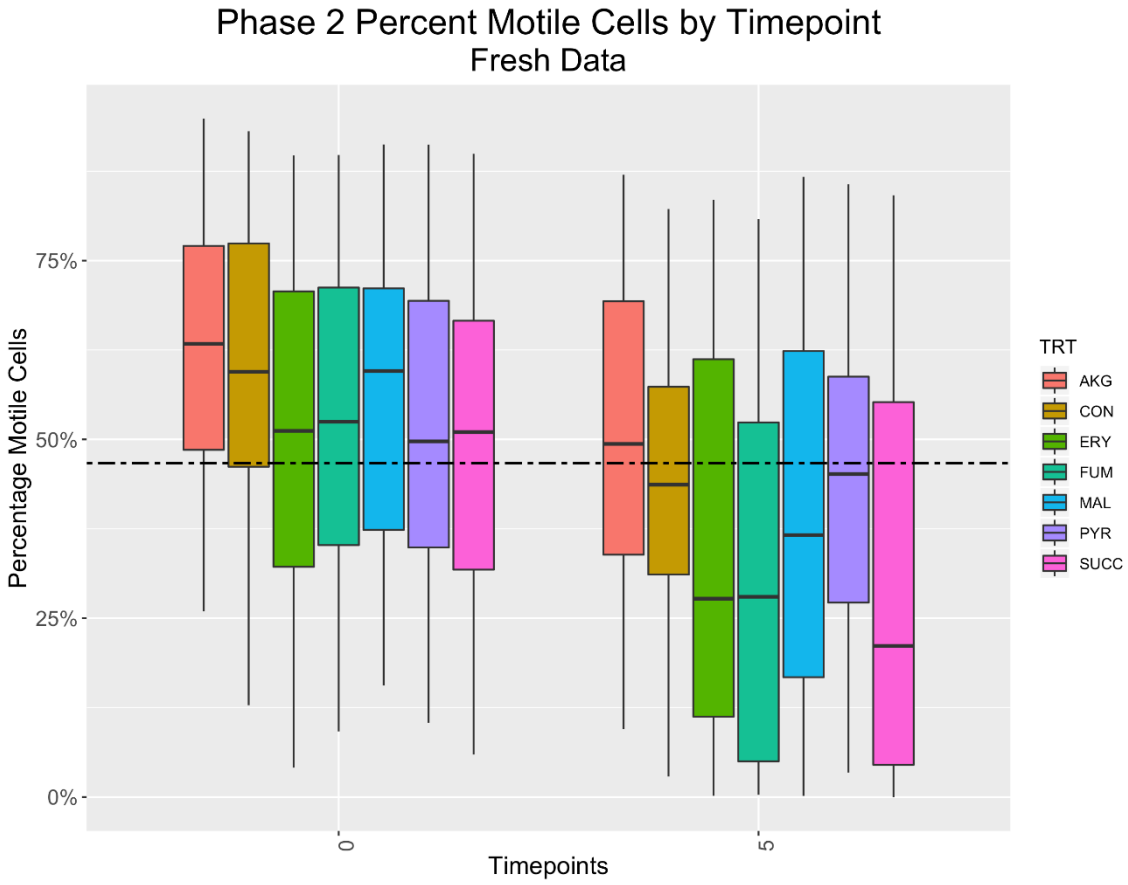

Figure 21 Phase 2 Percent Motile Cells by Timepoint – Fresh Data. The box plots signify the motile percentage of each treatment. It shows a trend over the two TP that data was recorded where motility decreases as time progresses, as expected. This figure agrees with the data presented in Phase 1 and Krzyzosiak et al. (2001) discoveries. The box plots illustrate that at TP 0, for both Cooling Methods, that all TRT medians are above the mean. AKG had the highest median and CON and MAL had the next highest medians. By TP 5, all TRTs became more variable in motile cell percentage. All TRT medians were below the mean at TP 5, except for AKG.

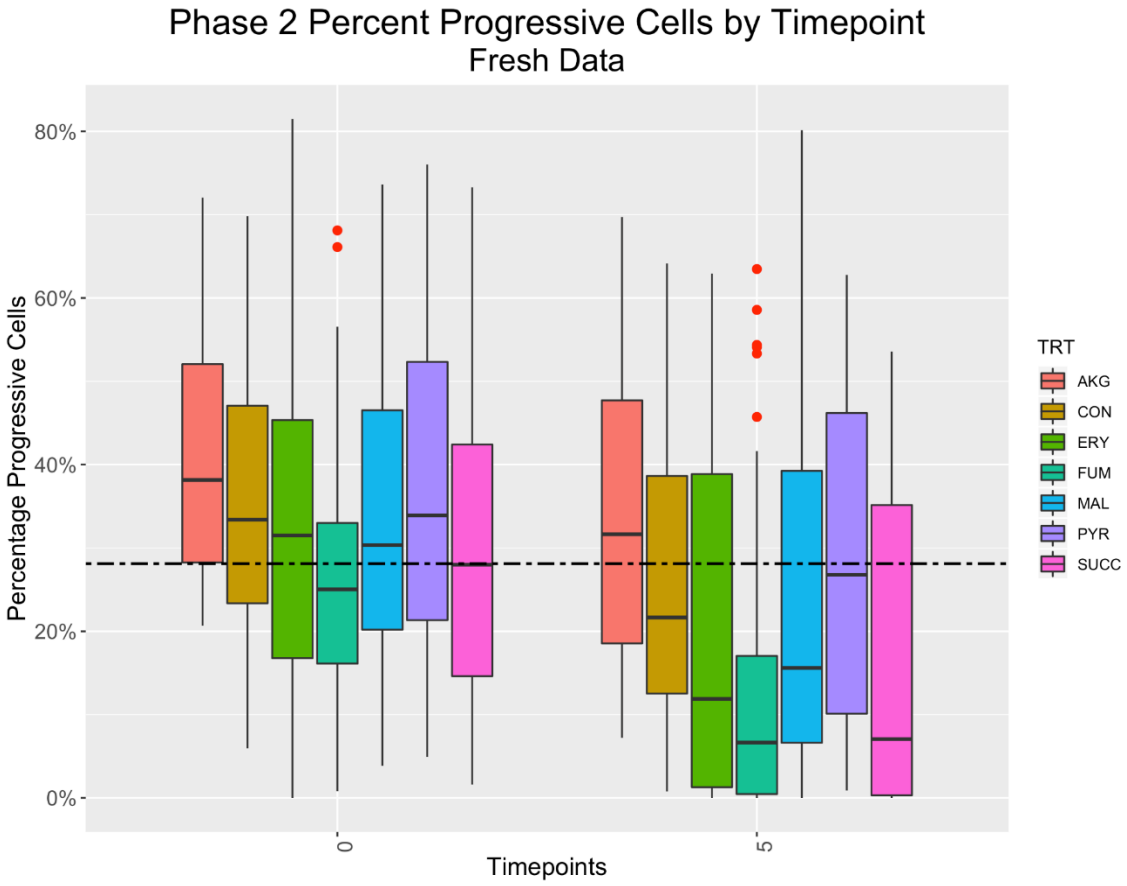

Figure 22 Phase 2 Percent Progressive Cells by Timepoint – Fresh Data. The box plots show the progressive motile percentage over the two TP that fresh data was collected. It also expresses an expected trend over the two TP that progressive motility decreases as time progresses. The box plots illustrate that at TP 0, for both Cooling Methods, that AKG has the highest median above the mean. CAMERY, PYR, and SUCC had the largest percent progressive cell range, and FUM is the only TRT that has a median below the mean. TP 5 shows that AKG is the only TRT with a median above the mean, which is the same as TP 5 in Figure 21. FUM has some outliers, but it has too many low percent progressive cells to bring the interquartile range (IQR) any higher. MAL, CAMERY, PYR, and SUCC all had considerable ranges within TP 5.

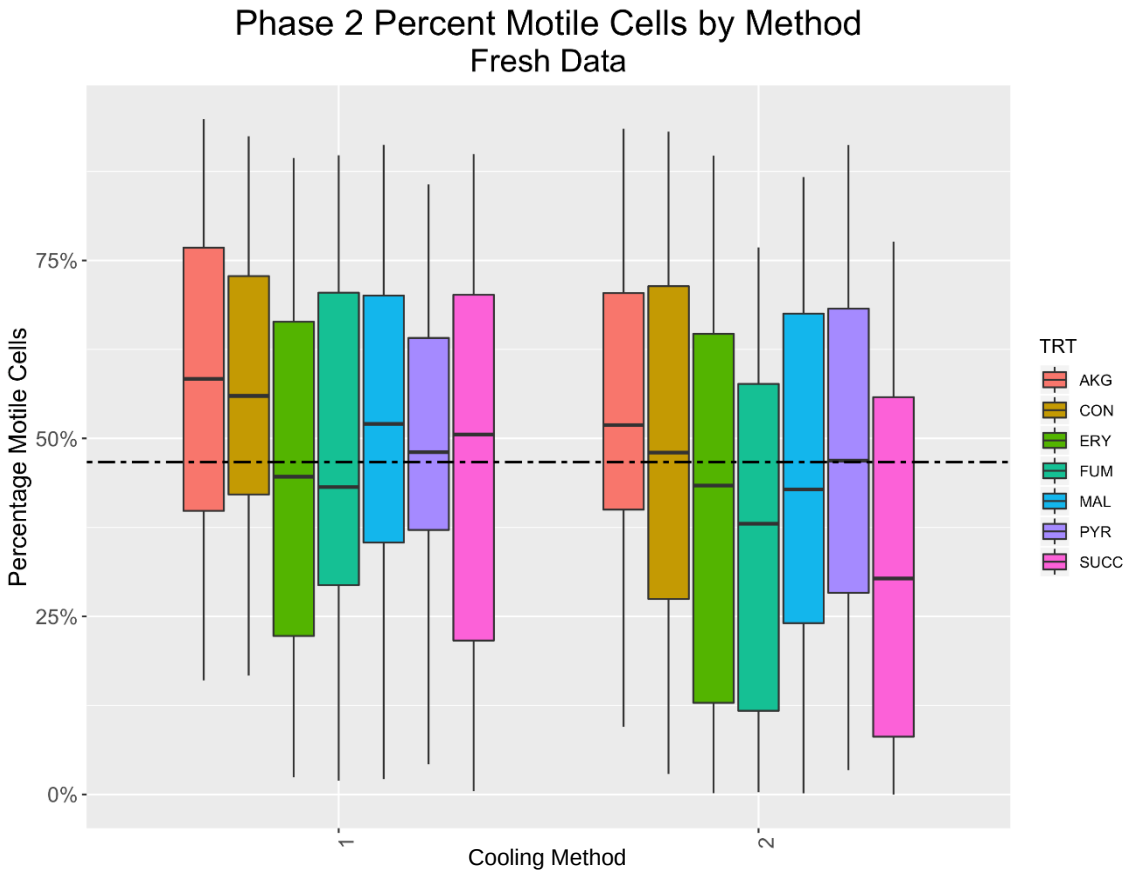

Figure 23 Phase 2 Percent Motile Cells by Method – Fresh Data. The figure depicts Cooling Method as an effect over the fresh data set of Phase 2. It shows that Cooling Method 1 has higher percent motile cells than Cooling Method 2, which was expected from previous research and data analysis in Phase 1. Cooling Method 1 slowed that AKG and CON had the highest percent motile cells, and they had the highest medians above the mean. CAMERY and FUM were the only two TRT with medians below the mean in Cooling Method 1, and SUCC has the largest range of percent motile cells. Cooling Method 2 depicts that AKG still has the highest median above the mean, with CON and PYR being right above or on the mean line. All other TRT medians were below the mean, and all TRT percent ranges increased, except for AKG.

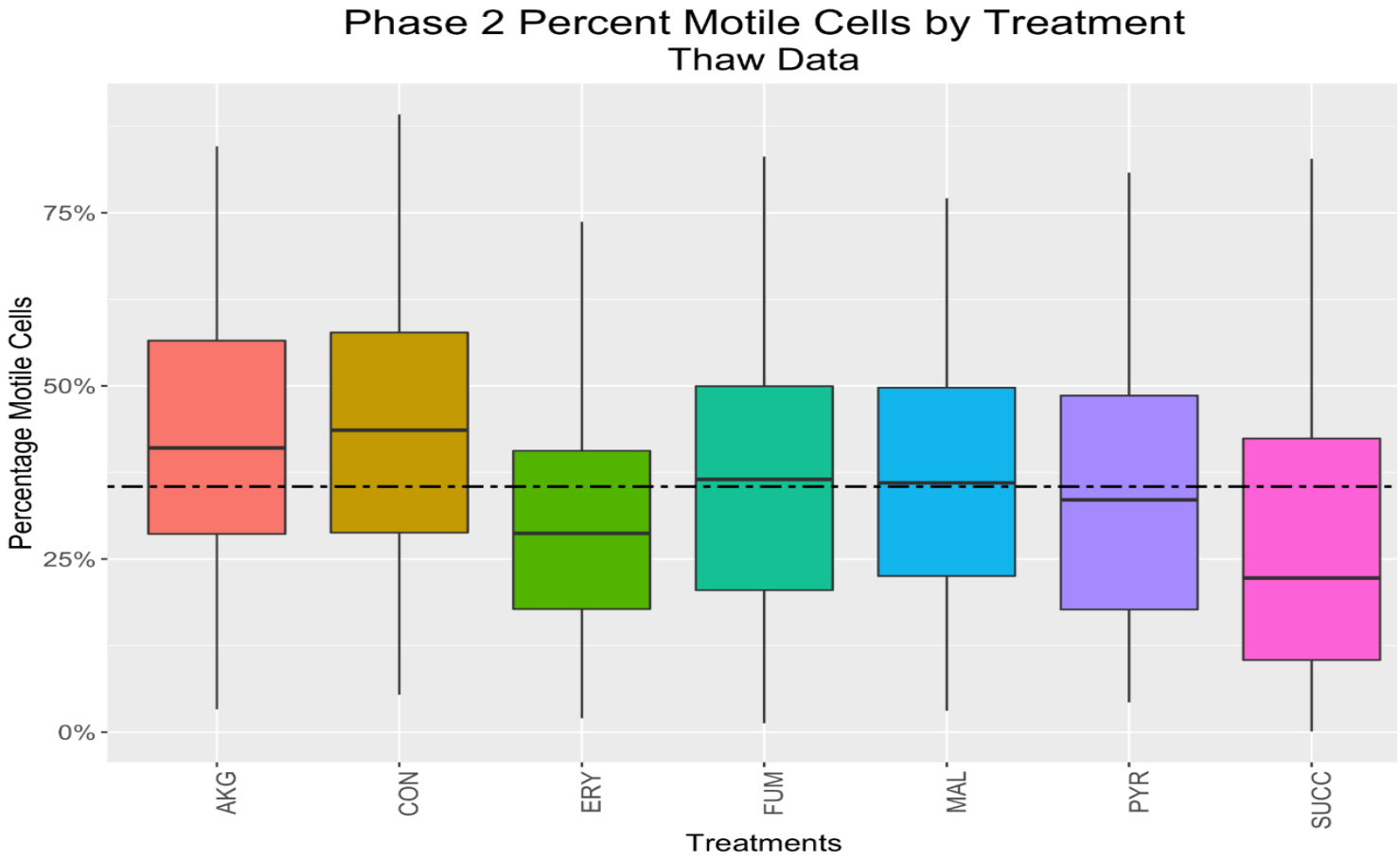

Figure 24 Phase 2 Percent Motile Cells by Treatment – Thaw Data. This figure shows percent motile cells by TRT across both times (Hour 0, and Hour 3) that thaw data was collected. Figure 24 illustrates that CON and AKG had the highest percent of motile cells in thawed media. Both TRT also had the highest medians above the mean. FUM and MAL had medians on the mean. All other TRT medians were below the mean, with SUCC having the lowest.

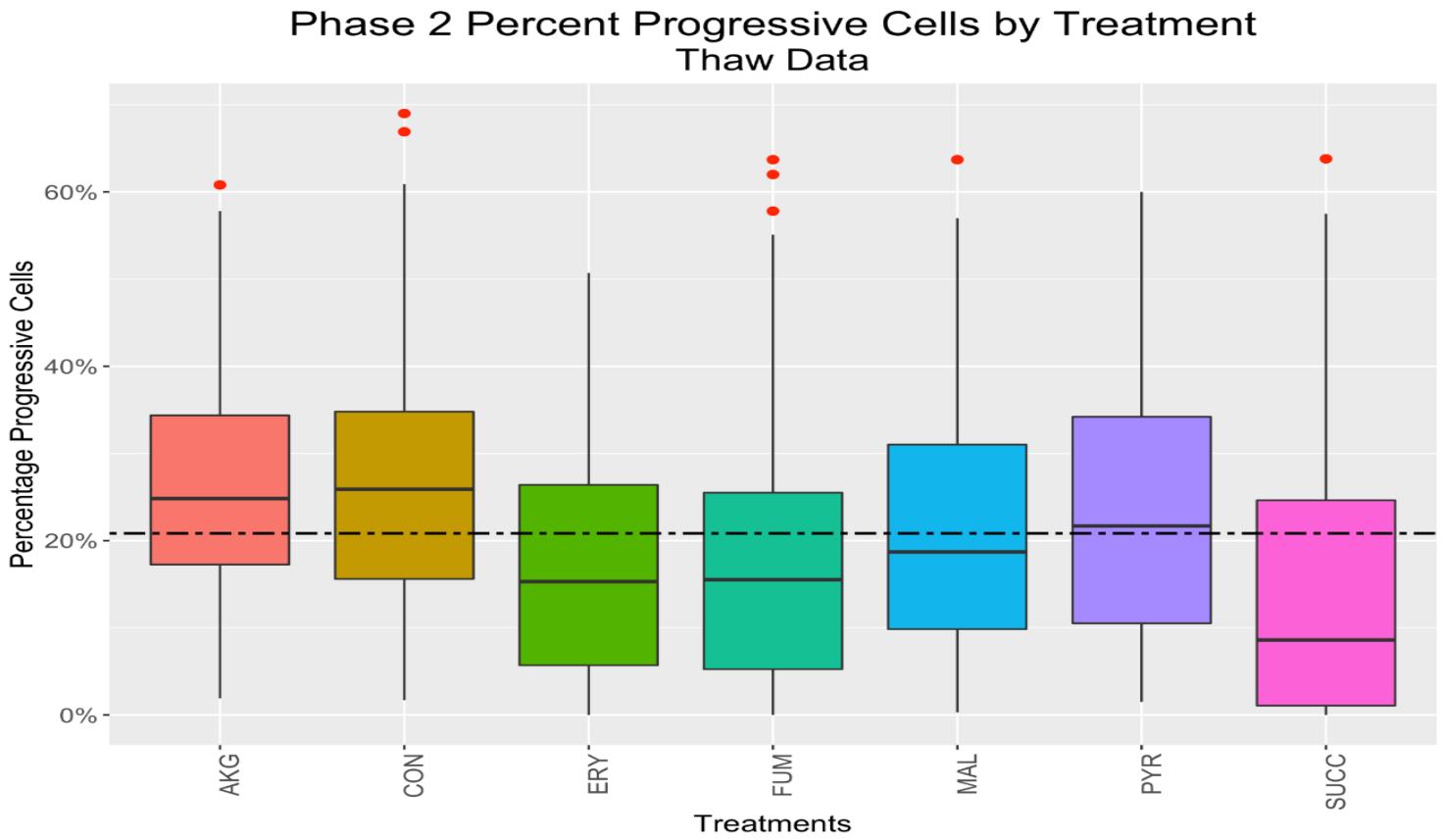

Figure 25 Phase 2 Percent Progressive Cells by Treatment – Thaw Data. This figure shows Percent progressive motile cells by TRT across both times (Hour 0, and Hour 3) that thaw data was collected. Figure 25 displays that CON and AKG had the highest percent of progressive motile cells in thawed media. Both TRT also had the highest medians above the mean, and the PYR median was just above the median. All other TRT had lower progressive motility percentages, and their medians were below the mean. SUCC had the lowest median, and there are a few high percentage outliers for most of the TRTs.

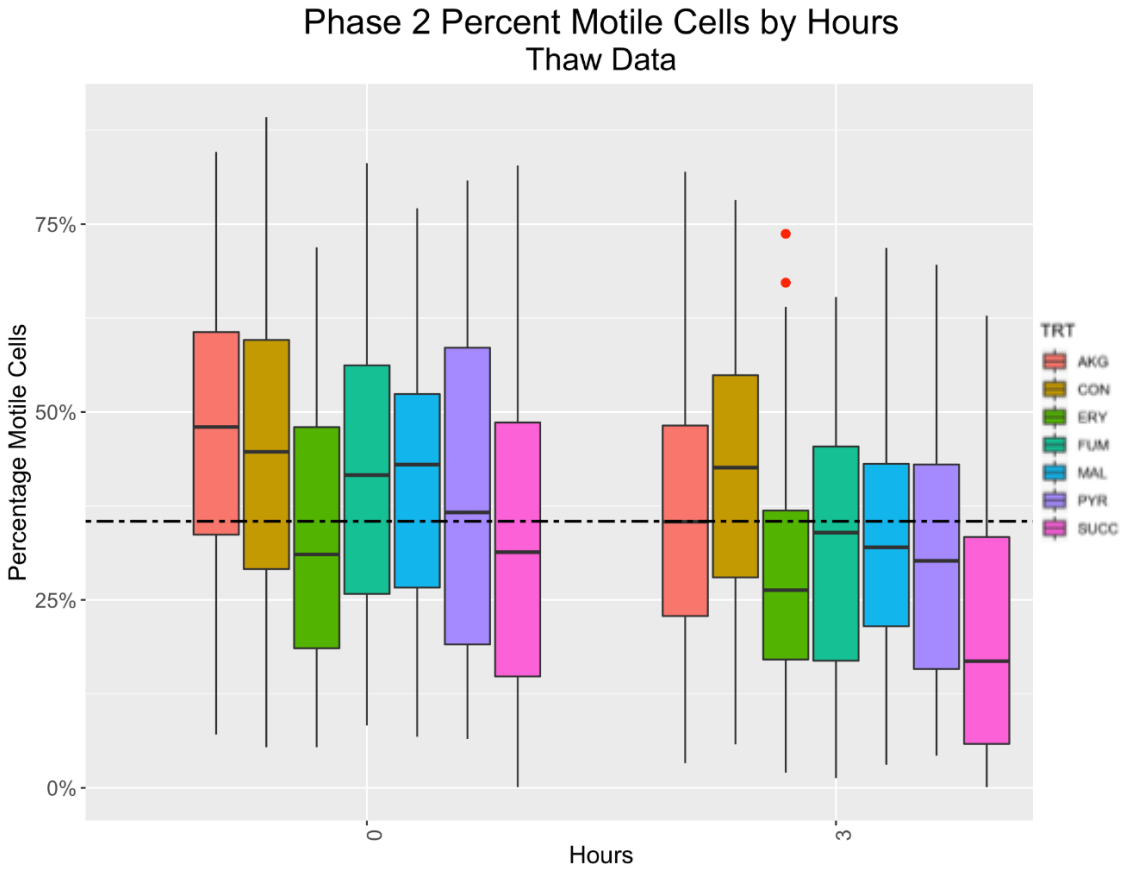

Figure 26 Phase 2 Percent Motile Cells by Hours – Thaw Data. The box plots represent the motile percentage of each treatment over the hours (HR 0 and HR 3) after thawing. It shows an expected trend of motility decreasing as time after thawing increased. This is due to the cells' metabolic rates resuming to normal rates after cryopreservation. The box plots illustrate that at HR 0, for both Cooling Methods, that all TRT medians are above the mean, except CAMERY and SUCC. AKG had the highest median and CON and MAL had the next highest medians. By HR 3, all TRTs were either at or below the mean, except for CON.

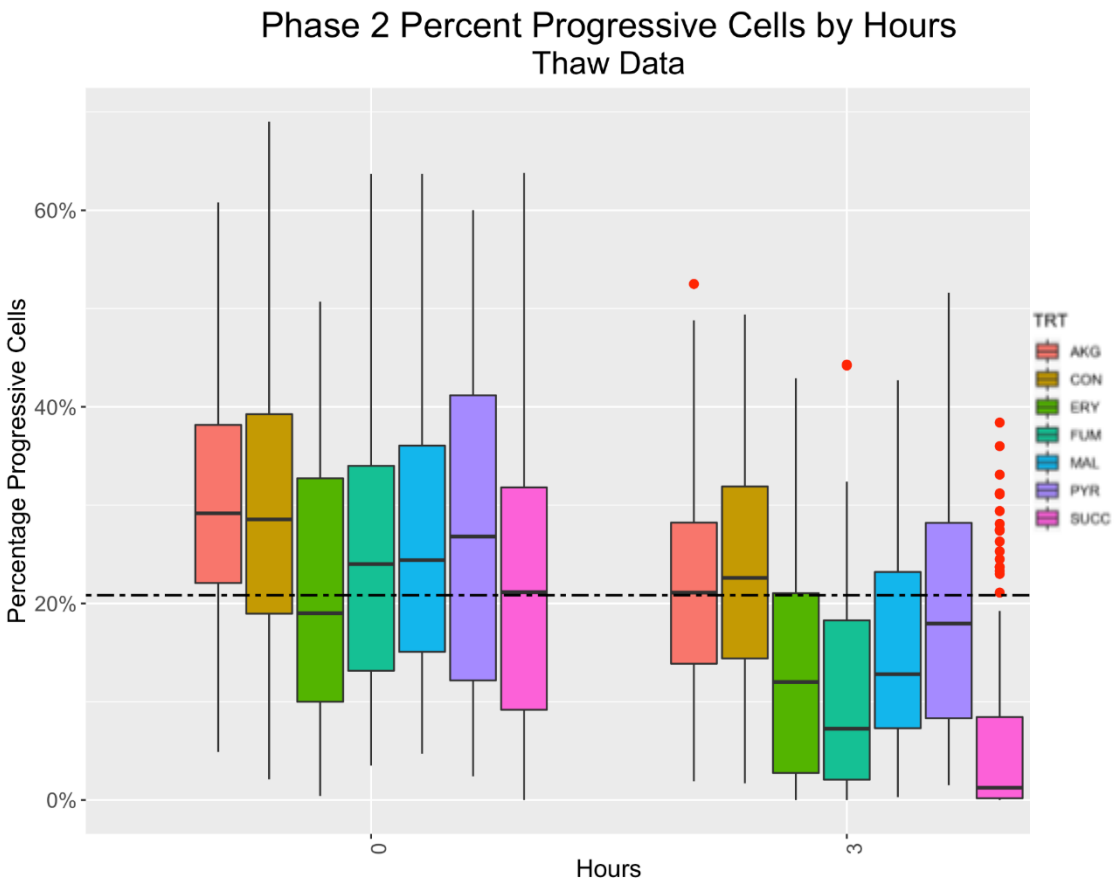

Figure 27 Phase 2 Percent Progressive Cells by Hours – Thaw Data. The box plots show the progressive motile percentage over the two HR that thawed data was collected. It also expresses the trend of progressive motility decreasing as time progresses. The box plots illustrate that at HR 0, for both Cooling Methods, that CON and AKG had the highest progressive motile cells and the greatest medians above the mean. CAMERY and SUCC were the only TRTs that either had a median at the mean or a median below it at HR 0. HR 3 shows that CON was the only TRT with a median above the mean, and AKG had a median at the mean. All of the other TRT medians were below the mean at HR 3, but SUCC had the lowest progressive cells and median when compared to the other TRTs, even though SUCC had many high progressive cell percent outliers.

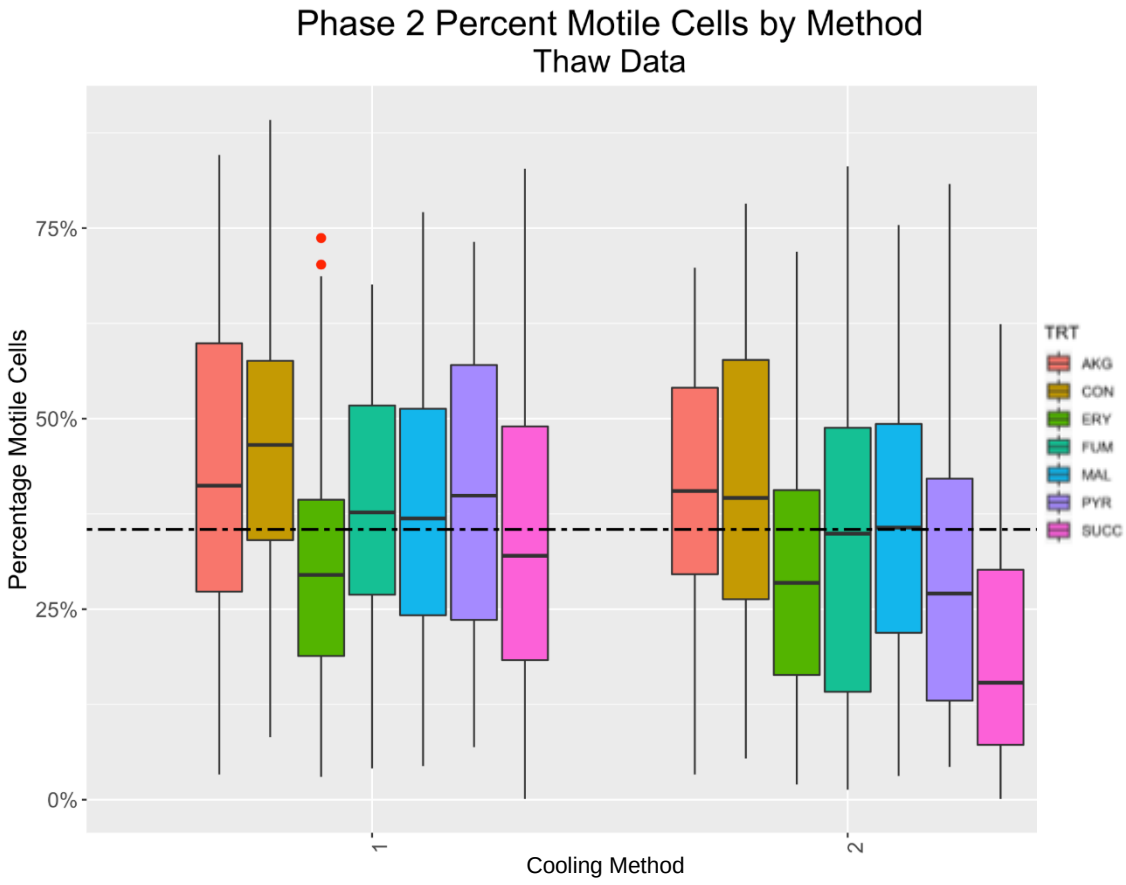

Figure 28 Phase 2 Percent Motile Cells by Method – Thaw Data. The figure depicts Cooling Method as an effect over the thawed data set of Phase 2. It shows that Cooling Method 1 has higher percent motile cells than Cooling Method 2, which was expected from previous research and data analysis in Phase 1. Cooling Method 1 illustrated that CON had the highest percent motile cells, and they had the highest median above the mean. CAMERY and SUCC were the only two TRT with medians below the mean in Cooling Method 1. Cooling Method 2 depicts that AKG and CON were the only two TRTs with medians above the mean. CAMERY, PYR, and SUCC were the TRTs with medians visibly below the mean for percent motile cells.

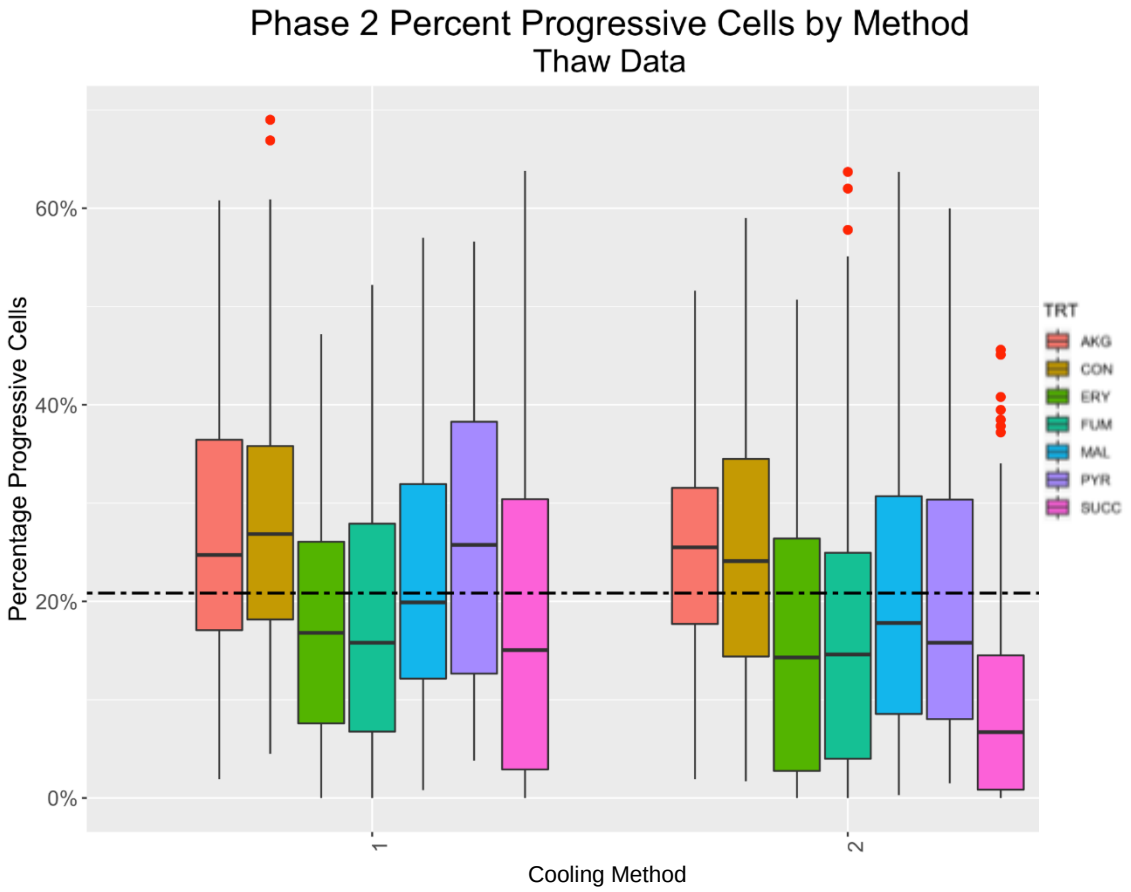

Figure 29 Phase 2 Percent Progressive Cells by Method – Thaw Data. This figure expresses that Cooling Method 1 has higher percent progressive motile cells than Cooling Method 2. This was expected from previous research and data analysis in Phase 1 and Phase 2. Cooling Method 1 showed that CON had the median above the mean for progressive motile cells. PYR and AKG were the only other TRTs with medians above the mean in Cooling Method 1. Cooling Method 2 reveals that AKG and CON were the only two TRTs with medians above the mean, with AKG having a slightly higher median than CON. All other TRT medians were well below the mean, and FUM and SUCC had high percent progressive motile outliers.

APPENDIX B

NO AUP REQUIRED MEMORANDUM

AUP Not Required

DIVISION OF RESEARCH

Offices of Research Compliance and Biosafety

May 9, 2018

MEMORANDUM

TO: Alexis Roach
Graduate Research & Teaching Assistant Department of Animal Science

FROM: Animal Welfare Office
Institutional Animal Care and Use Committee

SUBJECT: Animal Use Protocol not required

Thank you for informing the Institutional Animal Care and Use Committee (IACUC) of your research project which involves obtaining unfertilized chicken eggs from a bakery and utilizing bull sperm (not collected for the purposes of your research). As no manipulation of animals will be performed, nor samples collected for the purposes of your research, no Animal Use Protocol is required.

If the scope of your research changes or additional measures are added which may impact the health, safety, or lifestyle of vertebrate animals, or involve animal handling/manipulation, it is your responsibility to inform the IACUC office before the changes are implemented.

Thank you for keeping the IACUC informed of your research activities.

Best wishes on your research endeavors. 750 Agronomy Road, Suite 2701 1186 TAMU College Station, TX 77843-1186

Tel. 979.458.1467 Fax. 979.862.3176 http://rcb.tamu.edu

APPENDIX C

CONFIDENTIAL AND PROPRIETARY

Non-Disclosure agreement

All information is the property of Inguran LLC, STgenetics® Livestock Reproductive Facility in Navasota, TX. Please see the attached contract to this document for more information.

Mutual Nondisclosure Agreement

This Mutual Nondisclosure Agreement ("Agreement") is made by and between Texas A&M University, on behalf of its Department of Animal Science ("TAMU"), a member of The Texas A&M University System, and Inguran, LLC dba Sexing Technologies and is effective June 1, 2018 (the "Effective Date"). The parties wish to discuss information involving research conducted by Alexis Roach for her Thesis Dissertation (the "Purpose") and anticipate exchanging confidential information in the course of doing so. The parties agree as follows:

1. **Definitions.** For purposes of this Agreement, the following definitions apply:
   (a) "Confidential Information" means nonpublic information, other than Excluded Information, disclosed by one party (the "Discloser") to the other (the "Recipient") during the Disclosure Period under the procedures in Section 3;
   (b) "Disclosure Period" means either 2 years from the Effective Date or until either party gives written notice of termination of the Disclosure Period, whichever occurs first.
   (c) "Excluded Information" means information that:
      (1) Is or becomes publicly known or available other than as a result of a breach of this Agreement by the Recipient;
      (2) Was already in the possession of the Recipient or any of its Representatives as the result of disclosure by an individual or entity that was not then obligated to keep that information confidential;
      (3) The Discloser had disclosed or discloses to an individual or entity without confidentiality restrictions; or
      (4) The Recipient had developed or develops independently before or after the Discloser discloses equivalent information to the Recipient.
   Provided, however, that otherwise Confidential Information will not be deemed Excluded Information merely because a portion of such Confidential Information falls within the public domain or under a category of Excluded Information.
   (d) "Representative" means, as to either party, any of that party's directors, regents, officers, employees, agents, consultants, advisors, or other representatives.

2. **Maintaining Confidentiality.** During the Disclosure Period and for five (5) years thereafter, the Recipient may not:
   (a) Disclose Confidential Information except as permitted under this Agreement; or
   (b) Use Confidential Information except for the Purpose.
   Notwithstanding the foregoing where disclosure would be considered trade secret information (under applicable law), the obligations of this Section 2 will remain for as long as the information is a trade secret, but in no event for a period of less than the five (5) year period specified immediately above.

3. **Disclosure Procedures.**
   (a) If Confidential Information is disclosed in a printed document or otherwise fixed in a tangible medium, the Confidential Information must bear an appropriate and conspicuous marking unless such Confidential Information would reasonably be regarded as confidential by its nature.
   (b) If Confidential Information is disclosed orally, visually, or is not otherwise fixed in a tangible medium, the Discloser shall identify the Confidential Information as being such at the time of disclosure and confirm such in writing to the Recipient within 30 days after disclosure unless such Confidential Information would reasonably be regarded as confidential by its nature.

4. **Permitted Disclosure.** The Recipient may disclose Confidential Information only to the Recipient's Representatives having a need to know the Discloser's Confidential Information to fulfill the Purpose, provided that the Recipient remains responsible for its Representatives' compliance with the Recipient's obligations under this Agreement.

5. **Standard of Care.** The Recipient shall handle Confidential Information with the same care that the Recipient uses to protect its own information of comparable sensitivity, but not less than reasonable care.

6. **Inventions and Publications**. The parties understand and agree nothing in this Agreement, or done pursuant to this Agreement supersedes or replaces any obligations between Alexis Roach and Inguran, LLC, as set forth in set forth in the Employee Non-Disclosure/Assignment of Inventions Agreement. In furtherance of the Purpose, and consistent with said prior agreement, the parties agree that all right, title and interest in and to any intellectual property, including without limitation any inventions, patents, copyrights, trademarks, ideas, creations, conceived or reduced to practice by Alexis Roach in the scope of her employment with Inguran shall vest with Inguran. In furtherance of the Purpose, the parties agree and understand that certain research and research results may be published in peer reviewed journals or in a thesis and may contain Confidential Information provided by Inguran under this Agreement. Inguran shall have the right to review any publications contemplated for the Purpose at least 30 days prior to submission to evaluate whether patent protection is warranted and whether any Confidential Information provided by Inguran should be removed. In the event Inguran elects to pursue patent protection, submission of the publication containing the invention will be delayed 30 days. In the event Confidential Information provided by Inguran is identified in a potential submission, it will be removed at Inguran's request.

7. **Notification of Unauthorized Activities.** The Recipient shall promptly advise the Discloser of any known unauthorized disclosure, misappropriation, or misuse of Confidential Information and shall take prompt and effective steps to prevent a recurrence of such misappropriation or misuse.

8. **End of Disclosure Period.** The Recipient shall, within 15 days of the end of the Disclosure Period or upon request of the Discloser, promptly return to the Discloser or destroy all materials embodying Confidential Information other than materials in electronic backup systems or otherwise not reasonably capable of being readily located and segregated without undue burden or expense. The Recipient may securely retain one copy in its files solely for the purpose of identifying obligations with respect to Confidential Information. The Recipient's obligations under this Agreement survive the end of the Disclosure Period and continue until the end of the period specified in Section 2.

9. **Required Disclosure.** If the Recipient is legally required to disclose Confidential Information, the Recipient shall, to the extent allowed by law, promptly give the Discloser written notice of the requirement so as to provide the Discloser a reasonable opportunity to pursue appropriate process to prevent or limit the disclosure. If the Recipient complies with the terms of this Section 8, disclosure by the Recipient of that portion of the Confidential Information which the Recipient is legally required to disclose will not constitute a breach of this Agreement. The Recipient is not required to pursue any claim, defense, cause of action, or legal process or proceeding on the Discloser's behalf.

10. **Export Controlled Information.** Each party shall comply with U.S. export control regulations. If the Discloser desires to disclose to the Recipient any information, technology or data that is identified on any U.S. export control list, the Discloser shall advise the Recipient at or before the time of intended disclosure and may not provide export-controlled information to the Recipient without the written consent of the Recipient.

11. **No License.** Confidential Information remains the property of the Discloser and no rights or licenses are granted to the Recipient except the limited right to use the Confidential Information as provided above.

12. **Relationship of the Parties.** This Agreement does not obligate either party to enter into any transaction with the other except as specifically provided in this Agreement. This Agreement does not create an agency, partnership, joint venture, or exclusive relationship and each party may pursue other opportunities similar to those contemplated under the Agreement.

13. **Injunction.** In the event of breach or threatened breach or intended breach of this Agreement, each party, in addition to any other rights and remedies available to it, may seek injunctive or equitable relief.

14. **Disclaimer of Warranties.** The Discloser makes no representations or warranties, written or oral, express or implied, as to Confidential Information, including without limitation, any warranty of merchantability or of fitness for a particular purpose.

15. **General Provisions.**
    (a) TAMU is an agency of the State of Texas and nothing in this Agreement waives or relinquishes TAMU's right to claim any exemptions, privileges, and immunities as may be provided by law.
    (b) The substantive laws of the State of Texas (and not its conflicts of law principles) govern all matters arising out of or relating to this Agreement. Venue for any claim arising out of or related to this Agreement must be as provided by Texas law.
    (c) Any notices required or permitted under this Agreement will be deemed given (a) three business days after it is sent by certified or registered mail, return receipt requested, (b) the next business day after it is sent by overnight carrier, (c) on the date sent by facsimile or email transmission with confirmation of transmission and receipt, if sent during the recipient's normal business hours and if not, on the next business day, or (d) on the date of delivery if delivered personally, an in each case, addressed to the intended recipient at the address below or such other address as the intended recipient may specify in writing:

Texas A&M University Division of Research Attn: Travis Young 301 Old Main Drive, Ste. 3104 ILSB College Station, TX 77843-1260 Phone: 979-845-2426 Fax: 979-458-01474 Email: tyoung@tamu.edu	Inguran, LLC Attn: Thomas Gilligan, V.P. Proc. Dev. 22575 State Highway 6 S. Navasota, TX 77868 Phone: 970-310-0750 Email: tgilligan@stgen.com

    (d) Neither party waives a provision of this Agreement by failing to enforce that provision. Each provision of this Agreement is severable. If any provision is rendered invalid or unenforceable by statute or regulations or declared null and void by any court of competent

jurisdiction, the remaining provisions will remain in full force and effect if the essential terms of this Agreement remain valid, legal, and enforceable.

(e) This Agreement is the entire agreement of the parties relating to this subject matter and supersedes all prior and contemporaneous agreements and understandings relating to this subject matter. This Agreement may only be amended or superseded by a written agreement signed by authorized representatives of both parties.

(f) This Agreement may be executed in any number of counterparts, each of which shall be deemed to be an original and all of which together shall be deemed to be one and the same Agreement. An executed copy of this Agreement transmitted by facsimile transmission or e-mail delivery of a ".pdf" or similarly formatted data file shall be treated and accepted, in all manners and respects, as an original, legal and binding document, and the signature of any Party shall be, for these purposes, considered an original signature.

The parties have executed this Agreement on the date(s) written below.

Texas A&M University

By: Travis Young

Name: Travis Young, Ph.D.

Title: Assistant Director

Date: July 11, 2018

Inguran, LLC

By:

Name: Juan Moreno

Title: CEO

Date: 6-8-18

This Agreement has been read and acknowledged by:

Dr. David Forrest Date

Dr. Kathrin A. Dunlap Date

Dr. Jason T. Sawyer Date

Dr. Charles C. Love Date

jurisdiction, the remaining provisions will remain in full force and effect if the essential terms of this Agreement remain valid, legal, and enforceable.

(e) This Agreement is the entire agreement of the parties relating to this subject matter and supersedes all prior and contemporaneous agreements and understandings relating to this subject matter. This Agreement may only be amended or superseded by a written agreement signed by authorized representatives of both parties.

(f) This Agreement may be executed in any number of counterparts, each of which shall be deemed to be an original and all of which together shall be deemed to be one and the same Agreement. An executed copy of this Agreement transmitted by facsimile transmission or e-mail delivery of a ".pdf" or similarly formatted data file shall be treated and accepted, in all manners and respects, as an original, legal and binding document, and the signature of any Party shall be, for these purposes, considered an original signature.

The parties have executed this Agreement on the date(s) written below.

Texas A&M University	**Inguran, LLC**
By:_____	By:_____
Name: Travis Young, Ph.D.	Name: Juan Moreno
Title: Assistant Director	Title: CEO
Date:_____	Date:_____

This Agreement has been read and acknowledged by:

David Forrest	6-13-18
Dr. David Forrest	Date
	6/26/18
Dr. Kathrin A. Dunlap	Date
	6/22/18
Dr. Jason E. Sawyer	Date
	7/3/18
Dr. Charles C. Love	Date