DEVELOPMENT OF AN EXTENDER PROTOCOL TO ENHANCE THE VIABILITY OF FROZEN-THAWED BOVINE SPERMATOZOA

A Thesis

by

ERIN MICHELLE GRIFFIN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2004

Major Subject: Physiology of Reproduction

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Approved as to style and content by:

David Forrest (Chair of Committee)	Paul Harms (Member)
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ABSTRACT

Development of an Extender Protocol to Enhance the Viability of

Frozen-Thawed Spermatozoa.

(December 2004)

Erin Michelle Griffin, B.S., The Ohio State University

Chair of Advisory Committee: Dr. David Forrest

Determination of an extender protocol which will enhance the viability of frozen-thawed bovine spermatozoa will allow producers to obtain higher conception rates due to the increased survival rate of the spermatozoa. Ejaculates of six Brangus bulls (age=18 months) were evaluated for spermatozoal motility, acrosomal integrity, and morphological characteristics (collectively called spermatozoal viability) in two experiments to test our hypotheses that (1) the treatment combination of a 4 hr cooling duration and a 2 hr equilibration with glycerol will result in optimum spermatozoal characteristics after freezing and thawing and (2) rank of three selected extenders relative to their effects on spermatozoal viability after freezing and thawing will be egg yolk-citrate (EC), egg yolk-tris (IMV), and skim milk (milk). In experiment 1, an ejaculate from each bull was partially extended and cooled to 4 ºC for either 2 or 4 hr and then allowed to equilibrate with the glycerolated extender for 2, 4, or 6 hr. Spermatozoal viability was assessed at 0, 3, 6, and 9 hr after thawing. In experiment 1, 4 hr of cooling resulted in a higher percentage of motile spermatozoa than did 2 hr of

cooling. The 2 hr equilibration with glycerol yielded lower percentages of motile spermatozoa, acrosomal integrity, and morphologically normal spermatozoa than 4 and 6 hr equilibration durations with glycerol. In experiment 2, we observed a decrease in spermatozoal viability for all three extenders upon freezing and thawing. Viability of frozen-thawed spermatozoa extended in the milk was reduced for all incubation durations, and the IMV extender had a higher percentage of motile spermatozoa than the EC extender at 6 hr of incubation. A higher percentage of intact acrosomes was observed with the IMV extender; however, the EC extender had a higher percentage of morphologically normal spermatozoa than the IMV extender. Our results indicate that at cooling duration of 4 hr and a 4 hr equilibration with glycerol provide the highest level of spermatozoal viability post-thaw of the treatments evaluated and that the IMV extender enhances the percentage of spermatozoa with an intact acrosome for frozen-thawed spermatozoa over the EC and skim milk extenders.

This work is dedicated to my parents:

Michelle Denise Griffin and James Willard Griffin

You have guided me throughout my life on the wings of love and compassion.

You have given me the strength and courage to become something great.

You have encouraged me to fulfill my dreams and aspirations.

You have loved me for who I am and who I will become.

For this and so much more, I am and will be eternally grateful that I have you as my loving parents and ever-faithful supporters in all that I do.

Thanks, Mom and Dad for being my strength, my world.

Love Always,

Your Daughter,

Erin Michelle

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CHAPTER I

INTRODUCTION

The use of frozen semen for artificial insemination (AI) allows producers to utilize superior genetics, decrease the spread of disease, and obtain higher profits through the attainment of calf crops that are more desirable for market sales and consumer preferences. However, a majority of beef producers continue to use natural service mating programs due, at least in part, to the variability in conception rates to AI. If higher conception rates could be consistently achieved, then producers would be more inclined to incorporate the use of AI into their breeding programs to enhance genetic merit, thus producing a more desirable product.

However, advances in the cryopreservation of bull spermatozoa have not kept pace with the advances that have occurred in other reproductive technologies. Only minimal changes in protocols for processing and freezing bull semen have been implemented in the past 20 years. This limited rate of progress is due to a lack of complete understanding of the factors and interactions that alter spermatozoal viability upon processing, freezing, and thawing [1-3]. Some of these factors include membrane permeability, extender components, cooling duration of the spermatozoa prior to the addition of glycerol, equilibration time with glycerol prior to freezing, freezing process, thaw rate, collection frequency of the ejaculate, and seasonal effects. In general, the viability of the ejaculate determines how sensitive the spermatozoa will be to the freeze-

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This thesis follows the style and format of Theriogenology.

thaw process. The osmotic gradient of a sperm cell is determined by membrane permeability. If the spermatozoal membrane is damaged during the freeze-thaw process, intracellular components may be damaged or destroyed, thus rendering the spermatozoon useless for AI. Cryoprotectants such as glycerol are added to the extended ejaculate to protect the spermatozoa from damage during cryopreservation; however, due to variation among bulls [4,5], no exact concentration of glycerol [6,7] or equilibration time with glycerol [5,8] has been recommended for use by the semen collection facilities for freezing semen. The cooling duration functions to protect the spermatozoa against cold shock [2,9], and the length of cooling duration has an impact on the required length of glycerol equilibration [8]. Thaw rate is dependent upon the concentration of glycerol utilized in the extender, but practical considerations need to be taken into account. Producers will have a more difficult time accurately thawing straws without causing lethal damage for 7.5 sec at a high temperature than for 30 sec at a lower temperature [10]. To further complicate the process, environmental and management factors, such as seasonality, relative temperature, housing, and nutrition, also affect the quality and quantity of spermatozoa collected.

The optimum combination of variables that affect spermatozoal characteristics during the freezing-thawing process has not been clearly defined as illustrated above by the cascade of interdependent factors affecting spermatozoal post-thaw viability. This example is only one of the many possible combinations of variables that affect and protect spermatozoa viability post-thaw. Enhancing our knowledge of these variables is

imperative to increase the conception rates of beef cows and heifers following insemination with frozen-thawed semen.

The overall objective of this project was to identify methods for semen cryopreservation that will maximize post-thaw spermatozoan viability. Determining the optimal extender protocol for enhancing the viability of frozen-thawed spermatozoa will allow producers to more confidently utilize frozen semen for artificial insemination and obtain higher conception rates due to the increased survival rate of the spermatozoa that have been frozen and then thawed for breeding purposes.

1. Statement of Problem

1.1. Experiment 1

Artificial insemination is utilized worldwide by both beef and dairy cattle producers. However, conception rates by AI could be improved if the processing techniques during semen processing and freezing were more accurately defined. The length of time that is allowed for the partially-extended semen to cool to 5 ºC has an impact on the quality of the spermatozoa upon post-thaw examination. This "cooling duration" buffers the sperm cells against cold shock. Cold shock affects spermatozoal morphology, acrosomal integrity, membrane lipid ultrastructure, as well as many metabolic reactions required for cell survival [11]. However, cold shock can be reduced if the spermatozoa are given an adequate amount of time to slowly reach 5 ºC. Limited research has been conducted to determine the optimal cooling duration. Ennen et al. [8] demonstrated that either a 2 hr or 4 hr cooling duration resulted in the optimum

percentage of motile spermatozoa post-thaw, however these results hinged on the length of glycerol equilibration for the sample. For the 2 hr cooling duration, Ennen et al. [8] reported that 4 or 10 hr of glycerol equilibration was optimal for spermatozoal viability. In contrast, the 4 hr cooling duration required either 2 or 4 hr equilibration durations with glycerol for optimal spermatozoal viability post-thaw [8]. This brings to light the importance of glycerol equilibration as well as cooling duration for optimal spermatozoal viability post-thaw. Graham et al. [4] evaluated the effect of glycerol equilibration for 4, 8, and 12 hr at 4 ºC prior to freezing on non-return rates and observed a higher non-return rate for the 12 hr (67.8 %) equilibration over the 4 hr (63.4 %) equilibration, but not over the 8 hr (65.2 %). In contrast, Jondet [5] did not observe any significant differences in spermatozoal survival or non-return rates between 1 min (70 % survival and 80.60 % non-return) and 6 hr (70 % survival and 79.95 % non-return) equilibration durations with glycerol. Extensive research on equilibration with glycerol has continued to have great variability in the duration of glycerol equilibration that is recommended to semen collection facilities.

Further research is needed to obtain the optimum lengths for both cooling duration and equilibration with glycerol. The current study evaluated cooling duration and equilibration with glycerol in an attempt to make a recommendation as to the optimum durations for each by comparing the effects of cooling duration and the length of time for glycerol equilibration on the post-thaw motility, morphology, and acrosomal integrity of bovine spermatozoa.

1.2. Experiment 2

Several seminal extenders are available for use by semen collection facilities [12-14]. However, egg yolk and milk-based extenders have been most commonly utilized by the beef and dairy cattle industries for the past 50 years. While acceptable non-return rates, spermatozoal motility, acrosomal integrity, and morphological characteristics are achieved with egg yolk and milk-based extenders, extensive research has documented that differences exist between the two [15,16]. Schenk et al. [17] compared the effects of egg yolk-citrate, egg yolk-tes-tris, and homogenized milk extenders on spermatozoal motility and non-return rates. It was observed that at 0 hr post-thaw, the egg yolk-citrate extender resulted in a higher percentage of motile sperm than for the egg yolk-tes-tris or milk extenders [17]. Senger et al. [16] noted that the greatest degree of variation in spermatozoal motility was caused by the extender.

As with the cooling duration and equilibration time with glycerol, an optimal extender has yet to be established for commercial use in semen collection facilities. Further research needs to be conducted in order to recommend the optimal extender for semen cryopreservation. The current study evaluated three extenders in an attempt to make a recommendation as to the optimum extender for semen processing by comparing the effects of three extenders (egg yolk-citrate, IMV, and skim milk) on pre- and post-thaw motility, morphology, and acrosomal integrity of bovine spermatozoa.

CHAPTER II

LITERATURE REVIEW

1. Capacitation and the Acrosome Reaction

The fertilization of an ovum by a sperm cell is dependent upon the completion of capacitation and the acrosome reaction. Capacitation is the process whereby a sperm cell undergoes structural changes to the sperm head membranes, has a loss of seminal proteins, and an increased uptake of calcium causing hyperactivity [18]. Capacitation describes the changes that spermatozoa must undergo in the female reproductive tract for completion of the maturation process that allows for fertilization. This process is time dependent and requires approximately 8 hr in the bovine species. Capacitation is thought to be induced, at least in part, by the discharge of reactive oxygen species [19,20]. These reactive oxygen species alter the sulfhydryl groups in the head of spermatozoa, and are important in modulating cellular activities [19,20]. The mechanism whereby reactive oxygen species alter the sperm plasma membranes is unknown; however, alterations in the sulfhydryl groups appear to decrease the fertilizing capability of spermatozoa. The effects of cooling, freezing, and thawing on the sperm plasma membrane are thought to alter the sulfhydryl groups in such a way as to induce premature capacitation and/or hinder fertility by interfering with the fertilizing potential of the sperm cells [20]. The initiation of capacitation due to alterations of the plasma membrane cannot be easily assessed by semen collection facilities to estimate the fertilizing abilities of spermatozoa in a quick, reliable, and inexpensive fashion.

Therefore, visual estimates of fertility, such as spermatozoal motility, morphology, and acrosomal membrane integrity [21] are routinely used in the determination of spermatozoal viability post-thaw.

Saacke [22] demonstrated that a positive correlation exists between the percentage of sperm with an intact acrosomal membrane and the non-return rate in cattle. This positive correlation has given semen collection facilities an alternative means for evaluating spermatozoa viability beyond the evaluation for motility and spermatozoal morphological characteristics. Spermatozoa must maintain an intact acrosome throughout the freeze-thaw process in order to undergo the acrosome reaction in the female tract when breeding a cow or heifer by AI. The acrosome reaction is the final stage of maturation that spermatozoa undergo and is dependent upon the completion of capacitation for its occurrence [23]. The acrosome reaction occurs due to a fusion of the membranes located on the head region of a sperm cell and functions to allow the spermatozoa to penetrate the zona pellucida and results in exposure of the equatorial segment which is involved in sperm binding to the vitelline membrane of the ovum [23]. The fusion of these membranes allows for the release or leakage of acrosomal enzymes that function to open a channel through the zona pellucida, thus aiding in penetration of the egg [18]. An intact acrosome will encapsulate the acrosomal enzymes until the completion of capacitation. Upon contact with the zona pellucida of the ovum, the outer acrosomal membrane and the plasma membrane undergo vesiculation to release the enzymes required for the sperm to penetrate the zona pellucida. Damage to the acrosome prior to release of the acrosomal enzymes may result

in spermatozoa that cannot fertilize an ovum due to premature release of the enzymes or improper membrane fusion.

As with all biological systems, many factors influence slight, but significant changes in cellular competence. Mathevon et al. [24] attributed variability in spermatozoal characteristics to the environment, management, physiological status, and the genetics of the bull. More specifically, acrosomal membrane integrity can be influenced by sudden cooling and heating, changes in osmotic pressure, and or changes in pH [2,25,26]. Spermatozoa that are processed for freezing and AI are constantly being exposed to conditions that would not occur during natural mating, therefore every aspect of semen processing must be scrutinized in order to avoid damaging the spermatozoa beyond fertilizing potential. The delicate balance between increasing post-thaw spermatozoal survival and decreasing post-thaw spermatozoal incompetence is now beginning to be examined through research evaluating the induction of capacitation and the retention of the acrosome.

2. Spermatozoal Membrane Permeability

Successful cryopreservation depends on the survival of several cellular components including, but not limited to, the sperm plasma membrane, acrosome, and nucleus [3]. If the cellular components are damaged or destroyed during the freeze-thaw process, the spermatozoa will be rendered useless for fertilization if used for AI. Cryoprotectants are added to the semen to protect spermatozoa during the freeze-thaw process and are classified as penetrating or non-penetrating agents of the sperm plasma

membrane [1,3]. Penetrating cryoprotectants, such as glycerol, pass through the sperm plasma membrane to act both intracellularly and extracellularly to protect cell structures [3,27]. Non-penetrating cryoprotectants (generally termed the extender), such as egg yolk and milk, cannot pass through the sperm plasma membrane. The non-penetrating agents function to protect the outer lipid bi-layer of the cell against cold shock and aid in the movement of water out of the spermatozoa, resulting in dehydration and shrinkage of the cell [3,27,28]. The mechanisms by which the lipid-based, non-penetrating cryoprotectants protect the sperm plasma membrane are unknown [29]. However, low density lipoproteins (LDL) are thought to be involved by binding loosely to the outer sperm plasma membranes [29-31].

In addition to protecting membrane integrity, the osmotic characteristics of cryoprotectants function to alter the volume of intracellular water in order to decrease the formation of intracellular ice during the freezing process [28,32,33]. Freezing semen is the process whereby intracellular water concentrations of the spermatozoa are decreased due to the increasing amount of solutes outside the cell. The decrease in intracellular water allows the spermatozoon to dehydrate or shrink in response to the loss of water; this in turn prevents spermatozoal injury that would be caused by the formation of intracellular ice [3,34]. The extracellular solutes consist of the egg yolk or milk (lipid) portion of the extender as well as the ice crystals that are forming during the initial stages of the freezing process. As the extracellular water condenses into ice crystals, water diffuses from the spermatozoon to decrease the increasing solute concentration. Glycerol aids in the displacement of the intracellular water by diffusing

into the cell and functions to protect the spermatozoon by lowering the temperature at which water will begin to freeze, thus increasing the extracellular ice formation and diffusion of water out of the cell [1]. The change in osmotic pressure and subsequent loss of water out of the sperm cell that occurs as a result of the addition of seminal extenders and cryoprotectants allows spermatozoa to be successfully frozen, thawed, and used for artificial insemination [27].

As with all biological systems, spermatozoa cannot be altered beyond a specific osmotic tolerance without damaging motility, morphology, or acrosomal integrity. Previous studies have noted that moderate changes in cellular volume are tolerated by spermatozoa, in that motility is not greatly affected upon post-thaw evaluation of cryopreserved semen [28,32,33]. In accordance with previous research, Gilmore et al. [28] reported that bull spermatozoa must be kept within 92 – 103 % of their initial isosmotic volume to avoid reducing the motility percentage below 90 % of its original isosmotic value. A delicate balance between disrupting spermatozoal isotonicity with the addition of cryoprotective agents to preserve spermatozoa cryogenically needs to be studied in further detail to enhance the freeze-thaw process and subsequent motility, morphology, and acrosomal membrane integrity percentages for bovine spermatozoa.

3. Cooling Rate

Cooling rate is the process whereby the semen is slowly cooled from approximately 37 ºC (body temperature) to 5 ºC over several hours to prevent cold shock to the spermatozoa. Cold shock decreases spermatozoal viability by altering sperm cell

membrane integrity, thus affecting motility, morphology, and acrosomal membrane integrity [3,35]. Decreases in spermatozoal motility, morphologically normal spermatozoa, and acrosomal integrity decrease the potential fertility of a bull when breeding females by AI.

Jones and Stewart [25] reported that cooling semen to 5 ºC over 30 min primarily caused swelling of the acrosome, but not acrosomal rupture, and the freezing-thawing process caused damage to the acrosomal membranes as well as damage to the middle piece. Jones and Stewart [25] speculated that the swelling of the acrosome during the cooling process weakens the acrosomal membranes and facilitates increased damage or rupture of the acrosome during the freeze-thaw process. Gilbert and Almquist [36] studied the effects of cooling time from 25 ºC to 5 ºC for 0.5 or 3.5 hr. The authors reported higher percentages of motile spermatozoa after cooling the sample for 3.5 hr than for 0.5 hr (40 and 37 %, respectively) as well as a higher percentage of intact acrosomes (70 and 64 %, respectively). Results obtained from Ennen et al. [8] who studied the effects of cooling spermatozoa from 37 ºC to 5 ºC for 0.5, 2, or 4 hr on post-thaw spermatozoal motility support the observations of Gilbert and Almquist [36]. The authors also observed higher motility percentages for spermatozoa cooled for 2 or 4 hr than for 0.5 hr (17.1, 19.1, and 13.6 %, respectively). McFee and Swanson [9] evaluated the cooling rate of extended semen packaged in 0.5 mL plastic vials cooled directly on wire racks or in a 500 mL beaker of 32 ºC water. McFee and Swanson [9] reported the length of time required for the semen sample to reach 5 ºC for samples cooled on wire racks (< 25 min) and in the beaker (140 min). Their results illustrate the beneficial

effects of slowly cooling samples in some type of insulating water bath or bulk cooling to the desired storage temperature, as a higher percentage of motile sperm were observed following freezing and thawing for samples cooled slowly in the beaker of water than for samples cooled directly on the wire racks. Similarly, Dhami and Sahni [37] noted that a longer duration of cooling (2 vs 1 hr), once the semen reaches 4 ºC, was important for spermatozoal survival after the freezing-thawing process. Thus indicating that the damage caused to spermatozoa during the cooling process may not be solely dependent on the rate of cooling but also on the overall process and the total duration of cooling.

The rate and duration of cooling prior to freezing greatly influences post-thaw spermatozoal characteristics that are required for high non-return rates in females bred by AI. However, due to the variation in processing procedures among semen collection facilities, a recommendation for optimal cooling rates have yet to be determined for the semen collection industry.

4. Equilibration Time with Glycerol

The equilibration time with glycerol is a time-dependent process, where glycerol is slowly added to the partially-extended ejaculate to facilitate the displacement of water out of the sperm cell and the penetration of glycerol into the sperm cell. Commonly, semen collection facilities, allow for a period of glycerol equilibration upon addition of the final glycerolated extender components. Extensive research has been conducted on the optimum duration of equilibration with glycerol prior to freezing [6,38,39], however, a recommendation for the optimal equilibration duration has yet to be established.

Cragle et al. [40] evaluated equilibration durations ranging from 4 to 28 hr and then estimated that maximum post-thaw spermatozoal survival occurred with an equilibration duration of 14.9 hr. Jondet [5] reported that 6 hr of glycerol equilibration resulted in a higher percentage of progressively motile spermatozoa than did 8 min of glycerol equilibration (64 and 59 %, respectively). Jondet [5] also reported that almost equal percentages for progressive motility were observed for 6 hr vs 1 min equilibration with glycerol (70 %). The percentages obtained by Jondet [5] for progressively motile spermatozoa suggest that shorter durations of glycerol equilibration can be utilized to recover viable spermatozoa post-thaw. Graham et al [4] evaluated the effect of 4, 8, or 12 hr equilibration durations with glycerol on the non-return rate. The authors observed a higher non-return rate for cows inseminated with semen that was equilibrated with glycerol for 12 hr (67.8 %) than for the 4 hr (63.4 %), but not the 8 hr (65.2 %) equilibration. Foote and Kaproth [41] evaluated the effects of either a 4 or 18 hr equilibration with glycerol on the percentage of motile spermatozoa and an equilibration with glycerol for 4 or 28 hr on non-return rates. Foote and Kaproth [41] observed higher percentages of motile spermatozoa for 18 hr of equilibration (46 %) than for 4 hr of equilibration (44 %), but no significant difference between 4 or 28 hr of equilibration prior to freezing on the non-return rates. Miller and VanDemark [42] studied the effects of equilibration with glycerol for a duration of 2, 6, or 18 hr. The authors reported that higher spermatozoal survival rates, measured by percentage of motile sperm, were obtained with the 6 hr equilibration duration (48.8 % motile spermatozoa) than for the 2 or 8 hr durations (47.4 and 46.3 % motile spermatozoa, respectively).

As with all other aspects of semen processing, the duration for the equilibration with glycerol is dependent upon the semen collection facility, however, glycerol equilibration commonly ranges between 3 and 18 hr [43,44]. Further research is needed in order to determine the optimal duration for equilibration with glycerol to attain maximal spermatozoal motility post-thaw while maintaining processing protocols that fit within the production protocol of semen collection facilities.

5. Extender Components for Cryopreservation

Semen extenders increase the number of females that can be bred to a particular ejaculate and allow semen to be frozen, stored, shipped, and then thawed for artificial insemination. The accessibility of frozen semen to beef producers enables them to obtain superior genetics from multiple sires at a fraction of the cost of purchasing a bull for use in the cow herd. In addition to the greater access to superior genetics, producers can reduce disease in the cow herd, due to the antibiotic component of the extender and the rigorous health testing that most bulls must go through in order for their semen to be collected and processed for freezing. Freezing semen cannot successfully occur without the addition of components to the ejaculate to protect the spermatozoa against cold shock, intracellular ice formation, the depletion of energy reserves, autotoxication, and microorganisms during the freeze-thaw process.

5.1. Lipids and lipoproteins

A source of lipid (generally egg yolk) or liproprotein (generally milk) or a combination of the two is required to protect the spermatozoa from cold shock [1,3,11].

Cold shock is the process whereby the lipid bi-layer of the spermatozoa is altered, due to rapid changes in temperature [1]. This rapid change in temperature can be due to many factors during semen processing. Cold shock occurs most frequently when undiluted semen at 30 ºC or above is (1) added to a pre-cooled (5 ºC) diluent, (2) allowed to come into contact with pre-cooled glassware or instruments, and (3) when transferring a small volume of semen in a holding container to a pre-cooled water jacket to cool the semen to 5 ºC [11]. This is supported based on research conducted by Foote and Bratton [45] who demonstrated the importance of extending semen prior to cooling and slowly cooling the sample to minimize cold shock and to increase the non-return rates of cows bred by AI with cooled spermatozoa. The results of the authors reported that for 3 hr of storage at 5 ºC after cooling, the pre-extended semen had higher motility percentages than semen that had been extended after cooling to 5 ºC (63 and 48 %, respectively). Egg yolk and milk have been proven to enhance spermatozoa viability, specifically motility, upon the freeze-thaw process by protecting spermatozoal membranes through the provision of lipids and lipoproteins, respectively [46-48]. Hammerstedt et al. [1] and Watson [11] speculated that the lipid and lipoprotein fractions of the egg yolk and milk component of the extenders function to alter membrane lipid fluidity in such a way as to protect the membranes from damage upon cooling and re-warming. Hammerstedt et al. [1] suggested that even though the sperm plasma membranes are altered upon the cooling, freezing, and thawing processes, the lipid and lipoprotein properties of egg yolk and milk will allow the sperm plasma membrane to be reconstituted to its original state if given adequate time to adjust to the changes during processing and freezing. Pace and

Graham [30], reported that freezing and thawing bovine semen in an egg yolk-based extender without glycerol yielded higher percentages for spermatozoal motility as compared to extenders composed of glycerol alone. Furthermore, use of an egg yolk-based extender in combination with glycerol resulted in higher percentages of spermatozoal motility than for extenders with the use of either egg yolk or glycerol alone (40, 24, and 3 %, respectively). Their results suggest that both the egg yolk and the glycerol fractions of the extender are involved in protecting spermatozoa during cryopreservation and inclusion of both fractions is imperative for obtaining adequate spermatozoal motility post-thaw.

5.2. Glycerol

Since the discovery of glycerol as a cryoprotective agent for bovine spermatozoa [49], extensive research has been conducted to determine the optimal percentage (by volume) of glycerol to add to the extended semen in order to achieve maximum spermatozoal motility, acrosomal membrane integrity, and morphologically normal spermatozoa post-thaw [12,38,49]. Cragle et al. [40] compared three levels of glycerol in an egg yolk-citrate based extender. The authors determined the optimal value for glycerol to be 7.6 % by determining the maximum percentage of motile sperm and solving for the optimum level of glycerol corresponding to maximum motility. Saacke et al. [50] also evaluated the concentration of glycerol required to obtain maximum viability of spermatozoa post-thaw. Saacke et al. [50] reported the optimum concentration of glycerol for maximum spermatozoal survival to be 8.5 % when a thaw rate of 65 ºC for 7.5 sec was utilized. The authors noted that for practical purposes,

thawing frozen semen for 7.5 sec would be difficult for producers and AI technicians to achieve, therefore, they recommended utilizing a lower glycerol concentration, such as 7 % to allow for a thawing duration longer than 7.5 sec. Due to the constraint of thaw duration, many semen collection facilities, utilize a final glycerol concentration between 7 and 7.5 % by volume with a thaw rate of 35 ºC for 30 sec to achieve acceptable percentages for motility, acrosomal membrane integrity, and morphologically normal spermatozoa post-thaw [44,51].

5.3. Simple sugars

Energy obtained from simple sugars is required for cellular activity in all cell types of the body. For spermatozoa, energy is primarily required for motility and then cell maintenance [43]. Therefore, a source of energy must be abundant and easily accessible for spermatozoa that have been collected and extended for AI in order for them to successfully navigate the female reproductive tract and have an ample source of energy remaining to fertilize the ovum. In a study conducted by Blackshaw [38], the addition of arabinose to an extended glycerolated semen sample increased the revival of frozen-thawed spermatozoa. While the mechanism of action that simple sugars had on enhancing the viability of spermatozoa frozen and thawed in glycerol was unknown at that time, it has since been demonstrated that the simple sugars provide additional energy sources to guard against the energy-depleting process of glycolysis [44]. Glycolysis is a biological reaction that reduces glucose and other simple sugars to acidic toxic products such as lactic acid [44]. Without a way to provide additional sugars for energy and a method to prevent cellular death from autotoxication, spermatozoa will not

be able to survive the freeze-thaw process or harbor adequate energy reserves to fertilize the ovum.

5.4. pH

Maintaining a near neutral pH (6.5-6.9) of the spermatozoa and the seminal extender is imperative for protecting the spermatozoa against autotoxication caused by glycolysis [43,44]. Cragle et al. [40] studied the effects of various levels of sodium citrate on the viability of frozen-thawed spermatozoa. The authors estimated the level of sodium citrate required for the attainment of optimal motility in an egg yolk-based extender to be 2.9 %. Their findings more accurately defined the optimum percentage of sodium citrate (by volume) required for enhancing spermatozoal motility than the percentages utilized by previous researchers, as this level of sodium citrate is most commonly utilized by semen collection and processing facilities today. However, the optimum level of pH will vary with the extender components utilized; therefore, pH levels should be evaluated and modified as necessary with every change in extender component [44].

5.5. Antibiotics

Controlling microbial growth is an important step in preventing the spread of reproductive diseases through the use of semen and for enhancing reproductive efficiency of the cow herd. Extender components, such as egg yolk and milk, enhance microbial growth by providing the microorganisms with a favorable environment for their proliferation [43]. The addition of antibiotics to the neat semen as well as the extender provides semen collection facilities with an effective means at controlling

microbiological growth [44,51]. A multitude of antibiotics exist to control pathogenic microorganisms, however, the antibiotic regimen utilized by Certified Semen Services (CSS) participants is the most widely adopted protocol today. CSS requires the addition of gentamicin sulfate, tylosin, and linco-spectin to the neat semen and extender in order to provide effective microbiological control of Mycoplasmas, Ureaplasmas, Haemophilus somnus, and Campylobacter fetus subsp. venerealis [51]. Regardless of the antibiotics chosen for semen processing, preventing the spread of microbial reproductive diseases is necessary for promoting herd health.

As with many of the components of semen processing, individual semen collection facilities utilize their own combination of ingredients to create their extenders. However, as a general rule of thumb, extenders utilized for cryopreservation should contain the following components: (1) egg yolk or milk, (2) glycerol at a final concentration of approximately 7 %, (3) simple sugars, (4) sodium citrate dihydrate or tris (hydroxymethyl) amminomethane, and (5) antibiotics [3,43,44]. The efficacy of each extender must be evaluated to determine its effectiveness in enhancing post-thaw spermatozoal viability. Without determining the efficacy of each newly developed extender, semen collection facilities and producers alike may be reducing the potential for spermatozoal fertilization.

6. Freezing Process

Semen samples that have been extended, cooled, and packaged into straws can be frozen by various methods such as nitrogen vapor and mechanical freezing. Mechanical

freezers can be used to produce a consistent freeze by evenly distributing liquid nitrogen vapor throughout the freezing chamber at programmable rates [27,44]. However, nitrogen vapor is commonly utilized for freezing semen as it is a simple yet inexpensive technique to employ. Forgason et al. [52] and Roussel et al. [53] demonstrated that semen frozen in liquid nitrogen vapor produced spermatozoa with adequate post-thaw motility percentages (65.7 and 43.8 %, respectively) to impregnate a cow or heifer by AI. The rate of freezing varies among samples, however, as long as the extended semen within the straws achieves a temperature of at least –80 ºC in the liquid nitrogen vapor prior to plunging them into liquid nitrogen, spermatozoal viability remains adequate for fertilization post-thaw [54].

The facilities and equipment that are available for freezing determine the technique that will be utilized by each bull stud facility as well as govern any alterations to the freezing technique that are necessary in order to ensure an accurate match between the freezing process and the extender components. Currently, with the nitrogen vapor technique, semen-filled straws are held approximately 3 cm above liquid nitrogen in the vapor from 7 to 10 min to cool the sample to –100 ºC, then the straws are plunged into liquid nitrogen for storage at –196 ºC [44].

7. Thaw Rate

Thaw rate is the process of thawing straws of semen at a specific temperature (thaw temperature) for a specific amount of time (thaw duration). Extensive research has been conducted on thaw temperature and thaw duration to determine the thaw rate

that produces the highest percentage of viable spermatozoa post-thaw [7,10,55]. In a study conducted by Robbins et al. [56], the effects of thaw temperature and duration on frozen spermatozoa at 5 ºC for 4 min, 20 ºC for 1 min, 35 ºC for 30 sec, 75 ºC for 6 sec, and 75 ºC for 12 sec were evaluated. The authors observed higher percentages for motility and for acrosomal membrane retention with a thaw temperature of 35 ºC and a thaw duration of 30 sec than for any other treatment combination tested. Similar findings were reported by Pace et al. [55], who evaluated the effects of motility and acrosomal membrane integrity of semen frozen and thawed in iced water (1-3 ºC) for a minimum of 60 sec, ambient (5-20 ºC) for a minimum of 60 sec, or between 35-37 ºC for a minimum of 30 sec. The authors observed higher percentages for motility and acrosomal membrane integrity with samples thawed between 35-37 ºC for 30 sec than all other treatment groups.

Robbins et al. [7] demonstrated a positive correlation between the concentration of glycerol in the extender and the thaw rate and temperature required for maximum percentages of intact acrosomes and of motile spermatozoa upon post-thaw. Their results help to explain the optimal thaw temperature and duration (between 35-37 ºC and 30-60 sec, respectively) obtained from Pace et al. [55] and Robbins et al. [56]. Robbins et al. [7] evaluated the effects of thaw temperature and rate on frozen spermatozoa at 5 ºC for 2 min, 20 ºC for 1 min, 35 ºC for 30 sec, 50 ºC for 15 sec, and 65 ºC for 7.5 sec for final glycerol concentrations of 1, 4, 7, 10, and 13 %. Maximum percentages for motility and intact acrosomal membrane were extrapolated from the data and final glycerol concentration and thaw rate were calculated to be 8.5 % and 65 º C for 7.5 sec,

respectively. In general, the authors observed an inverse relationship between the final glycerol concentration and the thaw rate. As the glycerol concentration increased, the thaw temperature needed to increase, and the thaw duration needed to decrease in order to attain the highest percentage of post-thaw motility and acrosomal membrane integrity.

While increasing spermatozoal viability upon post-thaw is desirable, decreasing the thaw time is challenging for producers to achieve with the level of accuracy required to recover live motile spermatozoa thawed at high temperatures [10]. Therefore, semen is commonly thawed at a temperature between 33 ºC and 37 ºC with a thaw duration of 30 to 40 seconds [44]. Since thaw rate is dependent upon the equilibration time with glycerol, extender components (including final glycerol percent), and freezing technique utilized by individual semen collection facilities, it remains an unmarked piece of the freeze-thaw puzzle, fitting into any number of spaces to produce an overall picture that is not quite complete.

8. Collection Frequency

Obtaining the maximum number of progressively motile spermatozoa for the use of artificial insemination is the primary goal of any AI organization. Therefore, selecting a collection frequency that maximizes the output of normal progressively motile spermatozoa from a bull is imperative to the success of each individual semen collection facility. Almquist [57] demonstrated that continuous high frequency ejaculations, (collections were obtained six times per week), resulted in a 3.3 fold increase in motile sperm collected per week when compared with ejaculates collected

once per week (30.8 and 11.0 X 10^9, respectively). Lorton et al., [58] observed no significant differences in the quantity and quality of spermatozoa when three ejaculates were collected twice-weekly versus the collection of two ejaculates three days per week (33.9 X $10^9$and 33.2 X 10^9, respectively). Furthermore, the total number of spermatozoa collected two to three times per week was similar to that of the bulls collected six times per week in the study performed by Almquist [57]. This suggests that the same number of total spermatozoa can be obtained when collections are performed 2-3 times per week vs. 6 times per week. Decreasing the collection frequency has the potential to increase the cryopreservation of spermatozoa from multiple sires, in that a higher number of bulls could be collected per week at facilities utilizing a rotational collection schedule and that collect 4-5 times per week. Studies conducted by Everett et al., [59], Everett and Bean [60], and Mathevon et al., [24] has proven that shorter periods between collection times decreased the volume, concentration, and total number of sperm per ejaculate, and suggested several days of sexual rest between collections. By combining these results, researchers and AI organizations are provided with insight to the frequency at which bulls can be collected to maximize the production of quality spermatozoa within the time constraints that plague semen collection facilities.

In general, placing the bulls on a collection schedule of two to three times per week will allow these facilities to obtain the maximum number of progressively motile spermatozoa per ejaculate, while providing sexual rest between collections to increase the volume, concentration, and total number of spermatozoa per ejaculate in a timely fashion for production purposes. Varying processing procedures and company time

constraints will demand that semen collection facilities continue to utilize a basic collection regimen that has been altered to fit their specific needs in order to maximize individual facility production potential.

CHAPTER III

EFFECTS OF COOLING DURATION AND EQUILIBRATION WITH GLYCEROL ON PERCENTAGE OF SPERMATOZOAL MOTILITY, ACROSOMAL INTEGRITY, AND MORPHOLOGICAL CHARACTERISTICS

1. Introduction

Cooling duration and equilibration with glycerol have an impact on post-thaw spermatozoal viability as measured by the percentage of spermatozoal motility, acrosomal integrity, and morphological characteristics [25,39]. Research demonstrates that the viability of spermatozoal characteristics is dependent upon the length of time allotted for the partially-extended sample to cool from 37 ºC to 5 ºC as well as the length of time that the fully-extended sample is equilibrated with glycerol [40,61]. Determining the optimal lengths of time for cooling duration and equilibration with glycerol will allow semen collection facilities to produce a greater volume of straws that yield higher percentages of post-thaw spermatozoan viability, which in turn will increase the non-return rate of females bred by AI.

Numerous studies have evaluated the effects of the cooling duration and equilibration with glycerol on the non-return rates as a means of economic assessment for the value of frozen thawed spermatozoa. Jondet [5] conducted two studies to compare the effect of glycerol equilibration lengths (8 min vs 6 hr and 1 min vs 6 hr) on the non-return rate of females bred with frozen-thawed spermatozoa. The author

reported non-return rates of 58.70 vs 60.06 % and 80.60 vs 79.95 % for equilibration durations of 8 min vs 6 hr and for 1 min vs 6 hr, respectively. Pinpointing and eliminating, or at least decreasing, the factors that adversely affect post-thaw spermatozoal viability, and ultimately the non-return rate, will enable semen collection facilities to process and freeze semen that will enhance the ability of the producers to attain higher non-return rates with the use of frozen-thawed semen.

This experiment was designed to evaluate the effects of cooling duration, equilibration with glycerol, and inherent interactions between cooling duration and equilibration with glycerol in order to provide a recommendation to the semen collection industry as to the optimum combination of the two.

1.1. Hypothesis

The treatment combination of a 4 hr cooling duration and a 2 hr equilibration time with glycerol will result in the optimal viability of spermatozoal characteristics after freezing and thawing.

1.2. Objective

1. Compare the effects of cooling duration and the glycerol equilibration time on the post-thaw motility, morphology, and acrosomal integrity of bovine spermatozoa, and
2. Determine the effectiveness of each combination of cooling duration and equilibration with glycerol in order to make a recommendation to semen collection facilities as to which cooling duration and equilibration with

glycerol combination will provide the highest percentage of viable spermatozoa post-thaw.

2. Materials and Methods

A 2 x 3 factorial arrangement was designed to determine which of two cooling durations and three glycerol equilibration times was most effective in preserving bovine spermatozoa upon freezing and thawing. The treatment groups were as follows: (1) 2 hr cooling duration and 2 hr equilibration with glycerol, (2) 2 hr cooling duration and 4 hr equilibration with glycerol, (3) 2 hr cooling duration and 6 hr equilibration with glycerol, (4) 4 hr cooling duration and 2 hr equilibration with glycerol, (5) 4 hr cooling duration and 4 hr equilibration with glycerol, and (6) 4 hr cooling duration and 6 hr equilibration with glycerol. Each ejaculate was used in every treatment combination. The average monthly temperatures for March, April, and May, 2003 were 16, 21, and 26 ºC, respectively. The average percentages for relative humidity for March, April, and May, 2003 were 73, 68, and 70 %, respectively.

2.1. Extender preparation

2.1.1. IMV International CSS two step extender

The IMV International CSS two step extender (IMV International MN, USA) is a commercially available tris-based extender that is packaged as a complete kit including the CSS concentrate (containing the tris buffer), glycerol, and CSS antibiotics (Tylosin; 100 mg/mL Gentamyacin; 500 mg/mL Linco-Spectin; 300/600 mg/mL). The IMV extender was prepared as instructed by the manufacturer.

Eggs used to provide the yolk portion of the extender were purchased fresh on a weekly basis from Feather Crest Farms (Kurten, TX, USA) and stored at 4 ºC until use. Eggs were broken midway and the yolk was held in one half of the shell to allow most of the egg white to separate from the yolk and be discarded. The remaining egg white and yolk were then transferred to filter paper (Ahlstrom Corporation, Mt. Holly Springs, PA, USA) and gently rolled to further separate the egg white from the yolk. The yolk was rolled to a clean spot on the filter paper and slight pressure was applied to the yolk by folding the paper around the yolk, causing the yolk to rupture. The yolk was then collected in a graduated cylinder. Yolk membranes, discolored yolks, or yolks containing blood spots were not used for the extender.

The extender was prepared by making two fractions, A, the non-glycerol portion containing egg yolk and CSS bovine buffer concentrate and fraction B, the glycerol portion containing egg yolk, CSS bovine buffer concentrate, and glycerol. The IMV International CSS two step extender was prepared the night before collection and stored at 4 ºC until use. Table 1 provides a description of the volumes utilized to prepare the IMVextender.

Table 1
Experiment 1: Preparation of the IMV International CSS two step extender.

Component	**Fraction A (nonglycerol)**	**Fraction B (glycerol)**	**Final % of 1:1 Fractions**
Distilled Water	350 mL	350 mL	64.1
CSS Antibiotics	10 mL	--------	0.9
CSS Bovine Buffer Concentrate	50 mL	50 mL	9.2
Egg Yolk	100 mL	100 mL	18.3
Glycerol	--------	82 mL	7.5
Total Volume for Fractions A and B	510 mL	582 mL	--------

2.2. Semen collection

Single ejaculates from six 18-month-old Brangus bulls were collected two times per week until a total of six ejaculates had been collected from each bull. Bulls were loaded into the holding stalls approximately 15 min prior to the start of collections. An experienced handler led a haltered steer around the collection arena, stopping abruptly to mimic the behavior of a cow in estrus, that will stand to be mated by the bull, to increase sexual stimulation of the bulls. The collector released one bull at a time into the arena to be collected in a free-style fashion via an artificial vagina, prepared as previously described [44]. Artificial vaginae were prepared the night prior to collection and stored in an incubator overnight at 38 ºC until use. An insulated jacket was used to protect semen in the collection tube against temperature shock upon collection. The bull was allowed to false mount the steer up to three times prior to collection to enhance the concentration of the ejaculate via increasing the sexual stimulation of the bull. During the false mounting procedure, contact of the penis of the bull with the teaser animal was prevented by gently diverting the bull's sheath toward the collector. When the collector

determined that the bull was ready for collection, the bull was allowed to mount the steer again, however, this time the collector diverted the bull's penis into the AV for the collection of the ejaculate. Once the bull had successfully ejaculated into the AV, the ejaculate was taken into the lab for semen processing.

2.3. Semen processing

The neat semen volume was determined by use of a 15 mL graduated plastic vial and CSS antibiotics (Tylosin; 100 mg/mL Gentamyacin; 500 mg/mL Linco-Spectin; 300/600 mg/mL) were added to the neat semen based upon total volume (0.02 mL antibiotics to 1 mL neat semen). Spermatozoal concentration was determined by spectrophotometric assay. Total extender volume divided into two fractions, A and B, was based upon spermatozoal concentration and volume. Fraction A was allowed to equilibrate to 37 ºC before collections began, to prevent the spermatozoa from undergoing cold shock upon the combination of fraction A to the neat semen in a polystyrene tube within 5 min of collection. Prior to cooling, the partially-extended spermatozoa (neat semen plus fraction A) was equally divided into two polystyrene conical tubes to facilitate handling of the samples. Tubes were identified with bull name, time entered into cold room, duration of cooling (i.e., 2 or 4 hr cooling period prior to addition of fraction B), and volume of fraction A. Samples were cooled to and maintained at 4 ºC for either 2 or 4 hr. The semen was fully extended with the addition of the fraction B component of the extender by slowly dripping it into the samples (average time of 2 minutes) via a plastic funnel cup with a hole punctured in the bottom of the funnel cup by a 21 ga needle. Fully extended samples were equally divided into

one of six polystyrene conical tubes with lids, denoting the treatment group, bull name, and time entered into the cold room, then allowed to equilibrate with the fraction B portion of the extender for 2, 4, or 6 hr prior to being loaded into straws. The addition of fraction A and B to the raw sample extended the neat semen sample to a final concentration of at least 45 x 10^6 spermatozoa/mL (final volume includes permeable and non-permeable extender components and the spermatozoa).

Straws were labeled with the Minitub straw printer (Verona, WI, USA), identifying the stud facility, breed, individual bull identification code for the stud facility, bull name, private herd number, registration number, and collection date. Straws were cooled to 4 ºC to prevent the spermatozoa from warming above the cold room temperature. The fully extended semen was loaded into 0.5 mL French straws and heat sealed via the IMV MRSI straw filler (Maple Grove, MN, USA) with treatment and bull-specific metal needles and disposable tubing, eliminating the possibility of cross-contamination between treatment groups and/or bulls. The semen had a 30 min equilibration time in the straws on horizontal freezing racks prior to freezing. The extended semen was frozen by suspending the straws approximately 3 cm above the liquid nitrogen in liquid nitrogen vapor for 7 min before plunging them into the liquid nitrogen. Samples were then stored in a liquid nitrogen refrigerator (-196 ºC until the post-thaw evaluation was conducted.

2.4. Post-thaw evaluations

The frozen-thawed semen was evaluated for progressive motility, percentage of intact acrosomes, and any morphological abnormalities using a Nikon Eclipse E600

phase-contrast microscope (Melville, NY, USA). For evaluation of the frozen-thawed semen, two straws were chosen at random, thawed together in a Cito Thaw Unit between 35 ºC and 37 ºC for 30 sec, blotted dry, contents pooled in a graduated plastic vial with lid, and held in a dry bath between 35 ºC and 37 ºC for the duration of the 9-hr period. The percentage of progressively motile spermatozoa was evaluated at 0 (immediate), 3, 6, and 9 hr post-thaw and was visually estimated for gross motility (within 5%) at a low magnification in the phase-contrast setting of the microscope by averaging several fields of view. The percentage of intact acrosomes and morphological abnormalities were evaluated once between 0 and 6 hr post-thaw. The percentage of intact acrosomes was determined by observing 100 sperm cells for the presence of an apical ridge and/or damage to the apical ridge with the use of the differential interference contrast (DIC) setting on the microscope. The percentage of primary, secondary, and tertiary morphological abnormalities were determined by counting 100 sperm cells under high magnification with the use of the phase-contrast setting of the microscope and classifying the spermatozoa as either normal or as having a primary, secondary, or tertiary abnormality. Primary abnormalities are abnormalities of the head, secondary abnormalities are abnormalities of the middle piece, and tertiary abnormalities are abnormalities of the tail [44,62]. The percentage of morphologically normal spermatozoa was obtained by subtracting the sum of primary, secondary, and tertiary abnormalities from 100. The percentage of progressively motile spermatozoa and of morphological characteristics was evaluated by one individual throughout the entire

experiment and the percentage of spermatozoa with an intact acrosome was evaluated by one of two individuals on any given collection date.

2.5. Statistical analyses

Data retrieved from this study were analyzed using analysis of variance and all percentage data were transformed using arcsin before analysis. The effects of bull, collection date, treatment, and two-way interactions were studied as independent variables on progressively motile sperm, normal sperm, and acrosome integrity through the General Linear Model procedure of SAS (8.2, SAS Institute, Cary, NC, USA). Least squares mean separations were performed by the PDIFF procedure of SAS (two-tailed t-tests). The experimental design was a 2 X 3 factorial arrangement with two cooling durations and three equilibration times; however, the design also allowed for the study of potential interactions of treatment effects with collection date and individual bull.

3. Results

Table 2 provides a summary of P-values and the R-Square value for the effect of the independent variables on percentage of progressively motile frozen-thawed spermatozoa at 0, 3, 6, and 9 hr of incubation. Table 3 provides a summary of P-values and the R-Square value for the effect of the independent variables on percentage of intact acrosomal membrane, of primary, secondary, and tertiary abnormalities, and of morphologically normal spermatozoa.

Table 2
Experiment 1: Effect of independent variables (P-values and R-square value) on percentage of progressively motile, frozen-thawed spermatozoa at 0 (M0), 3 (M3), 6 (M6), and 9 (M9) hr of incubation.

Variable	M0	M3	M6	M9
CD	0.0078	0.0043	<.0001	0.0033
EG	0.0539	0.1835	0.079	0.0354
CD x EG	0.2477	0.0015	0.0259	0.1429
CDate	0.1618	<.0001	0.0008	<.0001
Bull	0.0002	<.0001	<.0001	<.0001
CDate x Bull	<.0001	<.0001	<.0001	<.0001
CDate x CD	0.2831	0.4766	0.8434	0.0025
CDate x EG	0.3009	0.8408	0.1884	0.7229
Bull x CD	0.4317	0.4856	0.4524	0.0257
Bull x EG	0.2624	0.5911	0.9541	0.5571
R-Square	0.5457	0.6473	0.7083	0.817

CDate = date of ejaculate collection, where, 1 through 6 correspond to 5-13-2003, 5-16-2003, 5-19-2003, 5-22-2003, 5-27-2003, and 5-30-2003, respectively.
CD = cooling duration at 4ºC in hours.
EG = equilibration with glycerol at 4ºC in hours.

Table 3
Experiment 1: Effect of independent variables (P-values and R-square value) on percentage of intact acrosomal membrane (PIA), percentage primary (1º), secondary (2º), and tertiary (3º) morphological abnormalities, and percentage morphologically normal (N) spermatozoa.

Variable	PIA	1º	2º	3º	N
CD	0.5911	0.1516	0.334	0.288	0.3185
EG	0.006	0.6616	0.0304	0.5624	0.0702
CDate	<.0001	0.0081	<.0001	<.0001	<.0001
Bull	<.0001	<.0001	<.0001	<.0001	<.0001
CD x EG	0.4699	0.4812	0.2693	0.627	0.2054
CDate x Bull	<.0001	<.0001	0.0038	0.0005	0.002
CDate x CD	0.1793	0.4786	0.6363	0.7998	0.916
CDate x EG	0.254	0.4886	0.9047	0.949	0.9411
Bull x CD	0.5121	0.4437	0.3003	0.2497	0.2523
Bull x EG	0.57	0.3029	0.6114	0.8104	0.5032
R-Square	0.739	0.7025	0.6215	0.5479	0.6413

CDate = date of ejaculate collection, where, 1 through 6 correspond to 5-13-2003, 5-16-2003, 5-19-2003, 5-22-2003, 5-27-2003, and 5-30-2003, respectively.
CD = cooling duration at 4ºC in hours.
EG = equilibration with glycerol at 4ºC in hours.

3.1. Cooling duration

Mean percentage of motile spermatozoa at 0 through 9 hr post-thaw differed ($P < 0.01$) between cooling durations (CD) of either 2 or 4 hr prior to cryopreservation (Table 2). Cooling spermatozoa at 4 ºC for a 2 hr duration prior to cryopreservation resulted in lower ($P < 0.05$) motility percentages at 0, 3, 6, and 9 hr post-thaw than a CD of 4 hr at 4 ºC (Table 4; Figure 1). However, upon post-thaw evaluation, there were no differences between the 2 and 4 hr CD at 4 ºC on mean percentage of intact acrosomes, primary, secondary, and tertiary morphological abnormalities, or percentage of morphologically normal spermatozoa (Table 5).

3.2. Equilibration with glycerol

Mean percentage of motile spermatozoa differed ($P < 0.05$) at 9 hr, but not at 0 through 6 hr post-thaw by duration of equilibration with glycerol (EG, Table 2). A 2-hr EG at 4 ºC resulted in lower ($P < 0.05$) motility percentages at the 9 hr post-thaw evaluation than a 6 hr EG (Table 4; Figure 2). Mean percentage of intact acrosomal membrane and secondary morphological abnormalities differed ($P < 0.05$) between the 2, 4, and 6 hr EG (Table 3). For spermatozoal morphological characteristics, the percentage of intact acrosomes was lower ($P < 0.05$) for 2 vs. 6 hr EG (Table 5; Figure 3), but secondary morphological abnormalities were higher ($P < 0.05$) when equilibrated for 4 hr vs 6 hr (Table 5; Figure 4). Percentage of primary and tertiary abnormalities and of morphologically normal spermatozoa were not different ($P > 0.07$) among the 2, 4, or 6 hr EG (Table 3). Percentages of morphologically normal spermatozoa by glycerol equilibration duration are graphically presented in Figure A-1.

Table 4
Experiment 1: Mean percentage of progressively motile, frozen-thawed spermatozoa at 0 (M0), 3 (M3), 6 (M6), and 9 (M9) hr of incubation by cooling duration, by equilibration with glycerol, and for cooling duration X equilibration with glycerol.

Variable	M0	M3	M6	M9
CD				
2	30[b]	21[b]	9[b]	3[b]
4	32[a]	23[a]	12[a]	4[a]
Pooled SEM	0.53	0.60	0.53	0.29
EG				
2	30	21	9	3[b]
4	31	22	11	4[a,b]
6	32	23	12	4[a]
Pooled SEM	0.65	0.73	0.65	0.36
CD x EG				
2 x 2	28	18[b]	6[c]	2
2 x 4	30	21[a]	10[b]	3
2 x 6	31	23[a]	10[a,b]	4
4 x 2	32	24[a]	13[a]	4
4 x 4	31	22[a]	11[a,b]	4
4 x 6	33	23[a]	13[a]	4
Pooled SEM	0.91	1.04	0.92	0.51

[a,b,c] LSMeans within a column by variable with different superscripts differ ($P < 0.05$).
CD = cooling duration at 4ºC, with 2 representing a 2 hr cooling duration and 4 representing a 4 hr cooling duration.
EG = equilibration with glycerol at 4ºC, with 2, 4, and 6 representing 2 hr, 4hr, and 6hr equilibration times with glycerol, respectively.

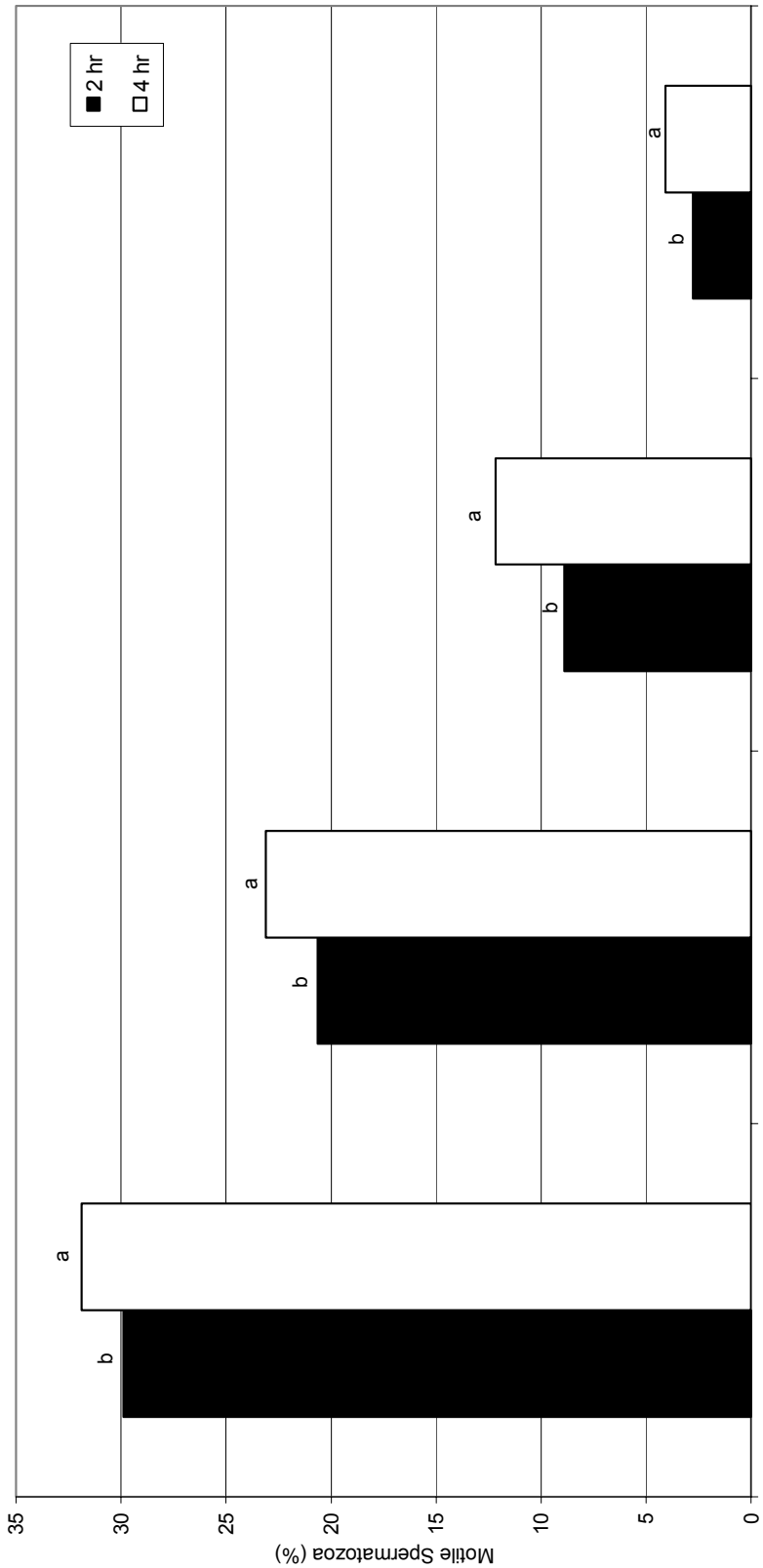

Fig. 1. Experiment 1: Mean percentage motile spermatozoa by cooling duration (2 or 4 hr) at 0, 3, 6, and 9 hr of incubation. Pooled SEM is 0.53, 0.60, 0.53 0.29, and 0.51 for 0, 3, 6, and 9 hr of incubation, respectively. ^{a,b} LSMeans within incubation time with different superscripts differ ($P < 0.05$).

Table 5
Experiment 1: Mean percentage of intact acrosomal membrane (PIA), percentage primary (1º), secondary (2º), and tertiary (3º) morphological abnormalities, and percentage morphologically normal (N) spermatozoa by cooling duration, by equilibration with glycerol, and for cooling duration X equilibration with glycerol.

Variable	PIA	1º	2º	3º	N
CD					
2	64	6	27	2	72
4	65	6	26	2	74
Pooled SEM	0.79	0.28	0.74	0.17	0.96
EG					
2	62 [b]	6	26 [a,b]	2	73
4	65 [a,b]	6	28 [a]	2	71
6	67 [a]	6	25 [b]	2	75
Pooled SEM	0.97	0.34	0.91	0.20	1.18
CD x EG					
2 x 2	61	6	28	2	71
2 x 4	65	6	28	2	71
2 x 6	67	5	25	2	75
4 x 2	63	6	24	2	76
4 x 4	64	6	29	1	71
4 x 6	66	7	25	2	75
Pooled SEM	1.37	0.48	1.28	0.29	1.67

[a,b] LSMeans within a column by variable with different superscripts differ ($P < 0.05$).
CD = cooling duration at 4ºC, with 2 representing a 2 hr cooling duration and 4 representing a 4 hr cooling duration.
EG = equilibration with glycerol at 4ºC, with 2, 4, and 6 representing 2 hr, 4hr, and 6hr equilibration times with glycerol, respectively.

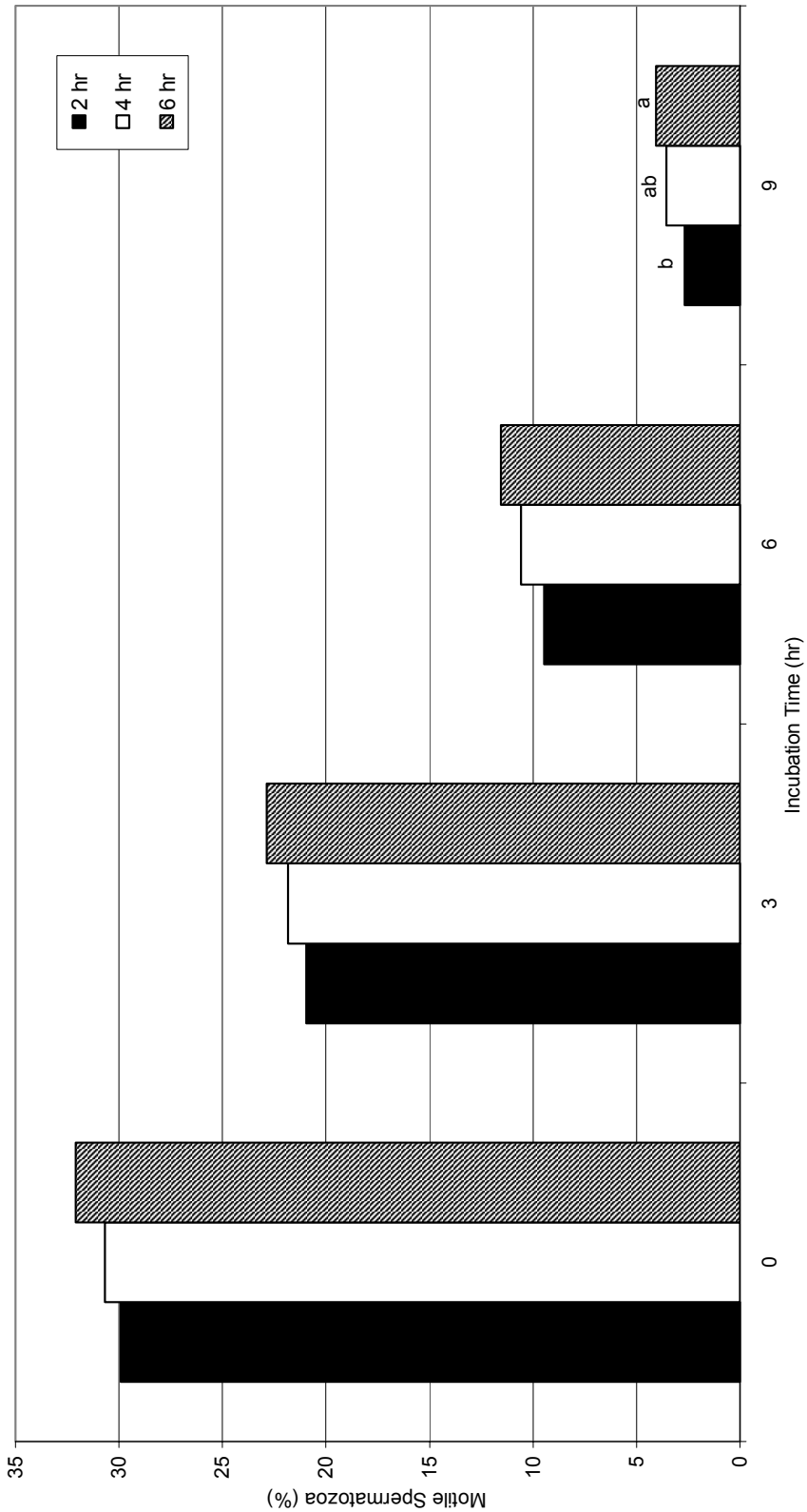

Fig. 2. Experiment 1: Mean percentage motile spermatozoa by duration of equilibration with glycerol (2, 4, or 6 hr) at 0, 3, 6, and 9 hr of incubation. Pooled SEM is 0.65, 0.73, 0.65, and 0.36 for 0, 3, 6, and 9 hr of incubation, respectively. ^{a,b}LSMeans within incubation time with different superscripts differ ($P < 0.05$).

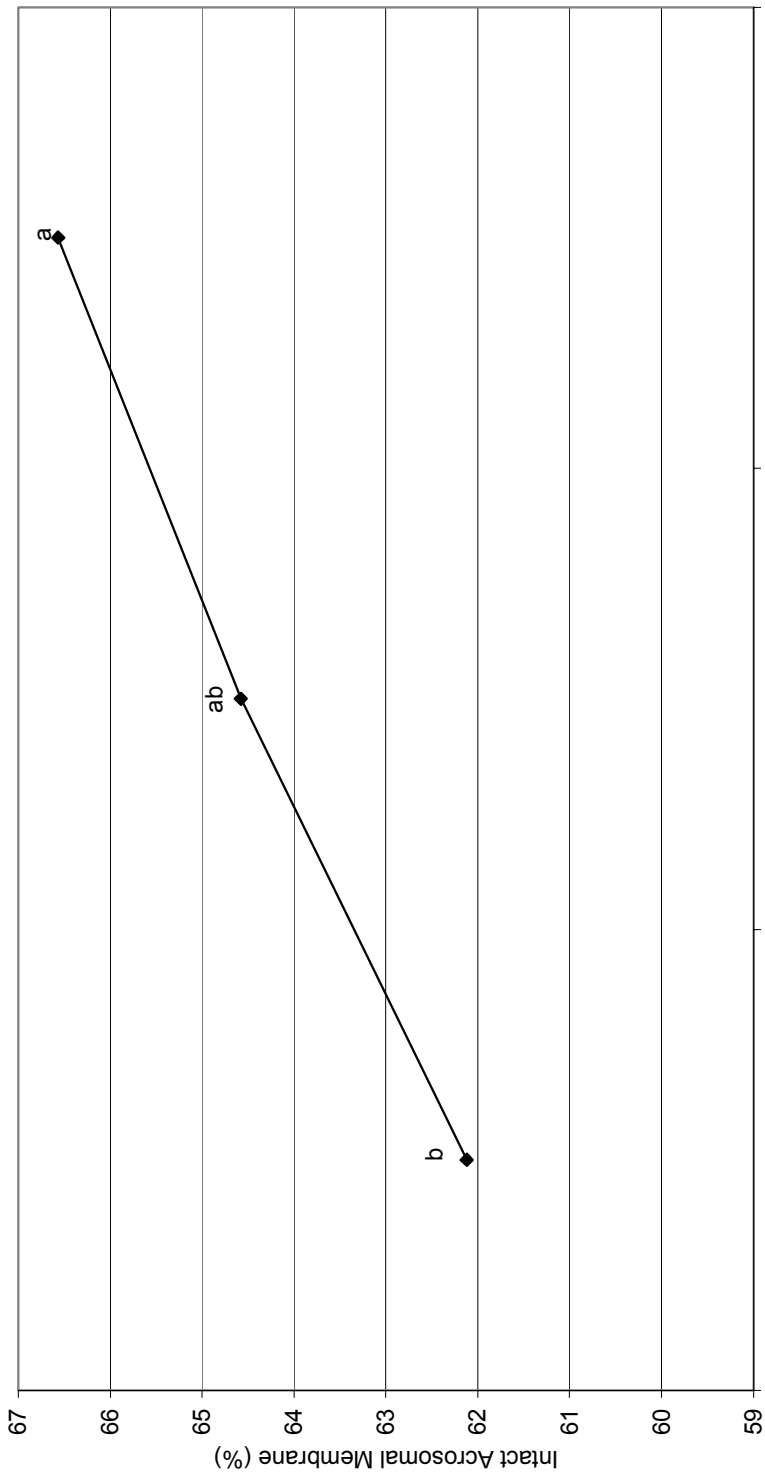

Fig. 3. Experiment 1: Mean percentage of spermatozoa with an intact acrosomal membrane by duration of equilibration with glycerol (2, 4, or 6 hr). Pooled SEM is 0.97. ^{a,b} LSMeans with different superscripts differ ($P < 0.05$).

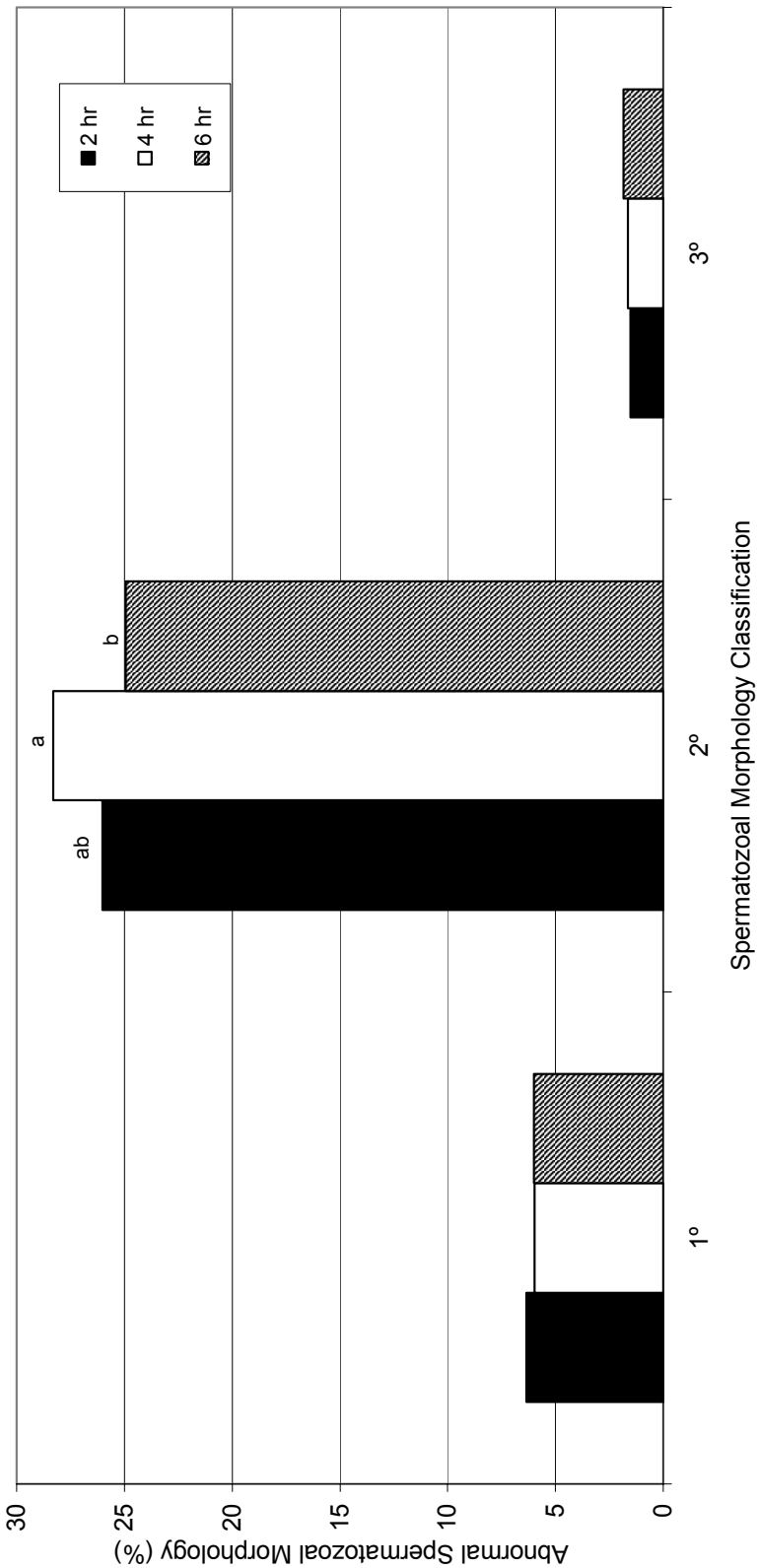

Fig. 4. Experiment 1: Mean percentage morphologically abnormal spermatozoa (1° = primary, 2° = secondary, or 3° = tertiary abnormalities) by duration of equilibration with glycerol (2, 4, or 6 hr). Pooled SEM is 0.34, 0.91, and 0.20 for 1°, 2°, and 3°, respectively. ^{a,b} LSMeans with different superscripts within abnormality classification differ ($P < 0.05$).

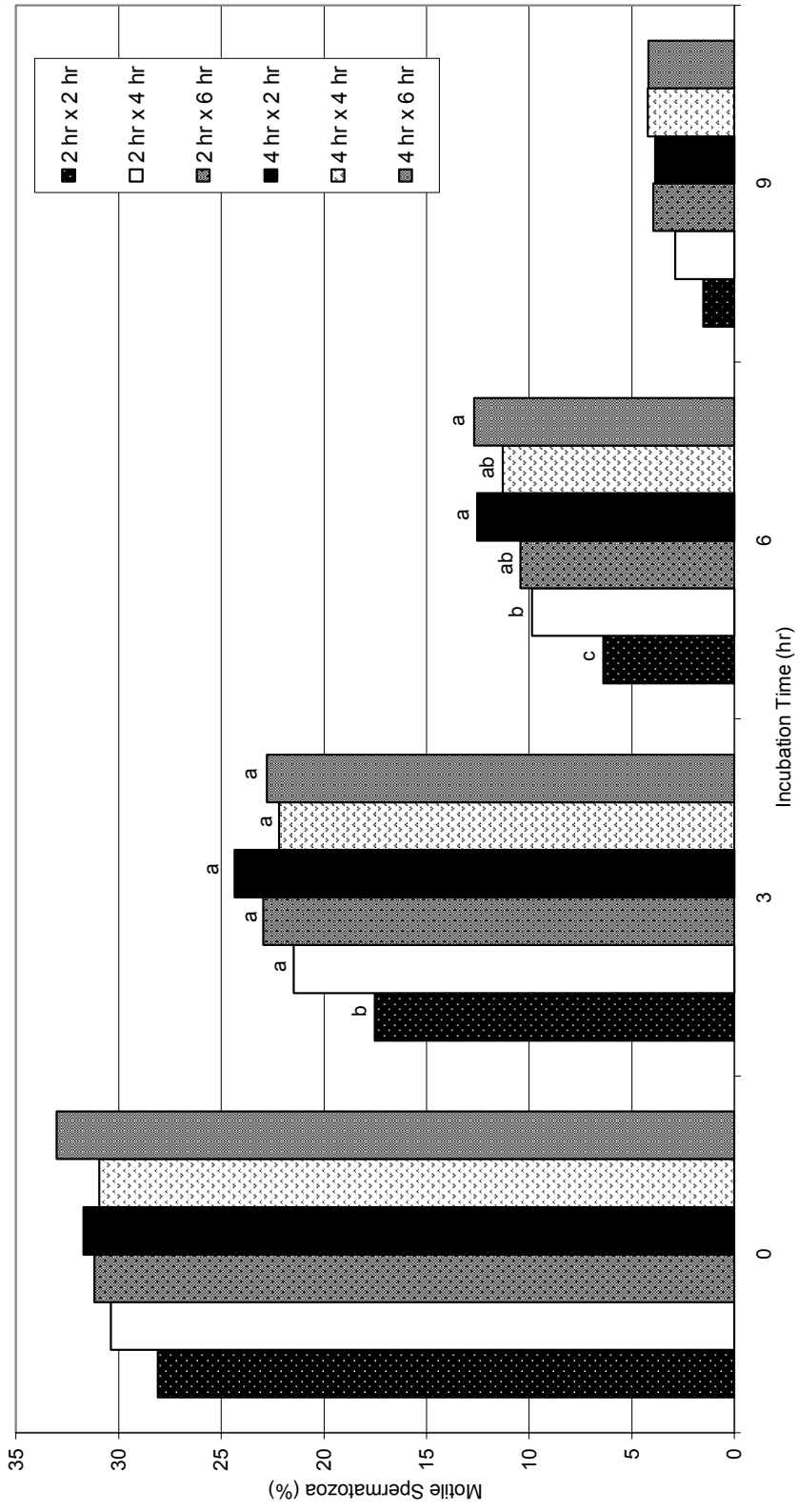

Fig. 5. Experiment 1: Mean percentage motile spermatozoa by cooling duration (2 or 4 hr) and duration of equilibration with glycerol (2, 4, or 6 hr) at 0, 3, 6, and 9 hr of incubation. Pooled SEM is 0.91, 1.04, 0.92, and 0.51 for 0, 3, 6, and 9 hr of incubation, respectively.
[a,b,c]LSMeans within incubation time with different superscripts differ ($P < 0.05$).

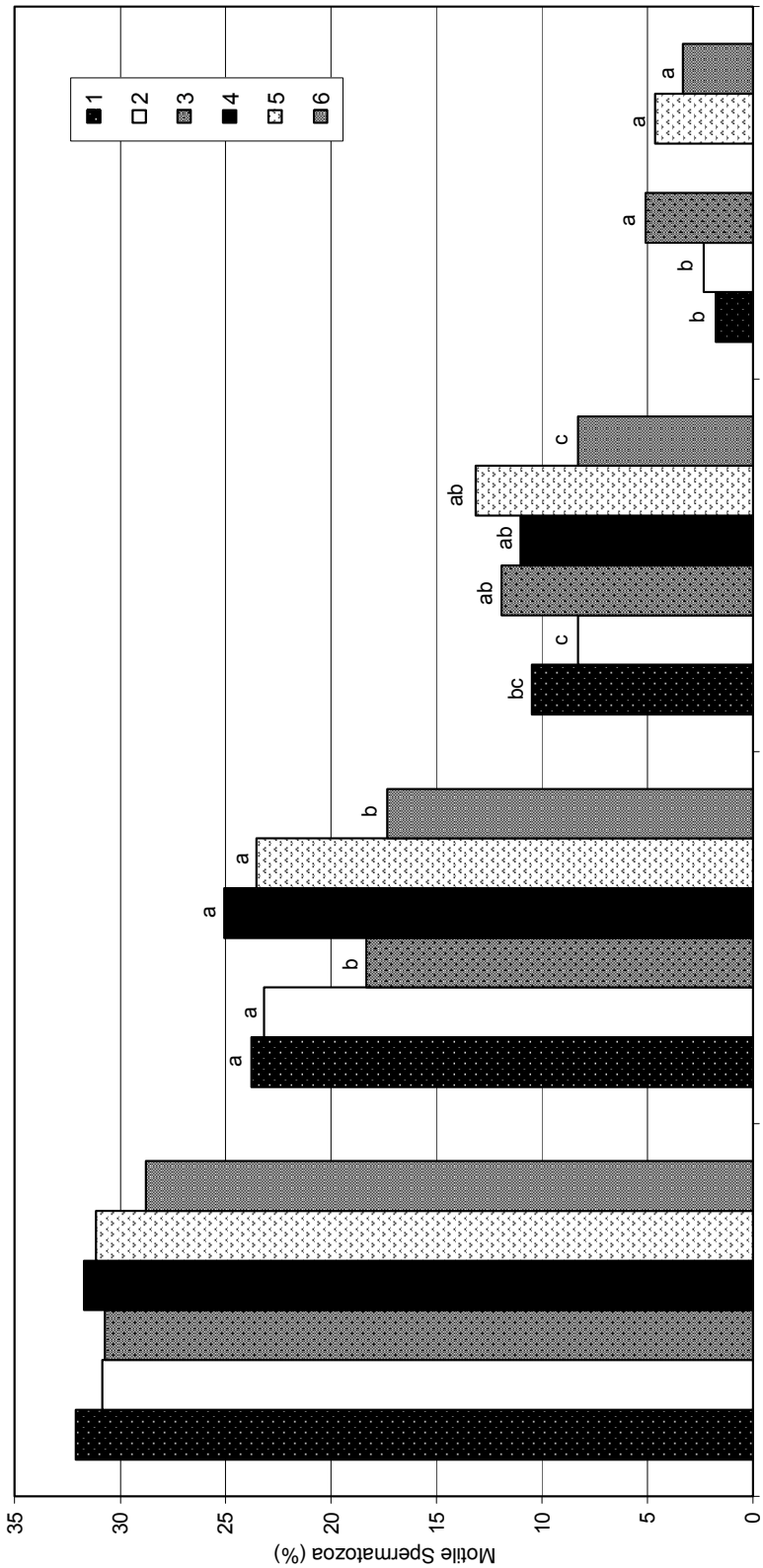

Fig. 6. Experiment 1: Mean percentage motile spermatozoa by collection date (1 to 6) at 0, 3, 6, and 9 hr of incubation. Pooled SEM is 0.91, 1.04, 0.92, and 0.51 for 0, 3, 6, and 9 hr of incubation, respectively. $^{a,b,c.}$ LSMeans within incubation time with different superscripts differ ($P < 0.05$).

3.3. Interaction of cooling duration X equilibration with glycerol

Mean percentage of motile spermatozoa at 3 hr post-thaw was affected ($P < 0.01$) by an interaction of CD X EG (Table 2). Spermatozoal motility at the 3 hr post-thaw evaluation was reduced ($P < 0.05$) for the 2 hr CD X 2 hr EG when compared with all other treatment combinations (Table 4; Figure 5). Mean percentage of motile sperm was also lowest ($P < 0.05$) at the 6 hr post-thaw evaluation for the 2 hr CD X 2 hr EG treatment, however, there was greater variability among treatments at the 6 hr than the 3 hr incubation (Table 4; Figure 5). The interaction between CD X EG did not affect ($P < 0.05$) mean percentage of intact acrosomes, primary, secondary, or tertiary morphological abnormalities, or percentage of morphologically normal spermatozoa (Table 3; Table 5).

3.4. Collection date

Mean percentage of motile spermatozoa at 3 through 9 hr post-thaw differed ($P < 0.01$) by collection date (CDate, Table 2). Collection dates 1, 2, 4, and 5 had higher spermatozoal motility percentages at 3 hr of incubation than CDates 3 and 6 (Table A-1; Figure 6). For the 6 hr incubation, CDates 3 through 5 had higher ($P < 0.05$) spermatozoal motility percentages than CDates 2 and 6 (Table A-1; Figure 6). Motility percentages at 9 hr of incubation were higher ($P < 0.05$) on CDates 3, 5, and 6 than on CDates 1 and 2. Motility percentages at 0 hr of incubation were not affected($P > 0.16$) by CDate (Table 2; Figure 6). Mean percentage of intact acrosomal membrane, percentage of primary, secondary, and tertiary abnormalities, and percentage morphologically normal spermatozoa differed ($P < 0.01$) by CDate (Table 3). Collection

date 1 resulted in the highest ($P < 0.05$) percentage of intact acrosomal membranes, while CDate 6 resulted in the lowest percentage of intact acrosomal membranes (Table A-2; Figure A-2). A two-percentage point difference ($P < 0.05$) among CDates was observed for primary morphological abnormalities (Table A-2; Figure 7). The highest ($P < 0.05$) percentage of secondary morphological abnormalities was observed for CDate 6, while the lowest percentage of secondary morphological abnormalities was observed for CDate 2 (Table A-2; Figure 7). Tertiary morphological abnormalities resulted in a two-percentage point difference ($P < 0.05$) among CDates (Table A-2; Figure 7). The highest ($P < 0.05$) value for percentage of morphologically normal spermatozoa was observed for CDate 2, while the lowest ($P < 0.05$) percentage of morphologically normal spermatozoa was observed for CDate 6 (Table A-2; Figure 8).

3.5. Bull

Mean percentage of motile spermatozoa at 0 through 9 hr post-thaw differed ($P < 0.01$) by bull (Table 2). Bulls B, D, and F had a higher ($P < 0.05$) motility percentage at 0 hr post-thaw than bulls A, C, and E (Table A-1; Figure 9). Bull F had the highest ($P < 0.05$) motility percentage at 3 hr post-thaw, while bulls A and E had the lowest ($P < 0.05$) motility percentage at 3 hr post-thaw (Table A-1; Figure 9). Bulls B and F had the highest ($P < 0.05$) motility percentage at 6 hr post-thaw, while bull A had the lowest ($P < 0.05$) motility percentage at 6 hr post-thaw (Table A-1; Figure 9). Ten percentage points separated bull F from bulls A and D for the highest ($P < 0.05$) percentage of motile spermatozoa at 9 hr post-thaw (Table A-1, Figure 9).

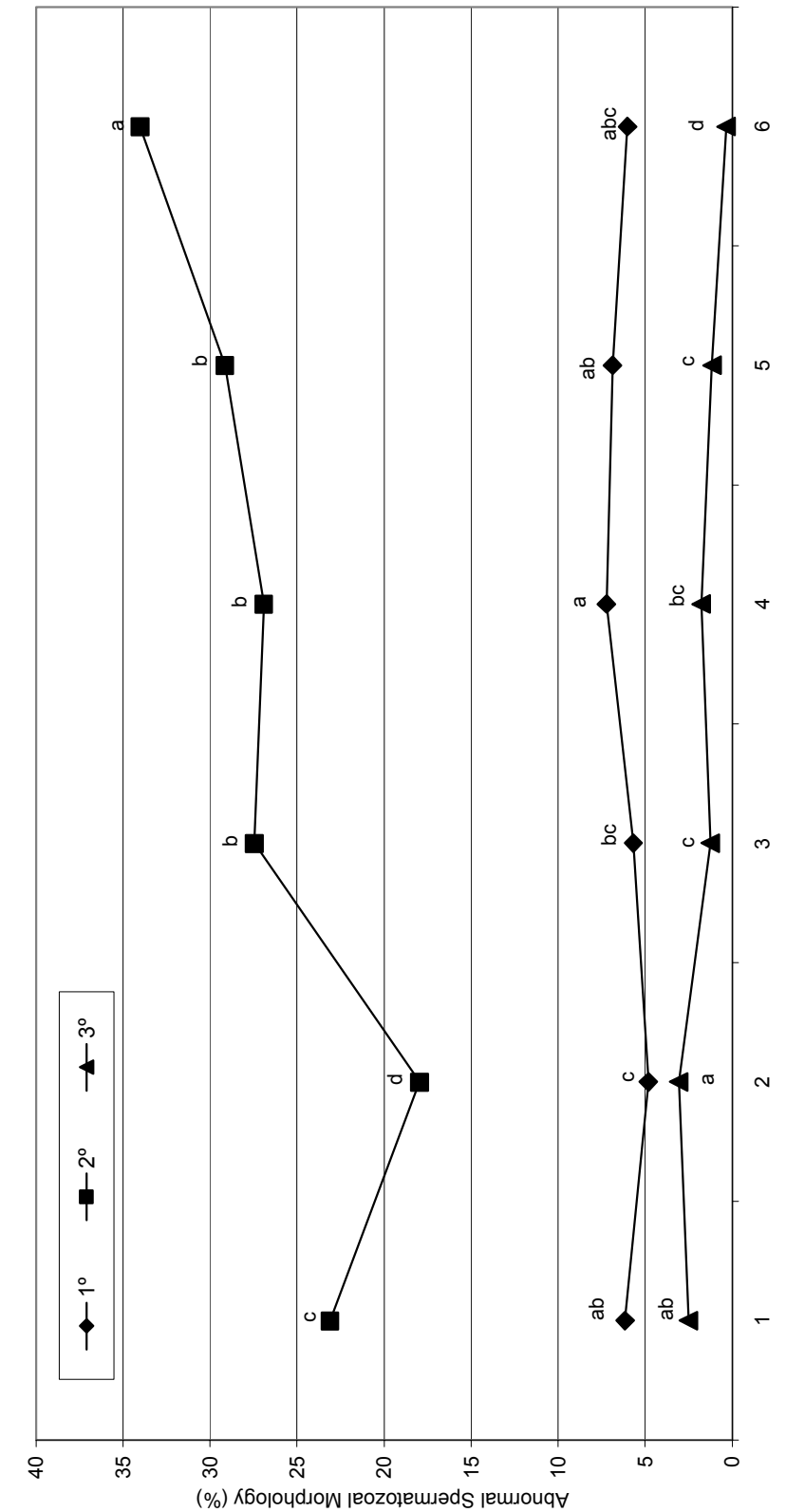

Fig. 7. Experiment 1: Mean percentage morphologically abnormal spermatozoa (1° = primary, 2° = secondary, or 3° = tertiary abnormalities) by collection date (1 to 6). Pooled SEM is 0.48, 1.28, and 0.29 for 1°, 2°, and 3°, respectively. ^{a,b,c,d} LSMeans with different superscripts within abnormality classification differ ($P < 0.05$).

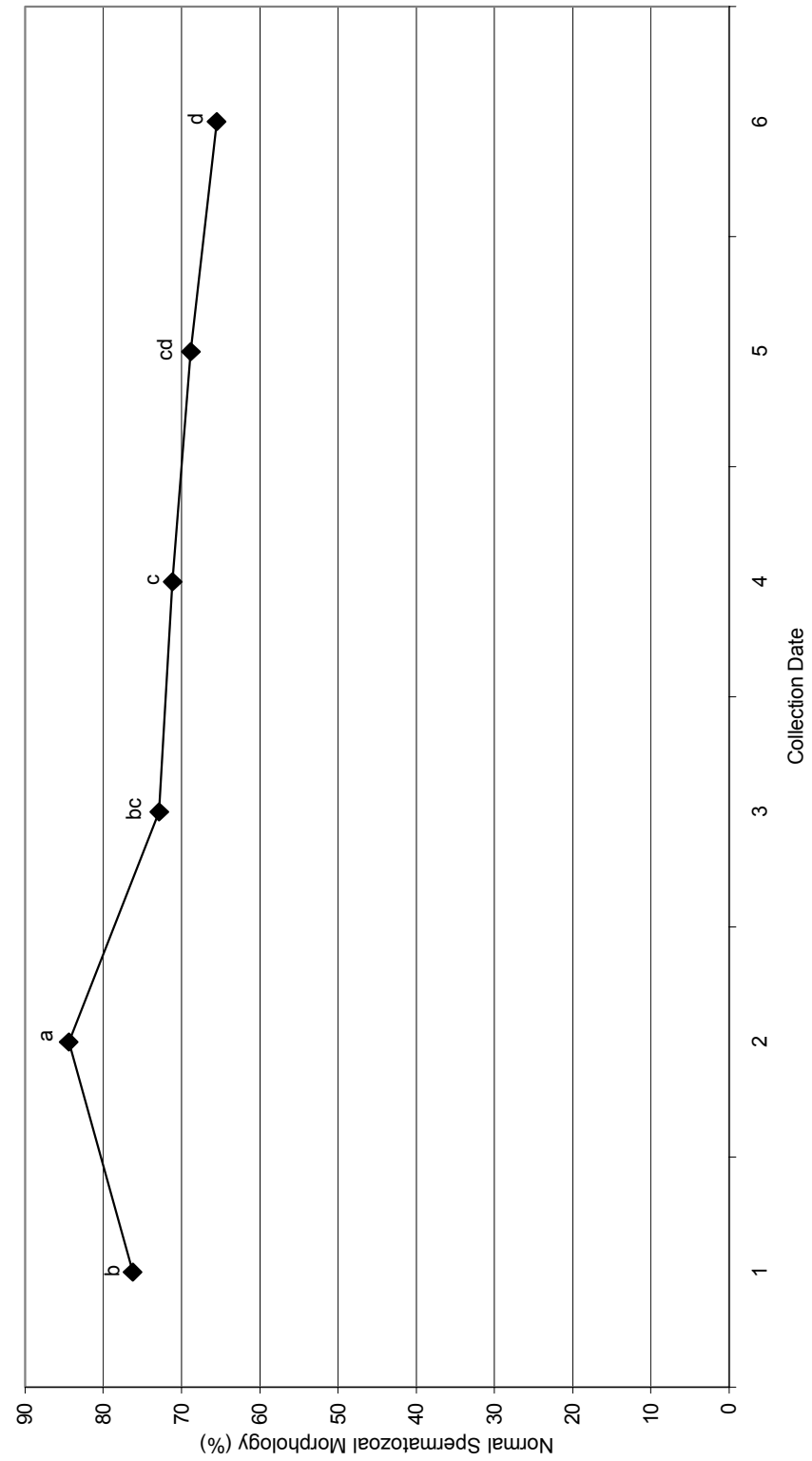

Fig. 8. Experiment 1: Mean percentage morphologically normal spermatozoa by collection date (1 to 6). Pooled SEM is 1.67. ^{a,b,c,d}LSMeans with different superscripts differ ($P < 0.05$).

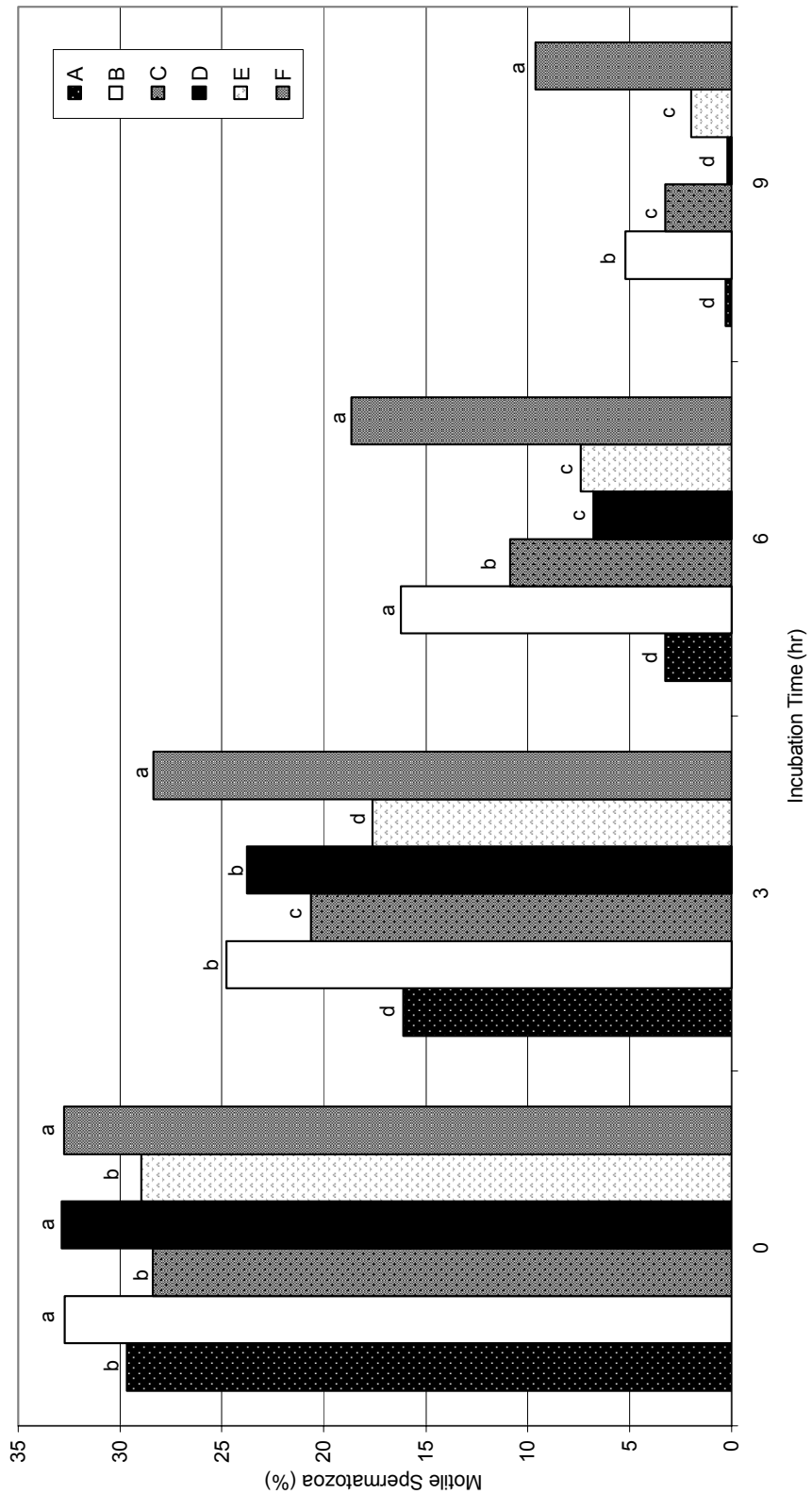

Fig. 9. Experiment 1: Mean percentage motile spermatozoa by bull (A to F) at 0, 3, 6, and 9 hr of incubation. Pooled SEM is 0.91, 1.04, 0.92, and 0.51 for 0, 3, 6, and 9 hr of incubation, respectively. [a,b,c,d] LSMeans within incubation time with different superscripts differ ($P < 0.05$).

The highest ($P < 0.05$) intact acrosomal membrane percentage was observed in bull B, while the lowest ($P < 0.05$) was observed in bull F (Table A-2; Figure A-3). Primary morphological abnormalities were highest ($P < 0.05$) in bull E, while bulls A, B, and C had the least (Table A-2; Figure A-4). The highest ($P < 0.05$) occurrence of secondary morphological abnormalities was observed in bull C and the lowest ($P < 0.05$) occurrence was observed in bull B (Table A-2; Figure A-4). The highest ($P < 0.05$) occurrence of tertiary morphological abnormalities was observed in bull E and the lowest occurrence was observed in bull C (Table A-2; Figure A-4). Bull B yielded the highest ($P < 0.05$) percentage of morphologically normal spermatozoa and bulls E and F yielded the lowest ($P < 0.05$) percentage of morphologically normal spermatozoa (Table A-2; Figure A-5).

3.6. Interaction of collection date X bull

Mean percentage of motile spermatozoa at 0 through 9 hr post-thaw differed ($P < 0.0001$) due to an interaction between CDate X bull (Table 2; Figures A-6 through A-9). Percentages of motile spermatozoa for the interaction between CDate X bull at 0 through 9 hr post-thaw are presented in Table A-3. Mean percentage of intact acrosomal membrane also differed ($P < 0.0001$) due to an interaction between CDate X bull (Table 3; Figure A-10). Mean percentage of primary, secondary, and tertiary morphological abnormalities differed ($P < 0.003$) due to an interaction between CDate X bull (Table 3; Figures A-11 through A-13). Mean percentage of morphologically normal spermatozoa differed ($P < 0.002$) due to an interaction between CDate X bull (Table 3; Figure A-14). Percentages of intact acrosomal membrane, of primary, secondary, and tertiary

morphological abnormalities, and of morphologically normal spermatozoa for the interaction between CDate X bull are presented in Table A-4.

3.7. Interaction of collection date X cooling duration

Mean percentage of motile spermatozoa at 9 hr post-thaw differed ($P < 0.002$) due to an interaction between CDate X CD (Table 2). Collection date 3 with a 4 hr CD and CDate 5 with a 4 hr CD resulted in a higher ($P < 0.05$) percentage of motile spermatozoa at 9 hr post-thaw than any of the other treatment combinations (Table A-5; Figure A-15). Mean percentage of motile spermatozoa did not differ ($P > 0.28$) for CDate X CD at 0, 3, and 6 hr post-thaw (Table 2). Mean percentage of intact acrosomal membrane, percentage primary, secondary, and tertiary morphological abnormalities, and percentage of morphologically normal spermatozoa did not differ ($P > 0.17$) for CDate X CD (Table 3; Table A-6).

3.8. Interaction of collection date X equilibration with glycerol

Mean percentage of motile spermatozoa at 0 through 9 hr post-thaw did not differ ($P > 0.18$) for the interaction between CDate X EG (Table 2; Table A-7). Mean percentage of intact acrosomal membrane, of primary, secondary, and tertiary morphological abnormalities, and of morphologically normal spermatozoa did not differ ($P > 0.25$) for the interaction between CDate X EG (Table 3; Table A-8).

3.9. Interaction of bull X cooling duration

Mean percentage of motile spermatozoa at 9 hr post-thaw differed ($P < 0.02$) due to an interaction between bull X CD (Table 2). Ten percentage points separated the highest ($P < 0.05$) spermatozoal motility percentage, bull F with CD of 2 and 4 hr, from

the lowest ($P < 0.05$) spermatozoal motility percentage, bulls A and D with CD of 2 and 4 hr (Table A-9; Figure A-16). Mean percentage of intact acrosomal membrane, of primary, secondary, and tertiary morphological abnormalities, and of morphologically normal spermatozoa did not differ ($P > 0.17$) for the interaction between bull X CD (Table 3; Table A-10).

3.10. Interaction of bull X equilibration with glycerol

Mean percentage of motile spermatozoa at 0 through 9 hr post-thaw did not differ ($P > 0.26$) for the interaction between bull X EG (Table 2; Table A-11). Mean percentage of intact acrosomal membrane, of primary, secondary, and tertiary morphological abnormalities, and of morphologically normal spermatozoa did not differ ($P > 0.30$) for the interaction between bull X EG (Table 3; Table A-12).

4. Discussion

4.1. Cooling duration

Spermatozoal post-thaw viability is dependent, at least in part, upon the length of time the extended sample is allotted to cool to 5 ºC prior to the addition of the glycerolated portion of the extender. Slowly cooling the partially extended semen over several hours reduces spermatozoa plasma membrane and mid-piece damage caused by cold shock [9,35]. Our study evaluated the effect of a 2 or 4 hr cooling duration on post-thaw spermatozoal motility, morphology, and acrosomal integrity.

Lower percentages for motility were observed for the 2 hr than 4 hr cooling duration in our study. These results concur with reports by Gilbert and Almquist [36]

and Ennen et al. [8] in which spermatozoal motility is increased as the cooling duration is increased. Interestingly, Ennen et al. [8] also reported that no differences in percentage of motile spermatozoa upon post-thaw evaluation between the 2 and 4 hr cooling durations were observed, which is not in agreement with our study. Ennen et al. [8] may not have observed an affect of cooling duration on post-thaw motility due to differences in breed (Angus and Hereford), bull age (2 to 5 years old), or packaging type (0.25 mL plastic straws) relative to the current study. A 1.5 hr difference in cooling durations between our experiment and those conducted by Gilbert and Almquist [36] and Ennen et al. [8], thus potentially explaining the lower motility percentages observed for the 0.5 hr cooling duration, but not between the 2 and 4 hr cooling durations. The difference between our observations and those of Ennen et al. [8] for percentage of spermatozoal motility post-thaw could be due to any number of factors involved in the processing, freezing, and or thawing procedures of the spermatozoa.

We observed no differences between the 2 and 4 hr cooling durations for percentage of intact acrosomal membranes, of primary, secondary, and tertiary abnormalities, and of morphologically normal spermatozoa. Our observations are not in agreement with a study [36] that reported differences in acrosomal retention and morphology with varying cooling durations. The discrepancy between our results and those of Gilbert and Almquist [36] could be due to differences in the final glycerol concentrations, straw volume, packaging technique, and or thaw rate, as those employed by Gilbert and Almquist [36] are significantly different from those utilized in our experiment.

4.2. Equilibration with glycerol

Glycerol is utilized in the extender to lower the freezing point of water, thus aiding in the reduction of intracellular ice formation [1]. However, the length of time required for maximum diffusion of glycerol into spermatozoa for optimal cryoprotection is unknown at this time. Our experiment evaluated the effect of a 2, 4, or 6 hr equilibration with glycerol on post-thaw spermatozoal motility, morphology, and acrosomal integrity percentages in an attempt to provide a recommendation for an optimal duration of glycerol equilibration.

Equilibration of the spermatozoa for 2, 4, or 6 hr with glycerol did not affect the percentage of motile spermatozoa when evaluated at 0, 3, or 6 hr post-thaw, however, the percentage of motile spermatozoa was higher for the 6 hr equilibration than the 2 hr equilibration when evaluated at 9 hr post-thaw. A potential explanation for the difference in motility at the 9 hr post-thaw check, but not at 0, 3, or 6 hr could be due to the stress of incubating the frozen-thawed spermatozoa at 37 ºC for the post-thaw examination, because spermatozoal viability, specifically motility and acrosomal integrity, decreases as the time of incubation post-thaw increases [1]. Miller and VanDemark [42] reported higher spermatozoal motility when spermatozoa were equilibrated with glycerol for 2 or 6 hr than 18 hr at 4.5 ºC. Even though we did not evaluate the effect of an 18 hr equilibration with glycerol, the findings of Miller and VanDemark [42] support our results. In contrast, Berndtson and Foote [6] reported the attainment of higher spermatozoal motility after 10 sec of glycerol equilibration as compared to10 min, 20 min, 30 min, or 6 hr of glycerol equilibration. However, the total

concentration of spermatozoa/mL was significantly higher in the experiment for Berndtson and Foote [6] than what was utilized in our experiment (300 x 10^6 and 45 x 10^6, respectively). In addition, we packaged our extended semen into 0.5 mL plastic French straws, while Berndtson and Foote [6] utilized the pellet method for freezing the spermatozoa.

We observed that the percentage of intact acrosomes was lower for the 2 hr than for the 6 hr equilibration with glycerol. Equilibration time with glycerol also affected the percentage of secondary morphological abnormalities. We observed a higher percentage of secondary morphological abnormalities when the sample was equilibrated for 4 hr vs 6 hr of equilibration. We did not however, observe any effects of equilibration of spermatozoa for 2, 4, or 6 hr with glycerol on the percentage of primary and tertiary morphological abnormalities or of morphologically normal spermatozoa.

4.3. Interaction of cooling duration X equilibration with glycerol

The time required for equilibration with glycerol to provide cryoprotective support to the spermatozoa is dependent upon the cooling duration [8]. Therefore, it can be speculated that an interaction between the cooling duration and equilibration with glycerol exists. We evaluated the effect of an interaction of CD X EG on spermatozoal motility, morphology, and acrosomal integrity to validate the potential of an interaction between the two.

Spermatozoal motility was decreased for the 2 hr CD X 2 hr EG relative to all other treatment combinations at the 3 and 6 hr post-thaw evaluations. Our results show that the maximum percentage for motility was obtained for a 4 hr CD X 2 or 6 hr EG.

Our results are supported by Ennen et al. [8], who also observed an effect on spermatozoal motility for interaction of CD X EG. The authors reported that the highest motility percentages were obtained for a 4 hr CD X 2 or 4 hr EG. Variations in final glycerol concentration, straw volume and or the procedure employed for thawing frozen spermatozoa could contribute to the differences observed in our experiment compared to those of Ennen et al. [8].

The percentages of acrosomal membrane retention, of primary, secondary, and tertiary morphological abnormalities, and of morphologically normal spermatozoa were not affected by the interaction of CD X EG in our study. Although others [8,61] have stated that the equilibration time with glycerol is dependent upon the cooling duration we are not aware of any reports of an effect on acrosomal integrity or sperm morphology due to an interaction between cooling duration and equilibration time with glycerol.

Decreasing the time from collection to freezing will increase the efficiency of semen collection facilities in their processing procedures. With this in mind, we recommend utilizing a cooling duration of 4 hr with a 4 hr equilibration with glycerol to achieve optimal percentages of motile spermatozoa post-thaw. The 4 hr equilibration time was chosen over 2 or 6 hr based on previous research [8,42], which reported that there were no significant differences between 2 and 6 hr equilibration durations with glycerol and from our own observations. Our research suggests that the utilization of a 4 or 6 hr equilibration with glycerol will provide maximal frozen-thawed spermatozoal viability post-thaw, as no significant differences were observed between the two for the

percentage of motile spermatozoa, of intact acrosomes, primary, secondary, and tertiary abnormalities, and of morphologically normal spermatozoa.

4.4. Collection date

Seasonality affects the quality and quantity of each ejaculate collected and processed for use by AI [60,63]. The effect of collection date was highly significant ($P < 0.01$), with the exception of motility at 0 hr post-thaw incubation. However, even though a high degree of variation exists, we observed a significant interaction only between CDate X Bull for all treatments and CDate X CD at 9 hr post-thaw incubation. Due to the known effects of bull variation on spermatozoal quality and a lack of significant interactions between CDate, CD, and EG, we chose not to perform separation of means across bull related interactions, with the exception of the interaction of CDate X CD for motility at 9 hr post-thaw incubation. We speculate that the variation in spermatozoal variability due to collection date and the interaction of collection date by bull observed in our study are primarily due to the collection personnel, order of collection, and seasonality.

4.4.1. Collection personnel and order of collection

The personnel involved in the collection process have an impact on the quantity and quality of the ejaculate upon collection [24]. Mathevon et al. [24] evaluated the effects of the bull handler and semen collector on ejaculate characteristics of Holstein bulls. The authors reported that the total number of cells and the total number of motile cells were affected ($P < 0.05$) by collection team and that the collection personnel accounted for less than 10 % of the variance observed in ejaculate characteristics among

bulls. Bulls in our experiment were not always collected by the same personnel; thus the current study was not designed to quantify the potential effect of the collection team. The order in which the bulls are collected has the potential to increase or decrease spermatozoal characteristics per ejaculate. This can be attributed to the degree of visual, olfactory, and or auditory stimulation required to sexually prepare each bull for semen collection [44]. The bulls in our study were not collected in any particular order. All six bulls were moved to the collection arena at the same time, loaded and then collected from individually. However, the first bull to load into the holding stalls was the first bull collected from and so on, providing each bull with equal opportunity to be collected first, last, or in the middle of the group. If a bull requiring a higher degree of sexual stimulation in order to achieve maximal sexual preparation prior to collection was one of the first bulls collected from, the time allotted for maximal sexual stimulation may not have been met, thus potentially reducing the quality of the ejaculate [64].

4.4.2. Seasonality

Seasonality has been reported to alter sperm output, production, motility, morphology, and acrosomal integrity [65-67]. Johnston and Branton [68] observed a decline in non-return rates for semen collected during the summer months of July, August, and September in Louisiana as the ambient temperature was increased as compared to all other months. In a comprehensive study in dairy bulls located in the mid western United States, Erb et al. [69] evaluated the effects of seasonality on ejaculate volume and concentration, spermatozoal motility, morphology, and viability upon storage at 4 ºC. The months were divided into 4 seasons, consisting of winter (January

through March), spring (April through June), summer (July through September), and fall (October through December). Overall, Erb et al. [69] reported that semen of inferior quality was produced during the summer months, while semen of superior quality was obtained during the spring months. Specifically, Erb et al. [69] reported, that volume, motility, and duration of viability upon storage were decreased during the summer months, and the percentage of abnormal spermatozoa increased by 25 % during the summer months. However, the authors observed maximal spermatozoal concentration and total sperm numbers during the spring months. As trends in spermatozoal quality and semen production followed the changes in temperature closely, Erb et al. [69] speculated that changes in temperature have the potential to have the largest impact on spermatozoal quality and production.

4.5. Bull

Individual variation among bulls can affect the cryoprotective properties that extenders and processing procedures have on the outcome of spermatozoal viability post-thaw [26,60]. Extensive reviews have examined the influence of animal individuality on the outcome of spermatozoal characteristics and spermatozoal viability post-thaw [3,70]. Graham et al. [4] studied the effects of glycerol equilibration time on non-return rate and observed differences among bulls on the equilibration with glycerol as well as the collection period. The observations of Graham et al. [4] were significant at the five percent level, which supports our observation that the effect of individual variation among bulls was highly significant ($P < 0.0003$). The interaction of CDate X bull affected all measures of spermatozoal variability. We observed a significant

interaction only for Bull X CD on spermatozoal motility at 9 hr post-thaw incubation. However, the percentage of progressively motile spermatozoa at 9 hr post-thaw was > 10 % for all bulls.

4.5.1. Variability among individual bulls

Reviews conducted by Hammerstedt et al. [1] and Watson [48] demonstrate that semen processing and thawing adversely affect spermatozoal viability, by altering and or damaging membrane structures throughout one or all of the aspects of spermatozoal evaluation, extension, cooling duration, equilibration with glycerol, and the freezing-thawing process. Several authors [26,40,53] have made note of the variation among bulls upon post-thaw evaluation, however, none speculated as to the cause of the variation among the bulls. Even though Graham et al. [4] and Jondet [5] did not evaluate spermatozoal characteristics prior to processing, their research illustrates that variation among bulls exists and can influence the percentage of cows that do not return to estrus after breeding by frozen-thawed semen. Graham et al. [4] observed variation among bulls when studying the effects of glycerol equilibration time on the non-return rate of cows bred by AI. Graham et al. [4] observed non-return rates as high as 78 % and as low as 61 % among bulls for the 12 hr equilibration with glycerol. The author observed an 11 percentage point spread for the 8 and 4 hr durations of equilibration with glycerol among the bulls, suggesting that differences among bulls and treatments exist. Jondet [5] also observed variations in spermatozoal motility and non-return rates among bulls for varying durations of glycerol equilibration. Jondet [5] observed differences in the percentage of motile spermatozoa and the non-return rate ranging from 1 to 13

percentage points between one bull and another. The author also speculated that the ejaculates vary from one to another for the same bull.

4.5.2. Age of bull

The age of the bull may also affect spermatozoal output and quality. Evertt and Bean [60] observed large differences between bulls in sperm producing ability from bulls of varying ages. Their research supported that of Amann et al. [71], who reported that spermatozoal concentration per ejaculate increased as the bull increased in age. Hallap et al. [72] observed no differences in spermatozoal motility or morphology on semen collected from bulls at 1 and 4 years of age, however, the authors did report that acrosomal membrane integrity was higher at 4 years of age than 1. The bulls utilized in our study were all approximately 18 months of age (± 2 months) and from the same contemporary group on one ranch in central Texas. Housing was identical for all bulls while on the ranch and while at the collection facility for all six bulls, thus eliminating or at least greatly reducing any environmental and or managerial differences among the bulls. The young age of the bulls in our study may have influenced the results obtained from our observations based on previous research [24,67], which demonstrated that younger bulls have decreased spermatozoal quality until they attain a plateau in sexual development. However, by utilizing bulls that appear to be more sensitive to all aspects of cryopreservation, we may have been able to more accurately demonstrate spermatozoal sensitivity to the cooling duration and or the equilibration with glycerol.

5. Conclusion

Cooling spermatozoa for 4 hr resulted in a higher percentage of motile spermatozoa than for a cooling duration of 2 hr. A higher percentage of motile spermatozoa at 9 hr post-thaw and of intact acrosomes was observed when equilibrated with glycerol for 6 hr than for 2 hr. The percentage of secondary morphological abnormalities was higher when equilibrated with glycerol for 4 hr than for 6 hr. Collection date and bull did affect motility, percentage of intact acrosomes, and morphological characteristics of spermatozoa. From our results, we conclude that maximal percentages of motility, of intact acrosomes, and of morphologically normal spermatozoa will be obtained when utilizing a cooling duration of 4 hr and equilibrating the sample with glycerol for 4 hr.

CHAPTER IV

EFFECTS OF SEMEN STORAGE TYPE AND EXTENDER TYPE ON PERCENTAGE OF SPERMATOZOAL MOTILITY, ACROSOMAL INTEGRITY, AND MORPHOLOGICAL CHARACTERISTICS

1. Introduction

Semen storage type (fresh or frozen-thawed) and extender type have an impact on spermatozoal viability as measured by the percentage of spermatozoal motility, acrosomal integrity, and morphological characteristics [14,17,73]. It is widely accepted by the beef and dairy cattle industries, semen collection facilities, and producers that frozen-thawed sepermatozoa have decreased percentages of spermatozoal motility, of acrosomal integrity, and morphological characteristics due to the stress of the freeze-thaw process [61]. Senger et al. [16] evaluated the effects of egg yolk-citrate, egg yolk-tris, and skim milk-based extenders on spermatozoal post-thaw viability. The authors reported that acrosomal integrity was higher for the egg yolk-tris-based extender than for egg yolk-citrate or skim milk (65, 54, and 48 %, respectively). Senger et al. [16] also reported that the percentage of motile spermatozoa was higher for semen frozen and thawed in the egg yolk-tris extender than for egg yolk-citrate or skim milk (52, 41, and 27 %, respectively). The work of Senger et al. [16] clearly demonstrates the variability of spermatozoal characteristics upon post-thaw, thus illustrating the need to determine an optimum extender to be utilized by semen collection facilities.

The primary goal of any semen collection facility is to efficiently and economically package and distribute quality spermatozoa for the use of AI in the beef and dairy cattle industries. Many extenders exist for processing semen, and spermatozoal viability fluctuates with the extender utilized. Determination of an extender composition which optimizes spermatozoal post-thaw viability will enable semen collection facilities to process and freeze semen that will yield higher non-return rates based upon post-thaw viability of the spermatozoa.

This experiment was designed to evaluate the effects of semen type (fresh or frozen-thawed), extender type, and inherent interactions between semen type and extender type in order to provide a recommendation to the semen collection industry as to the optimum extender to utilize for the processing and freezing of bovine spermatozoa.

1.1. Hypothesis

The rank of the three extenders relative to their beneficial effects on spermatozoal viability after freezing and thawing will be egg yolk-citrate, IMV, and skim milk.

1.2. Objective

1. Compare the effects of three extenders (egg yolk-citrate, IMV, and skim milk) on the motility, morphology, and acrosomal integrity (viability) of spermatozoa during a 9-hr incubation period either before or after freezing, and

2. Determine which of the three extenders utilized will provide the highest percentage of viable spermatozoa post-thaw.

2. Materials and Methods

A 3 x 2 factorial arrangement was designed to determine which of three cryopreservation extenders (egg yolk-citrate, IMV International CSS two step extender, or skim milk) was most effective in preserving bovine spermatozoa upon freezing and thawing. The semen was treated as follows: (1) fresh semen extended in egg yolk-citrate, (2) frozen-thawed semen extended in egg yolk-citrate, (3) fresh semen extended in IMV, (4) frozen-thawed semen extended in IMV, (5) fresh semen extended in skim milk, and (6) frozen-thawed semen extended in skim milk. Each ejaculate was used in every treatment combination. Samples were diluted to yield 45 x 10^6 spermatozoa/mL in one of the three extenders at 37 ºC, within 5 min of collection. The average monthly temperatures for March, April, May, and June, 2003 were 16, 21, 26, and 27 ºC, respectively. The average percentages for relative humidity for March, April, May, and June, 2003 were 73, 68, 70, and 73 %, respectively.

2.1. Extender Preparation

2.1.1. Egg yolk-citrate

The sodium citrate buffer ($Na_3C_6H_5O_7 \cdot H_2O$) was prepared by weighing out 29 g of practical grade, granular sodium citrate (Nasco, Fort Atkinson, WI, USA) on an analytical balance and adding the salt to 1 liter of distilled water. The solution was then swirled to completely dissolve the sodium citrate crystals and stored at 4 ºC until use.

Eggs used to provide the yolk portion of the extender were purchased fresh on a weekly basis from Feather Crest Farms (Kurten, TX, USA) and stored at 4 ºC until use. Eggs were broken midway and the yolk was held in one half of the shell to allow most of the egg white to fall out and be discarded. The remaining egg white and yolk were then transferred to filter paper (Ahlstorm Corporation, Mt. Holly Springs, PA, USA) and gently rolled to further separate the egg white from the yolk. The yolk was rolled to a clean spot on the filter paper and slight pressure was applied to the yolk by folding the paper around the yolk, causing the yolk to rupture. The yolk was then collected in a graduated cylinder. Yolk membranes, discolored yolks, or yolks containing blood spots were not used for the extender.

The extender was prepared by making two fractions, A, the non-glycerol portion containing egg yolk and the sodium citrate buffer and fraction B, the glycerol portion containing egg yolk, the sodium citrate buffer, and glycerol. The egg yolk-citrate extender was prepared the night before collection and stored at 4 ºC until use. Table 6 provides a description of the volumes utilized to prepare the egg yolk-citrate extender.

Table 6*
Experiment 2: Preparation of 250 mL of the egg yolk-citrate extender.

Component	**Fraction A (nonglycerol)**	**Fraction B (glycerol)**	**Final % of 1:1 Fractions**
Sodium Citrate Buffer (2.9%)	100 mL	82.5 mL	73
Egg Yolk	25 mL	25 mL	20
Glycerol	--------	17.5 mL	7
Total Volume for Fractions A and B	125 mL	125 mL	--------

*Adapted from Mitchell and Doak (2004)

2.1.2. Skim milk

Fresh pasteurized, homogenized skim milk (approximately ½ percent fat) was utilized for this experiment. Approximately 700 mL of skim milk were heated in a metal double boiler without a lid at 92 ºC to 95 ºC for 10 min. While heating, the milk was stirred constantly to keep it from sticking to the bottom of the double boiler. The milk was removed from the heat and allowed to cool to room temperature. Once the milk was cooled, it was strained through a disposable plastic funnel lined with sterile gauze into a graduated flask to remove the film. Any boiled, strained skim milk that was not utilized in the preparation of the skim milk extender was discarded.

The extender was prepared by making two fractions, A, the non-glycerol portion containing the boiled, strained skim milk and fraction B, the glycerol portion containing the boiled, strained skim milk and glycerol. The skim milk extender was prepared the night before collection and stored at 4 ºC until use. Table 7 provides a description of the volumes utilized to prepare the skim milk extender.

Table 7*
Experiment 2: Preparation of 250 mL of the skim milk extender.

Component	**Fraction A (nonglycerol)**	**Fraction B (glycerol)**	**Final % of 1:1 Fractions**
Skim Milk	125 mL	107.5 mL	93
Glycerol	--------	17.5 mL	7
Total Volume for Fractions A and B	125 mL	125 mL	--------

*Adapted from Mitchell and Doak (2004)

2.1.3. IMV International CSS two step extender

The IMV International CSS two step extender (IMV International MN, USA) was prepared as described in chapter III. Table 1 in chapter III provides a description of the volumes utilized to prepare the IMV CSS two step extender.

2.2. Semen collection

Single ejaculates from six 18-month-old Brangus bulls were collected two times per week until a total of six ejaculates had been collected from each bull. Bulls were loaded into the holding stalls approximately 15 min prior to the start of collections. An experienced handler led a haltered steer around the collection arena, stopping abruptly to mimic the behavior of a cow in estrus, that will stand to be mated by the bull, to increase sexual stimulation of the bulls. The collector released one bull at a time into the arena to be collected in a free-style fashion via an artificial vagina, prepared as previously described [44]. Artificial vaginae were prepared the night prior to collection and stored in an incubator overnight at 38 ºC until use. An insulated jacket was used to protect semen in the collection tube against temperature shock. The bull was allowed to false mount the steer up to three times prior to collection to enhance the concentration of the ejaculate via increasing the sexual stimulation of the bull. During the false mounting procedure, contact of the penis of the bull with the teaser animal was prevented by gently diverting the bull's sheath toward the collector. When the collector determined that the bull was ready for collection, the bull was allowed to mount the steer again, however, this time the collector diverted the bull's penis into the AV for the collection of

the ejaculate. Once the bull had successfully ejaculated into the AV, the ejaculate was taken into the lab for semen processing.

2.3. Semen processing

The neat semen volume was determined by use of a 15 mL graduated plastic vial and CSS antibiotics (Tylosin; 100mg/mL Gentamyacin; 500mg/mL Linco-Spectin; 300/600 mg/mL) were added to the neat semen based upon total volume (0.02 mL antibiotics to 1 mL neat semen). Spermatozoal concentration was determined by spectrophotometric assay. Total extender volume divided into two fractions, A and B, was based upon spermatozoal concentration and volume. Fraction A was allowed to equilibrate to 37 ºC before collections began to prevent the spermatozoa from undergoing cold shock upon the combination of fraction A to the neat semen in a polystyrene tube within 5 min of collection. Prior to cooling, the partially extended spermatozoa (neat semen plus fraction A) was equally divided into three polystyrene conical tubes to facilitate handling of the samples. Tubes were identified with bull name, time entered into cold room, extender type (i.e., egg yolk-citrate, IMV, or milk), and volume of fraction A. Samples were cooled to and maintained at 4 ºC for a total of 3 hr. The semen was fully extended with the addition of the fraction B component of the extender by slowly dripping it into the samples via a plastic funnel cup with a hole punctured in the bottom of the funnel cup by a 21 ga needle. Fully extended samples were then allowed to equilibrate with the fraction B portion of the extender for 1.5 hr prior to being loaded into straws. The addition of fraction A and B to the raw sample extended the neat semen sample to a final concentration of at least 45 x 10^6

spermatozoa/mL (final volume includes permeable and non-permeable extender components and the spermatozoa).

Straws were labeled with the Minitub straw printer (Verona, WI, USA), identifying the stud facility, breed, individual bull identification code for the stud facility, bull name, private herd number, registration number, and collection date. Straws were cooled to 4 ºC to prevent the spermatozoa from warming above the cold room temperature. The fully extended semen was loaded into 0.5 mL French straws and heat sealed via the IMV MRSI straw filler (Maple Grove, MN, USA) with treatment and bull specific metal needles and disposable tubing, eliminating the possibility of cross-contamination between treatment groups and/or bulls. The semen had a 30 min equilibration time in the straws on horizontal freezing racks prior to freezing. The extended semen was frozen by suspending the straws approximately 3 cm above the liquid nitrogen in liquid nitrogen vapor for 7 min before plunging them into the liquid nitrogen. Samples were then stored in a liquid nitrogen refrigerator until the post-thaw evaluation was conducted.

2.4. Fresh, partially-extended semen and post-thaw evaluations

The fresh, partially-extended semen and the frozen-thawed semen were evaluated for progressive motility, percentage of intact acrosomes, and any morphological abnormalities using a Nikon Eclipse E600 phase-contrast microscope (Melville, NY, USA). For the fresh, partially- extended semen, a 1 mL aliquot of semen was placed in a graduated plastic vial with lid and held in a dry bath between 35 ºC and 37 ºC for the duration of the 9-hr period. For the post-thaw sample, two straws were chosen at

random and thawed together in a Cito Thaw Unit between 35 ºC and 37 ºC for 30 sec and the contents pooled in a graduated plastic vial with lid and held in a dry bath between 35 ºC and 37 ºC for the duration of the 9-hr period. The percentage of progressively motile spermatozoa was evaluated at 0 (immediate), 3, 6, and 9 hr post-collection or post-thaw and was visually estimated for gross motility (within 5 %) at a low magnification in the phase-contrast setting of the microscope by averaging several fields of view. The percentage of intact acrosomes and morphological abnormalities were evaluated between 0 and 6 hr post-collection and post-thaw. The percentage of intact acrosomes was determined by observing 100 sperm cells for the presence of an apical ridge and/or damage to the apical ridge with the use of the differential interference contrast (DIC) setting on the microscope. The percentage of primary, secondary, and tertiary morphological abnormalities were determined by counting 100 sperm cells under high magnification with the use of the phase-contrast setting of the microscope and classifying the spermatozoa as either normal or as having a primary, secondary, or tertiary abnormality. Primary abnormalities are abnormalities of the head, secondary abnormalities are abnormalities of the middle piece, and tertiary abnormalities are abnormalities of the tail [44,62] for further descriptions of spermatozoal abnormalities. The percentage of morphologically normal spermatozoa was obtained by subtracting the sum of primary, secondary, and tertiary abnormalities from 100. The percentage of progressively motile spermatozoa and of morphological characteristics was evaluated by one individual throughout the entire experiment and the percentage of spermatozoa with an intact acrosome was evaluated by one of two individuals on any given collection date.

2.5. Statistical analyses

Data retrieved from this study were analyzed using analysis of variance, and all percentage data were transformed using arcsin before analysis. The effects of bull, collection date, treatment, and two-way interactions were studied as independent variables on progressively motile sperm, normal sperm, and acrosome integrity through the General Linear Model procedure of SAS (8.2, SAS Institute, Cary, NC, USA). Least squares mean separations were performed by the PDIFF procedure of SAS (two-tailed t-tests). The experimental design was a 3 X 2 factorial arrangement with three extenders and two storage temperatures.

3. Results

Table 8 provides a summary of P-values and the R-Square value for the effect of the independent variables on percentage of progressively motile frozen-thawed spermatozoa at 0, 3, 6, and 9 hr of incubation. Table 9 provides a summary of P-values and the R-Square value for the effect of the independent variables on percentage of intact acrosomal membrane, of primary, secondary, and tertiary abnormalities, and of morphologically normal spermatozoa.

Table 8
Experiment 2: Effect of independent variables (P-values and R-square value) on percentage of progressively motile, frozen-thawed spermatozoa at 0 (M0), 3 (M3), 6 (M6), and 9 (M9) hr of incubation.

Variable	M0	M3	M6	M9
Semen	<.0001	<.0001	<.0001	NS
Extender	<.0001	<.0001	<.0001	NS
Semen x Extender	0.4993	<.0001	<.0001	NS
CDate	0.0136	0.2221	0.5472	NS
Bull	0.0045	<.0001	<.0001	NS
CDate x Bull	0.0035	0.003	0.0658	NS
CDate x Extender	0.0193	0.007	0.9016	NS
Bull x Semen	0.0007	<.0001	0.0026	NS
Bull x Extender	0.8079	0.0036	0.0171	NS
CDate x Bull x Semen	0.0005	0.0196	0.0773	NS
CDate x Semen x Extender	0.3651	0.2491	0.246	NS
Bull x Semen x Extender	0.1719	0.0498	0.2778	NS
R-Square	0.8927	0.925	0.8365	0.5982

CDate = date of ejaculate collection, where, 1 through 6 correspond to 6-13-2003, 6-17-2003, 6-20-2003, 6-24-2003, 6-27-2003, and 7-1-2003, respectively.
Semen = semen storage type as either fresh or frozen-thawed semen.
Extender = seminal extender type as either egg yolk-citrate, skim milk, or IMV.
NS = Model did not account for a significant portion of variability in the percentage of progressively motile spermatozoa at 9 hr of incubation ($P > 0.09$).

Table 9
Experiment 2: Effect of independent variables (P-values and R-square value) on percentage of intact acrosomal membrane (PIA), percentage of primary (1º), secondary (2º), and tertiary (3º) morphological abnormalities, and percentage of morphologically normal (N) spermatozoa.

Variable	PIA	1º	2º	3º	N
Semen	<.0001	0.0013	<.0001	<.0001	<.0001
Extender	<.0001	0.9823	<.0001	0.6257	<.0001
Semen x Extender	0.3234	0.3044	0.0036	0.6257	0.0771
CDate	<.0001	0.0438	<.0001	0.0059	0.0004
Bull	0.0006	<.0001	0.0079	0.316	<.0001
CDate x Bull	0.006	0.0299	0.012	0.0222	0.0127
CDate x Extender	0.2961	0.0602	0.0426	0.58	0.3307
Bull x Semen	<.0001	0.2843	0.3922	0.316	0.3506
Bull x Extender	0.9269	0.1513	0.074	0.7601	0.2853
CDate x Bull x Semen	0.1034	0.2948	0.0638	0.0222	0.0441
CDate x Semen x Extender	0.032	0.0895	0.7856	0.58	0.4594
Bull x Semen x Extender	0.333	0.1176	0.1815	0.7601	0.1014
R-Square	0.9811	0.8365	0.8947	0.8122	0.8834

CDate = date of ejaculate collection, where, 1 through 6 correspond to 6-13-2003, 6-17-2003, 6-20-2003, 6-24-2003, 6-27-2003, and 7-1-2003, respectively.
Semen = semen storage type as either fresh or frozen-thawed semen.
Extender = seminal extender type as either egg yolk-citrate, skim milk, or IMV.

3.1. Semen storage type

Mean percentage of motile spermatozoa at 0 through 6 hr post-thaw differed ($P < 0.0001$) between semen storage type (semen) prior to cryopreservation (Table 8). Mean percentage of motile spermatozoa at 9 hr post-thaw did not differ ($P > 0.09$) between semen storage types prior to cryopreservation (Table 8). Cryopreservation decreased ($P < 0.05$) spermatozoal motility at 0, 3, and 6 hr of incubation as compared to spermatozoal motility at 0, 3, and 6 hr of incubation for fresh semen (Table 10; Figure 10). Mean percentage of intact acrosomal membrane, of primary, secondary, and tertiary morphological abnormalities, and of morphologically normal spermatozoa differed ($P < 0.01$) by semen storage type (Table 9). Semen stored fresh had higher ($P < 0.05$) percentages of intact acrosomal membrane, tertiary morphological abnormalities, and morphologically normal spermatozoa (Table 11). Secondary morphological abnormalities were two-fold higher ($P < 0.05$) for frozen-thawed than for fresh semen (Table 11). There was a difference of two percentage points ($P < 0.05$) in tertiary morphological abnormalities between fresh and frozen-thawed semen (Table 11). Percentages of primary, secondary, and tertiary morphological abnormalities stored in fresh or frozen-thawed semen are graphically presented in Figure A-17.

Table 10
Experiment 2: Mean percentage of progressively motile, frozen-thawed spermatozoa at 0 (M0), 3 (M3), 6 (M6), and 9 (M9) hr of incubation by semen, by extender, and semen X extender.

Variable	M0	M3	M6	M9
Semen				
1	52[a]	43[a]	24[a]	5
2	29[b]	17[b]	10[b]	4
Pooled SEM	0.72	0.69	0.88	0.76
Extender				
1	43[a]	34[a]	17[b]	3
2	36[b]	21[b]	9[c]	4
3	43[a]	36[a]	25[a]	6
Pooled SEM	0.89	0.84	1.08	0.93
Semen x Extender				
1 x 1	54	49[a]	25[b]	4
1 x 2	48	30[b]	10[c,d]	4
1 x 3	55	50[a]	38[a]	9
2 x 1	32	18[c,d]	10[c,d]	3
2 x 2	24	13[d]	8[d]	4
2 x 3	30	21[c]	12[c]	4
Pooled SEM	1.78	1.68	2.15	1.86

[a,b,c,d] LSMeans values within a column by variable with different superscripts differ ($P < 0.05$).
Semen = semen storage type, where, 1 refers to fresh and 2 refers to frozen-thawed semen.
Extender = seminal extender type, where, 1 through 3 correspond to egg yolk-citrate, skim milk, and IMV, respectively.

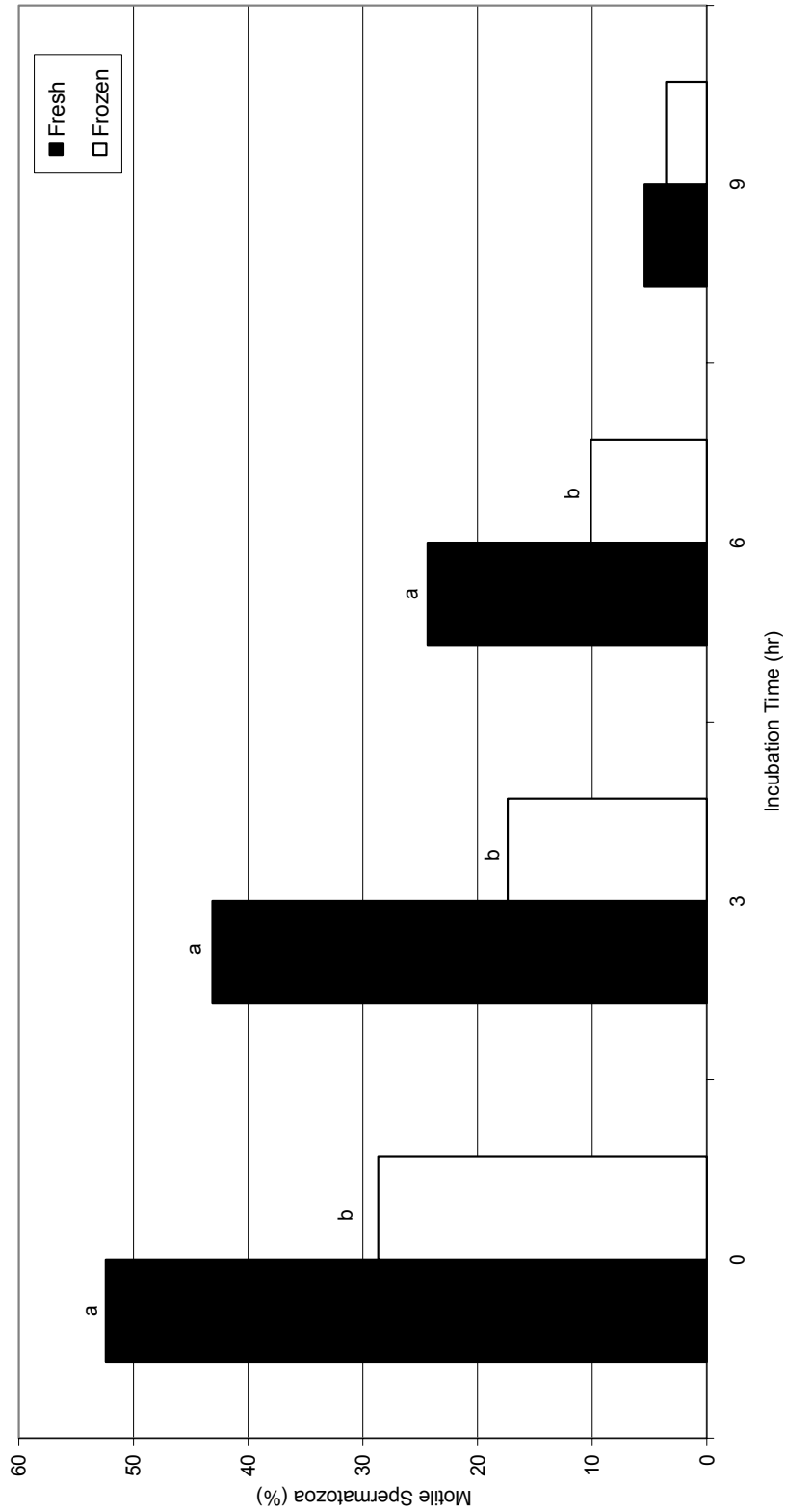

Fig. 10. Experiment 2: Mean percentage motile spermatozoa by semen storage type (fresh or frozen) at 0, 3, 6, and 9 hr of incubation. Pooled SEM is 0.72, 0.69, 0.88, and 0.76 for 0, 3, 6, and 9 hr of incubation, respectively. [a,b] LSMeans with different superscripts within incubation time differ ($P < 0.05$).

Table 11
Experiment 2: Mean percentage of intact acrosomal membrane (PIA), percentage of primary (1º), secondary (2º), and tertiary (3º) morphological abnormalities, and percentage of morphologically normal (N) spermatozoa by semen, by extender, and semen X extender.

Variable	PIA	1º	2º	3º	N
Semen					
1	92[a]	7	14[b]	2[a]	89[a]
2	27[b]	9	28[a]	0[b]	69[b]
Pooled SEM	0.81	0.30	0.62	0.13	0.95
Extender					
1	55[b]	8	18[b]	1	83[a]
3	64[a]	8	24[a]	1	75[b]
Pooled SEM	0.81	0.30	0.62	0.13	0.95
Semen x Extender					
1 x 1	88	7	13[d]	2	92
1 x 3	96	7	15[c]	2	87
2 x 1	22	9	24[b]	0	74
2 x 3	32	9	33[a]	0	64
Pooled SEM	1.15	0.43	0.87	0.19	1.34

[a,b,c,d] LSMeans values within a column by variable with different superscripts differ ($P < 0.05$).
Semen = semen storage type, where, 1 refers to fresh and 2 refers to frozen-thawed semen.
Extender = seminal extender type, where, 1and 3 correspond to egg yolk-citrate and IMV, respectively.

3.2. Extender type

Mean percentage of motile spermatozoa at 0 through 6 hr post-thaw differed ($P < 0.0001$) by extender type (extender, Table 8). Utilization of the skim milk (milk) extender as a cryoprotectant prior to freezing resulted in lower ($P < 0.05$) motility percentages after 0 and 3 hr of incubation post-thaw than the egg yolk-citrate (EC) and IMV extenders (Table 10; Figure 11). The 6 hr post-thaw had the highest ($P < 0.05$) motility percentage with the use of the IMV extender and the lowest ($P < 0.05$) motility percentage with the use of the milk extender. Mean percentage of motile spermatozoa at 9 hr post-thaw did not differ ($P > 0.09$) between extenders (Table 8). Due to the opaqueness of the semen extended in skim milk, mean percentage of intact acrosomal membrane, of primary, secondary, and tertiary abnormalities, and of morphologically normal spermatozoa were not evaluated for the milk extender throughout the experiment. Mean percentages of intact acrosomal membrane, of secondary morphological abnormalities, and of morphologically normal spermatozoa differed ($P < 0.0001$) by extender (Table 9). The EC extender had lower ($P < 0.05$) percentages for intact acrosomal membrane, and for secondary morphological abnormalities (Table 11; Figure 12). The EC extender had higher ($P < 0.05$) percentages of morphologically normal spermatozoa than the IMV extender (83 and 75 percent, respectively; Table 11). Mean percentages of primary and tertiary morphological abnormalities were not different ($P > 0.62$) by extender (Table 9).

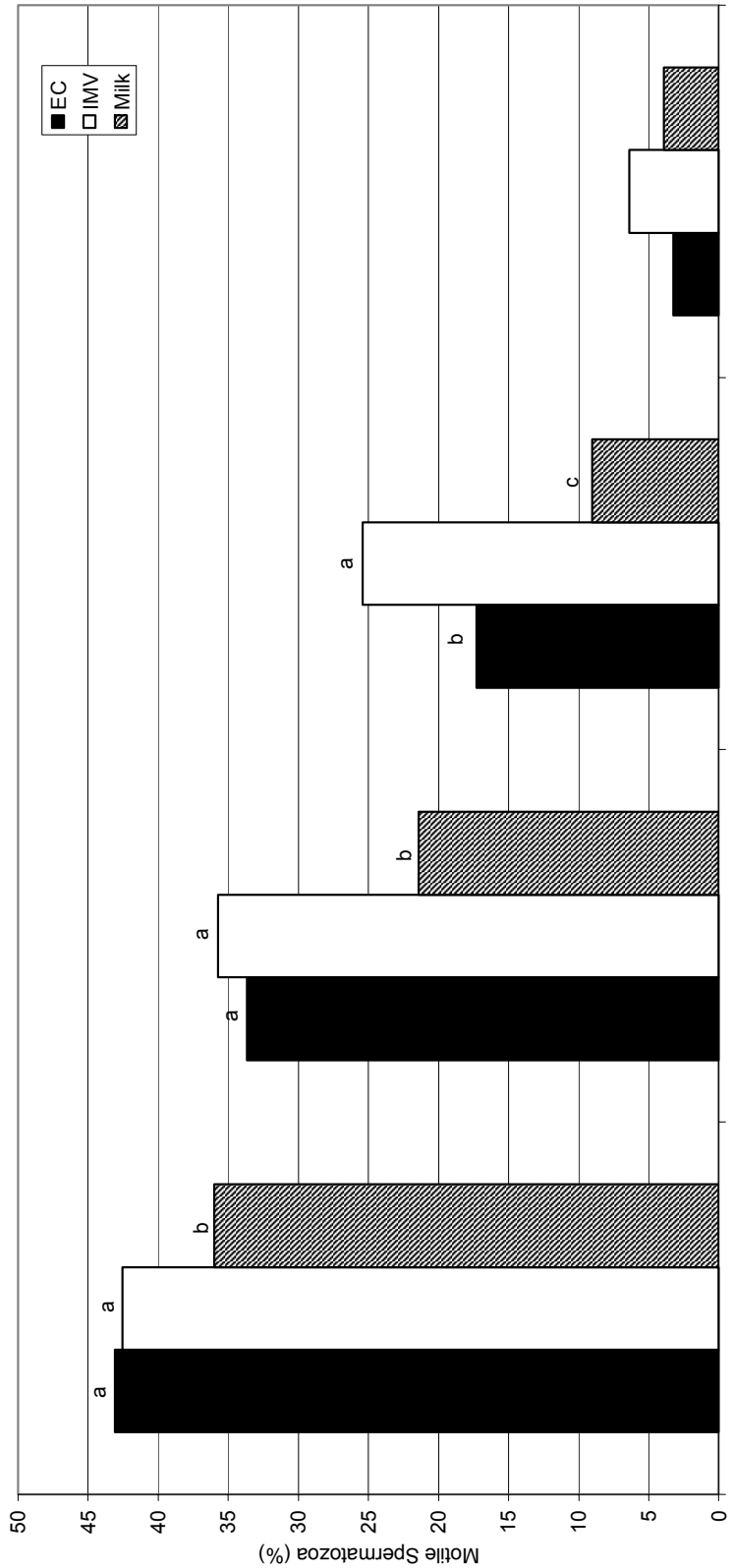

Fig. 11. Experiment 2: Mean percentage motile spermatozoa by extender (EC = egg yolk-citrate, Milk = skim milk, or IMV) at 0, 3, 6, and 9 hr of incubation. Pooled SEM is 0.89, 0.84, 1.08, and 0.93 for 0, 3, 6, and 9 hr of incubation, respectively. ^{a,b,c} LSMeans with different superscripts within incubation time differ ($P < 0.05$).

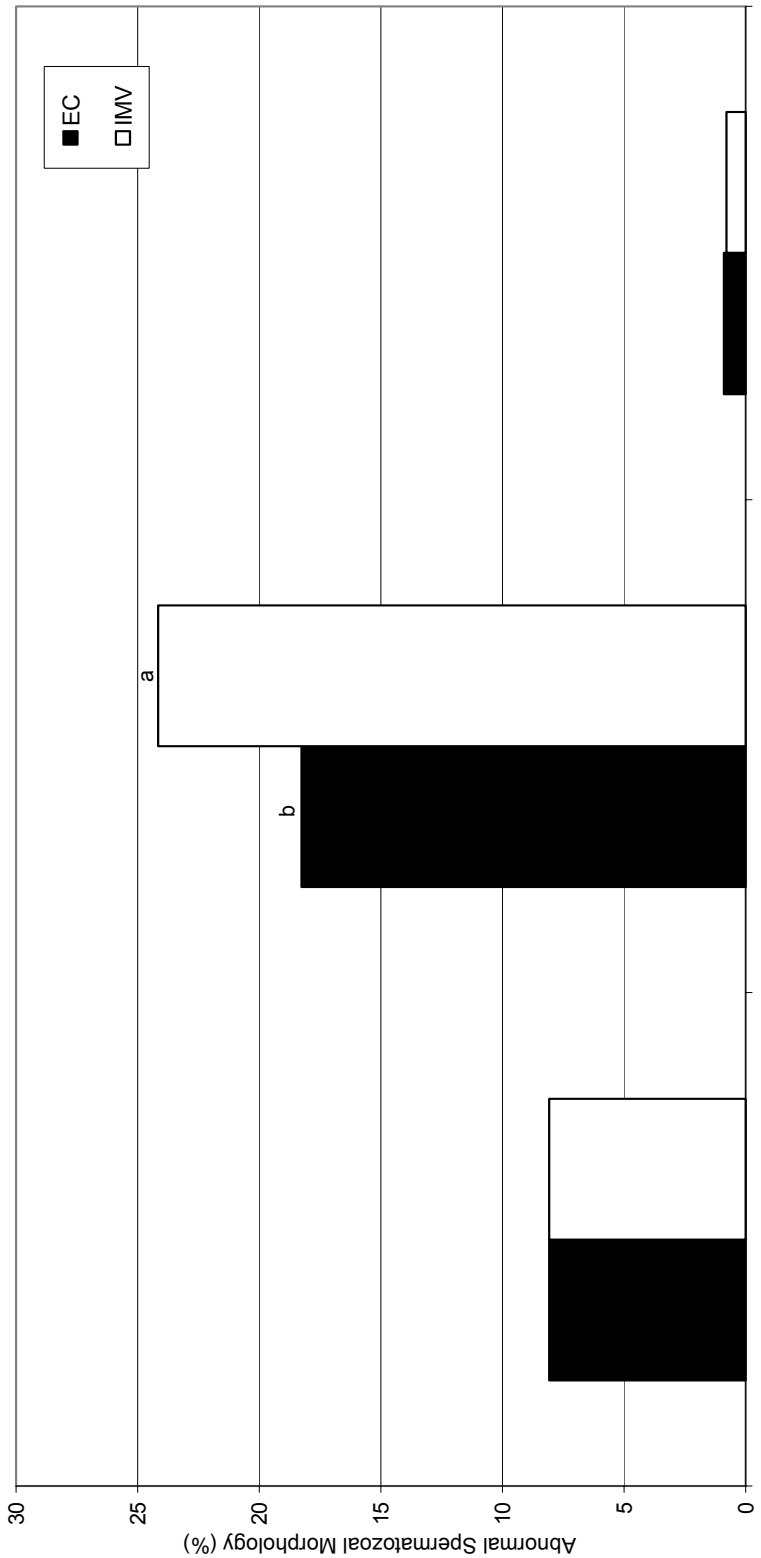

Fig. 12. Experiment 2: Mean percentage morphologically abnormal spermatozoa by extender (EC = egg yolk-citrate or IMV) for primary (1°), secondary (2°), or tertiary (3°) morphological abnormalities. Pooled SEM is 0.30, 0.62, and 0.13 for 1°, 2°, and 3°, respectively. ^{a,b} LSMeans with different superscripts within abnormality classification differ ($P < 0.05$).

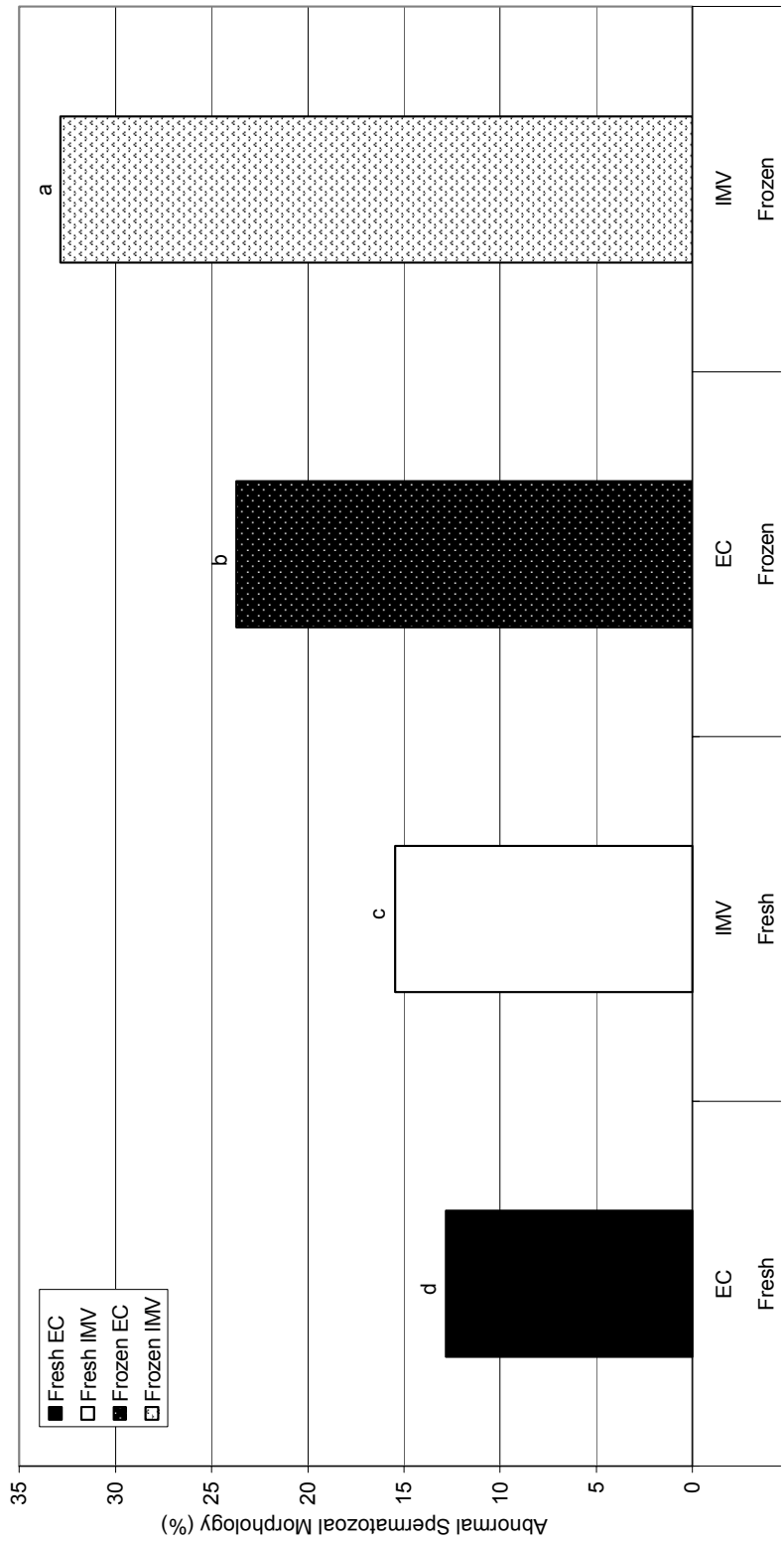

Fig. 13. Experiment 2: Mean percentage secondary morphologically abnormal spermatozoa by semen storage type (fresh or frozen) and extender (EC = egg yolk-citrate or IMV). Pooled SEM is 0.87. ^{a,b,c,d} LSMeans with different superscripts differ ($P < 0.05$).

3.3. Interaction of semen storage type X extender type

Mean percentage of motile spermatozoa at 3 and 6 hr post-thaw differed ($P < 0.0001$) due to an interaction between semen X extender (Table 8). At 3 hr post-thaw, highest ($P < 0.05$) motility was observed with the use of fresh semen and either EC or IMV as the extender, while, lowest ($P < 0.05$) motility was observed with the use of frozen-thawed semen and milk as the extender (Table 10; Figure A-18). At 6 hr post-thaw, highest ($P < 0.05$) motility was observed with the use of fresh semen and IMV as the extender, while, lowest ($P < 0.05$) motility was observed with the use of frozen-thawed semen and milk as the extender (Table 10; Figure A-18). Mean percentage of secondary morphological abnormalities differed ($P < 0.01$) due to an interaction between semen X extender (Table 9). Frozen-thawed semen extended in the IMV extender had a higher ($P < 0.05$) percentage of secondary morphological abnormalities than fresh semen extended in the EC (Table 11; Figure 13). Mean percentage of intact acrosomal membrane, of primary and tertiary morphological abnormalities, and of morphologically normal spermatozoa did not differ ($P > 0.07$) for the interaction between semen X extender (Table 9; Table 11).

3.4. Collection date

Mean percentage of motile spermatozoa at 0 hr differed ($P < 0.02$) by collection date (CDate, Table 8). Collection dates 1, 2, 5, and 6 had a higher ($P < 0.05$) percentage of motile spermatozoa at 0 hr post-thaw than CDate 3 (Table A-13; Figure A-19). Mean percentage of motile spermatozoa at 3, 6, and 9 hr did not differ ($P > 0.09$) by CDate (Table 8). Mean percentage of intact acrosomal membranes, of secondary and tertiary

morphological abnormalities, and of morphologically normal spermatozoa differed ($P < 0.01$) by CDate. The highest ($P < 0.05$) percentage of acrosomal membrane retention occurred on CDates 2 and 5, while, the lowest ($P < 0.05$) retention occurred on CDate 6 (Table A-14; Figure A-20). The highest ($P < 0.05$) percentage of secondary morphological abnormalities was observed for CDate 1 and the lowest ($P < 0.05$) percentage of secondary morphological abnormalities was observed for CDates 2 and 5 (Table A-14; Figure 14). There was a difference of two-percentage points ($P < 0.05$) in tertiary morphological abnormalities among CDates (Table A-14; Figure 14). The highest ($P < 0.05$) percentage of morphologically normal spermatozoa was observed for CDate 2 and the lowest ($P < 0.05$) percentage of morphologically normal spermatozoa was observed for CDate 1 (Table A-14; Figure 15).

3.5. Bull

Mean percentage of motile spermatozoa at 0, 3, and 6 hr post-thaw differed ($P < 0.01$) by bull (Table 8). Bull E had the highest ($P < 0.05$) percentage of motile spermatozoa at 0 hr post-thaw and bull B had the lowest ($P < 0.05$) percentage of motile spermatozoa at 0 hr post-thaw (Table A-13; Figure 16). Bulls E and F had the highest ($P < 0.05$) percentage of motile spermatozoa at 3 hr post-thaw and bulls A, B, C, and D had the lowest ($P < 0.05$) percentage of motile spermatozoa at 3 hr post-thaw (Table A-13; Figure 16). Bull E had the highest ($P < 0.05$) percentage of motile spermatozoa at 6 hr post-thaw and bull A had the lowest ($P < 0.05$) percentage of motile spermatozoa at 6 hr post-thaw (Table A-13; Figure 16). Mean percentage of motile spermatozoa at 9 hr did not differ ($P > 0.09$) by bull (Table 8).

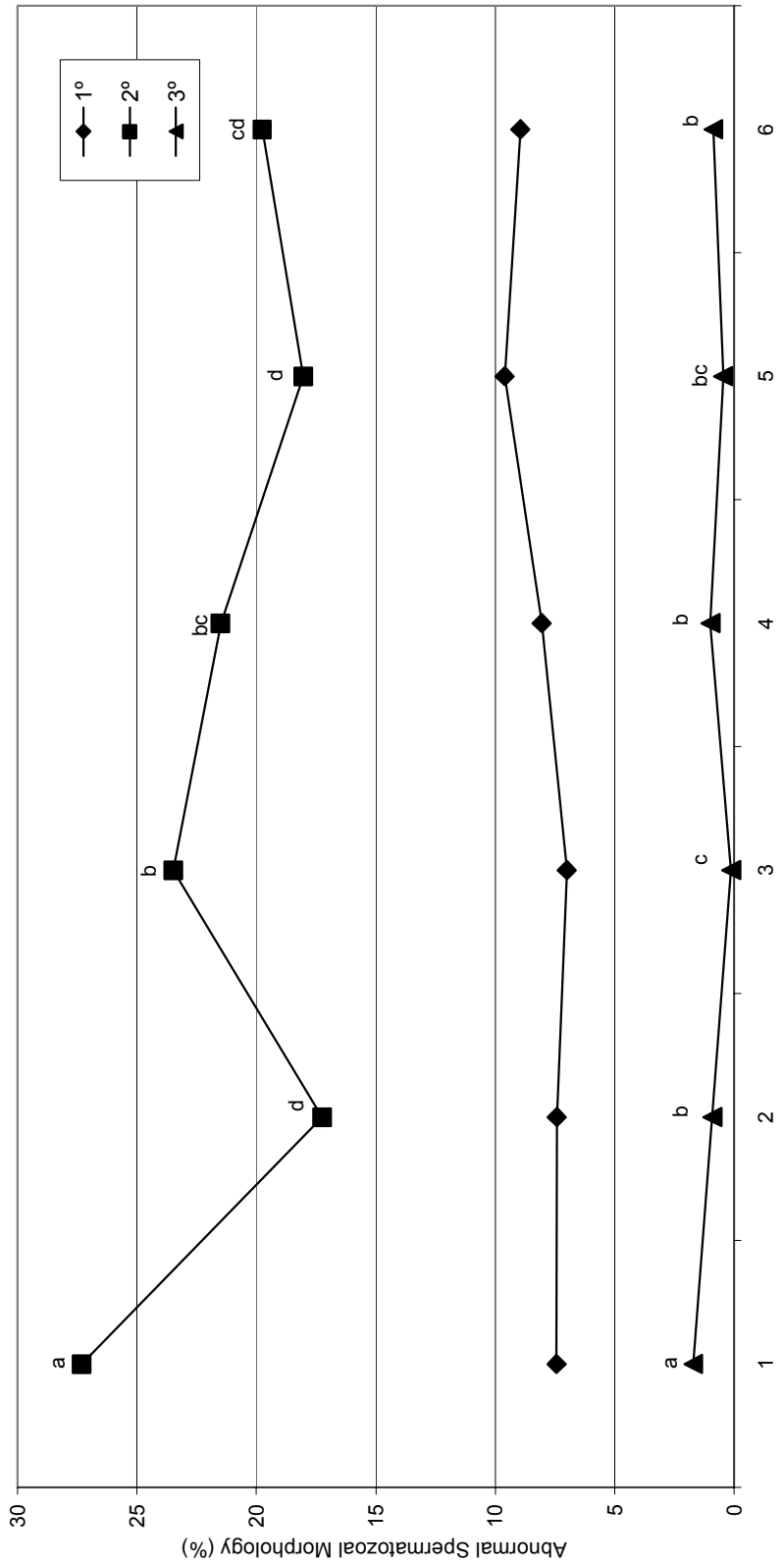

Fig. 14. Experiment 2: Mean percentage morphologically abnormal spermatozoa (1º = primary, 2º = secondary, or 3º = tertiary abnormalities) by collection date (1 to 6). Pooled SEM is 0.52, 1.07, and 0.23 for 1º, 2º, and 3º, respectively. ^{a,b,c,d} LSMeans with different superscripts within abnormality classification differ ($P < 0.05$).

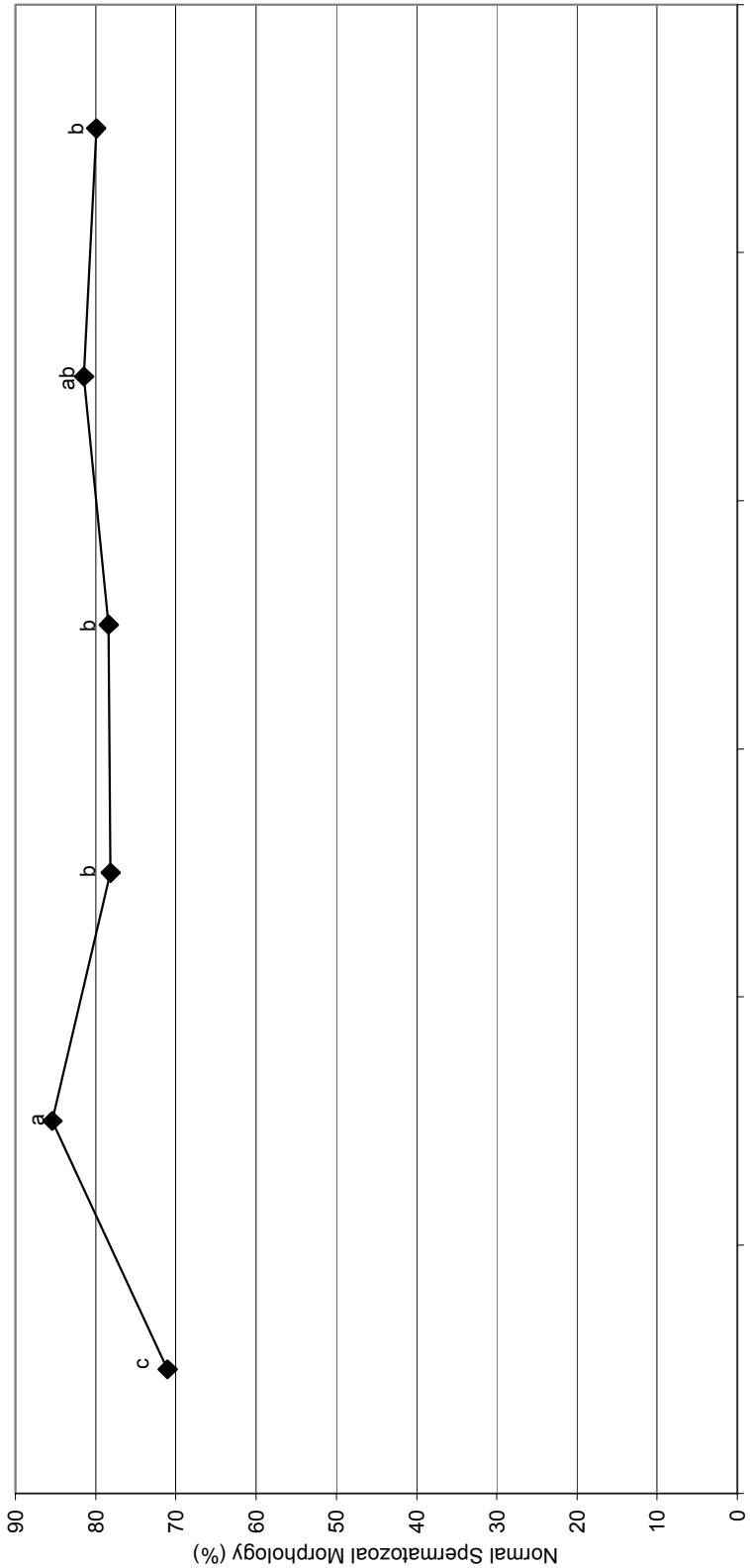

Fig. 15. Experiment 2: Mean percentage morphologically normal spermatozoa by collection date (1 to 6). Poole SEM is 1.64. ^{a,b,c} LSMeans with different superscripts differ ($P < 0.05$).

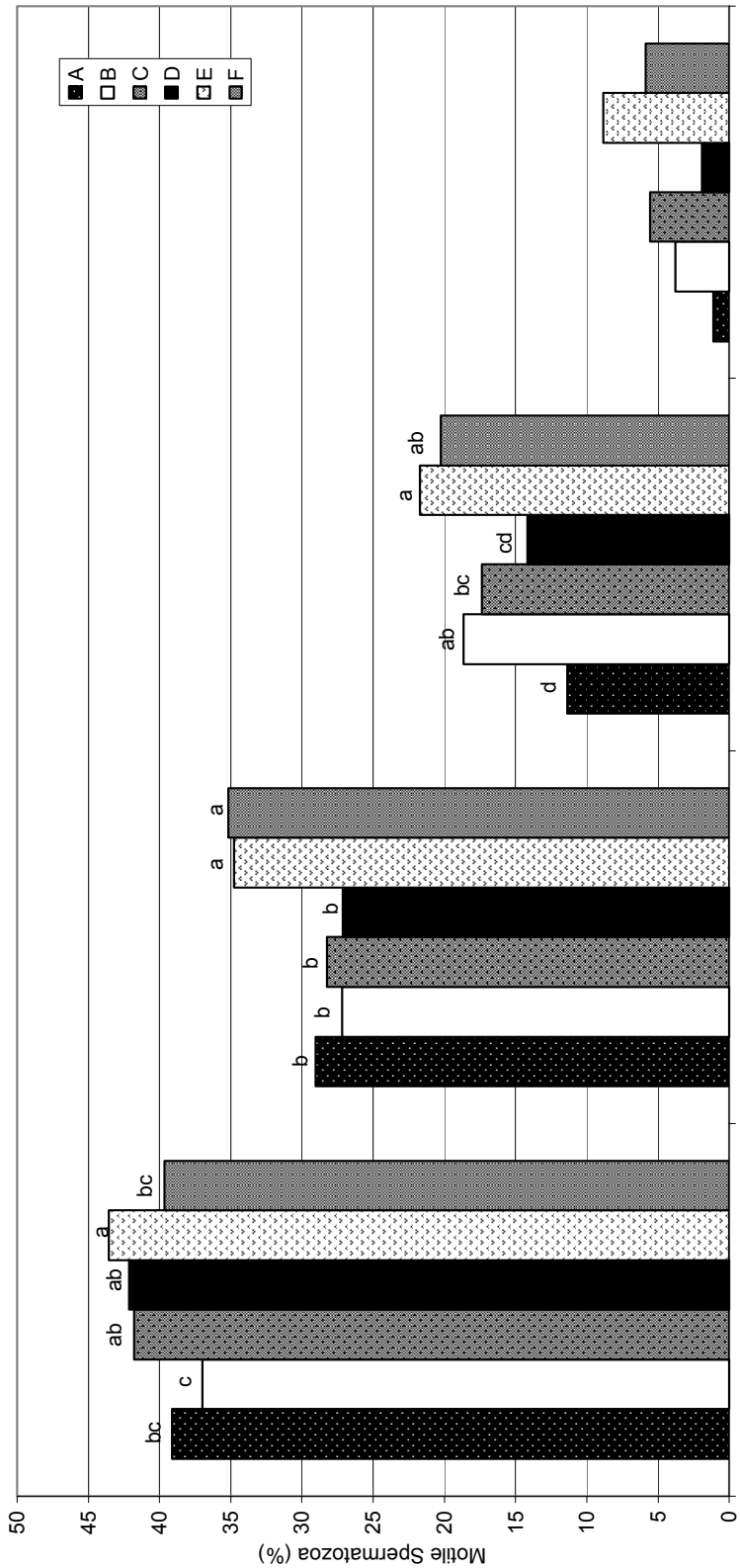

Fig. 16. Experiment 2: Mean percentage of motile spermatozoa by bull (A to F) at 0, 3, 6, and 9 hr of incubation. Pooled SEM is 1.26, 1.19, 1.52, and 1.31 for 0, 3, 6, and 9 hr of incubation, respectively. ^{a,b,c,d}LSMeans with different superscripts within incubation time differ ($P < 0.05$).

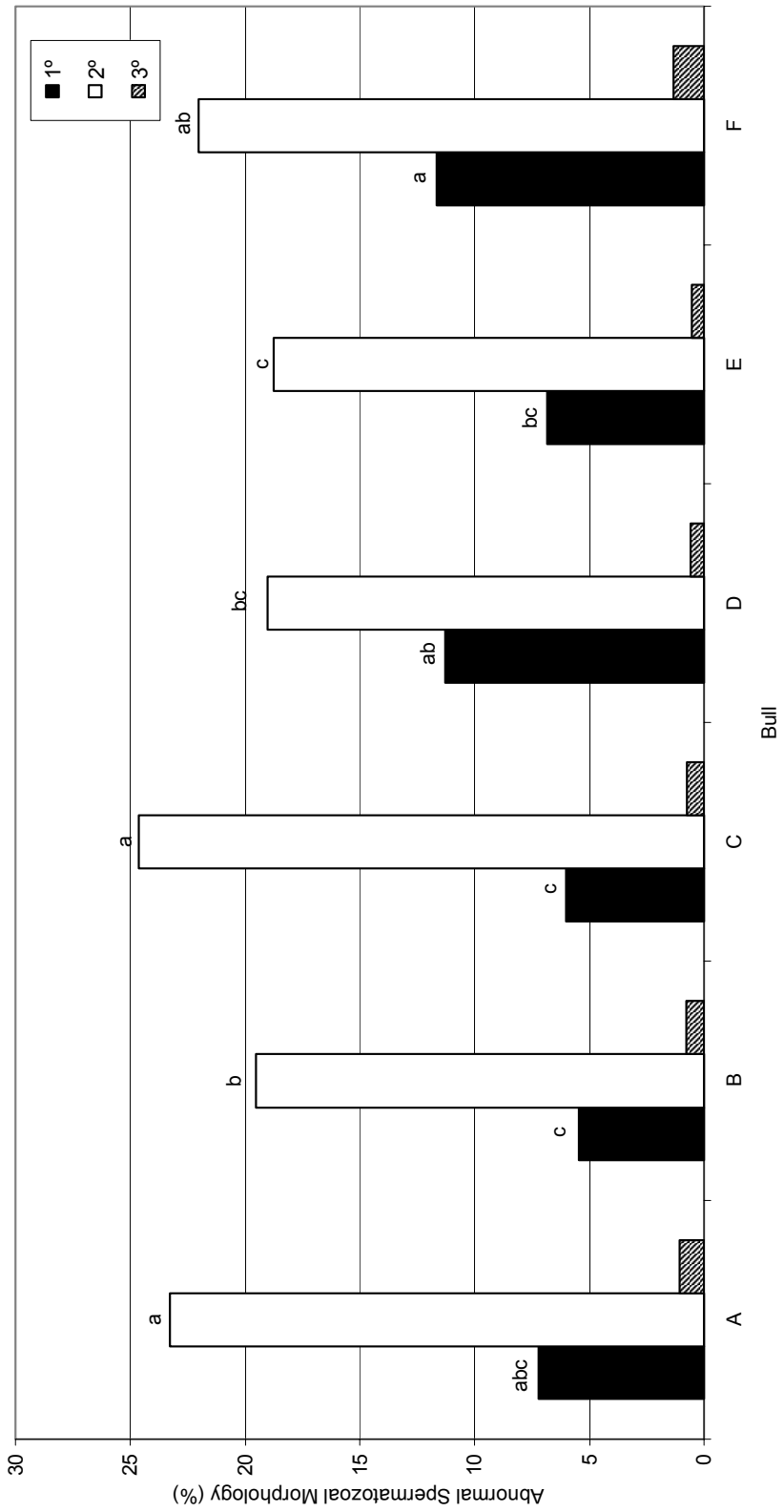

Fig. 17. Experiment 2: Mean percentage morphologically abnormal (1° = primary, 2° = secondary, or 3° = tertiary abnormalities) spermatozoa by bull (A to F). Pooled SEM is 0.52, 1.07, and 0.23 for 1°, 2°, and 3°, respectively. ^{a,b,c} LSMeans with different superscripts within abnormality classification differ ($P < 0.05$).

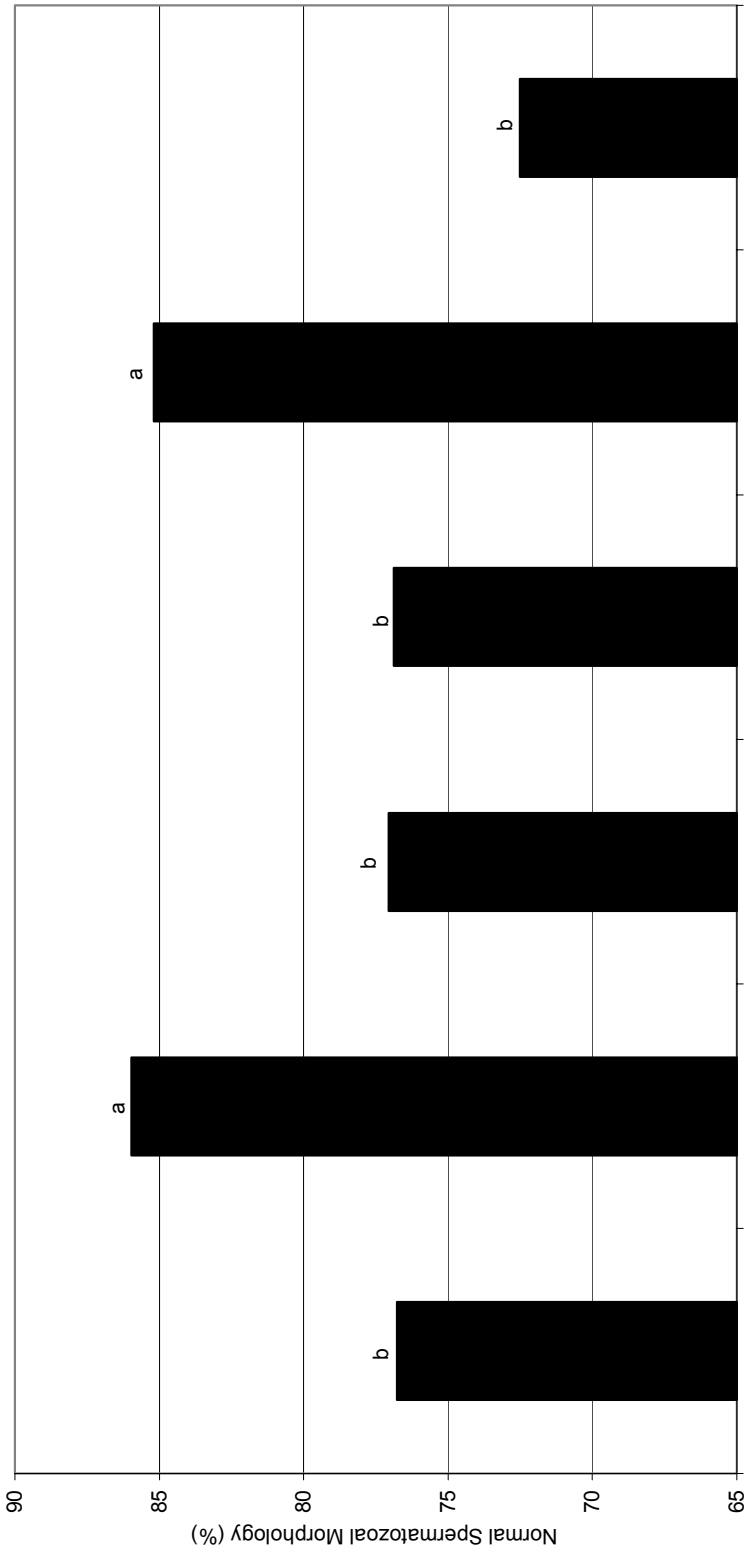

Fig. 18. Experiment 2: Mean percentage morphologically normal spermatozoa by bull (A to F). Poole SEM is 1.64. [a,b] LSMeans with different superscripts differ ($P < 0.05$).

Mean percentages of motile spermatozoa at 9 hr post-thaw are presented in Table A-13 and Figure 16. Mean percentage of intact acrosomal membrane, of primary and secondary morphological abnormalities, and of morphologically normal spermatozoa differed ($P < 0.01$) by bull (Table 9). The highest ($P < 0.05$) percentage of acrosomal membrane retention was observed for bull D while, the lowest ($P < 0.05$) percentage of acrosomal membrane retention was observed for bull A (Table A-14; Figure A-21). The highest ($P < 0.05$) percentage of primary morphological abnormalities was observed for bull F and the lowest ($P < 0.05$) percentage of primary morphological abnormalities was observed for bulls B and C (Table A-14; Figure 17). The highest ($P < 0.05$) percentage of secondary morphological abnormalities was observed for bulls A and C and the lowest ($P < 0.05$) percentage of secondary morphological abnormalities was observed for bull E (Table A-14; Figure 17). Bulls B and E were approximately ten-percentage points higher ($P < 0.05$) for morphologically normal spermatozoa than bulls A, C, D, and F (Table A-14; Figure 18).

3.6. Interaction of collection date X bull

Mean percentage of motile spermatozoa at 0 and 3 hr post-thaw differed ($P < 0.01$) due to an interaction between CDate X bull (Table 8; Figure A-22; Figure A-23). Mean percentage of motile spermatozoa at 6 and 9 hr post-thaw did not differ ($P > 0.09$) for the interaction between CDate X bull (Table 8). Percentages of motile spermatozoa for the interaction between CDate X bull at 0 through 9 hr post-thaw are presented in Table A-15. Mean percentage of intact acrosomal membrane differed ($P < 0.01$) due to an interaction between CDate X bull (Table 9; Figure A-24). Mean percentage of

primary, secondary, and tertiary morphological abnormalities differed ($P < 0.02$) due to an interaction between CDate X bull (Table 9; Figures A-25 through A-27). Mean percentage of morphologically normal spermatozoa differed ($P < 0.02$) due to an interaction between CDate X bull (Table 9; Figure A-28). Percentages of intact acrosomal membranes, of primary, secondary, and tertiary morphological abnormalities, and of morphologically normal spermatozoa for the interaction between CDate X bull are presented in Table A-16.

3.7. Interaction of collection date X extender type

Mean percentage of motile spermatozoa at 0 and 3 hr post-thaw differed ($P < 0.02$) due to an interaction between CDate X extender (Table 8; Figure A-29; Figure A-30). Mean percentage of motile spermatozoa at 6 and 9 hr post-thaw did not differ ($P > 0.09$) for the interaction between CDate X extender (Table 8). Percentages of motile spermatozoa for the interaction between CDate X extender at 0 through 9 hr post-thaw are presented in Table A-17. Mean percentage of intact acrosomal membrane, of primary and tertiary morphological abnormalities, and of morphologically normal spermatozoa did not differ ($P > 0.06$) for the interaction between CDate X extender (Table 9; Table A-18). Percentages of intact acrosomal membranes for the interaction between CDate X extender are graphically presented in Figure A-31. Mean percentage of secondary morphological abnormalities differed ($P < 0.5$) due to an interaction between CDate X extender (Table 8). Mean percentages of secondary morphological abnormalities by CDate X extender are presented in Table A-18 and Figure A-32.

3.8. Interaction of bull X semen storage type

Mean percentage of motile spermatozoa at 0, 3, and 6 hr post-thaw differ ($P < 0.01$) due to an interaction between bull X semen (Table 8). The highest ($P < 0.05$) percentage of motile spermatozoa at 0 hr post-thaw was observed for bulls A, C, and E with fresh semen and the lowest ($P < 0.05$) percentage of motile spermatozoa at 0 hr post-thaw was observed for bulls A and B with frozen-thawed semen (Table A-19; Figure 19). The highest ($P < 0.05$) percentage of motile spermatozoa at 3 hr post-thaw was observed for bulls A, E, and F with fresh semen and the lowest ($P < 0.05$) percentage of motile spermatozoa at 3 hr post-thaw was observed for bull A with frozen-thawed semen (Table A-19; Figure 20). The highest ($P < 0.05$) percentage of motile spermatozoa at 6 hr post-thaw was observed for bull E with fresh semen and the lowest ($P < 0.05$) percentage of motile spermatozoa at 6 hr post-thaw was observed for bull A with frozen-thawed semen (Table A-19; Figure 21). Mean percentage of motile spermatozoa at 9 hr post-thaw did not differ ($P > 0.09$) for the interaction between bull X semen (Table 8). Mean percentage of intact acrosomal membrane differed ($P < 0.0001$) due to an interaction between bull X semen (Table 9). The highest ($P < 0.05$) percentage of acrosomal membrane retention was observed for bull C using fresh semen and the lowest ($P < 0.05$) percentage of acrosomal retention was observed for bulls A, C, E, and F using frozen-thawed semen (Table A-20; Figure 22). Mean percentage of primary, secondary, and tertiary morphological abnormalities and of morphologically normal spermatozoa did not differ ($P > 0.28$) for the interaction between bull X semen (Table A-20).

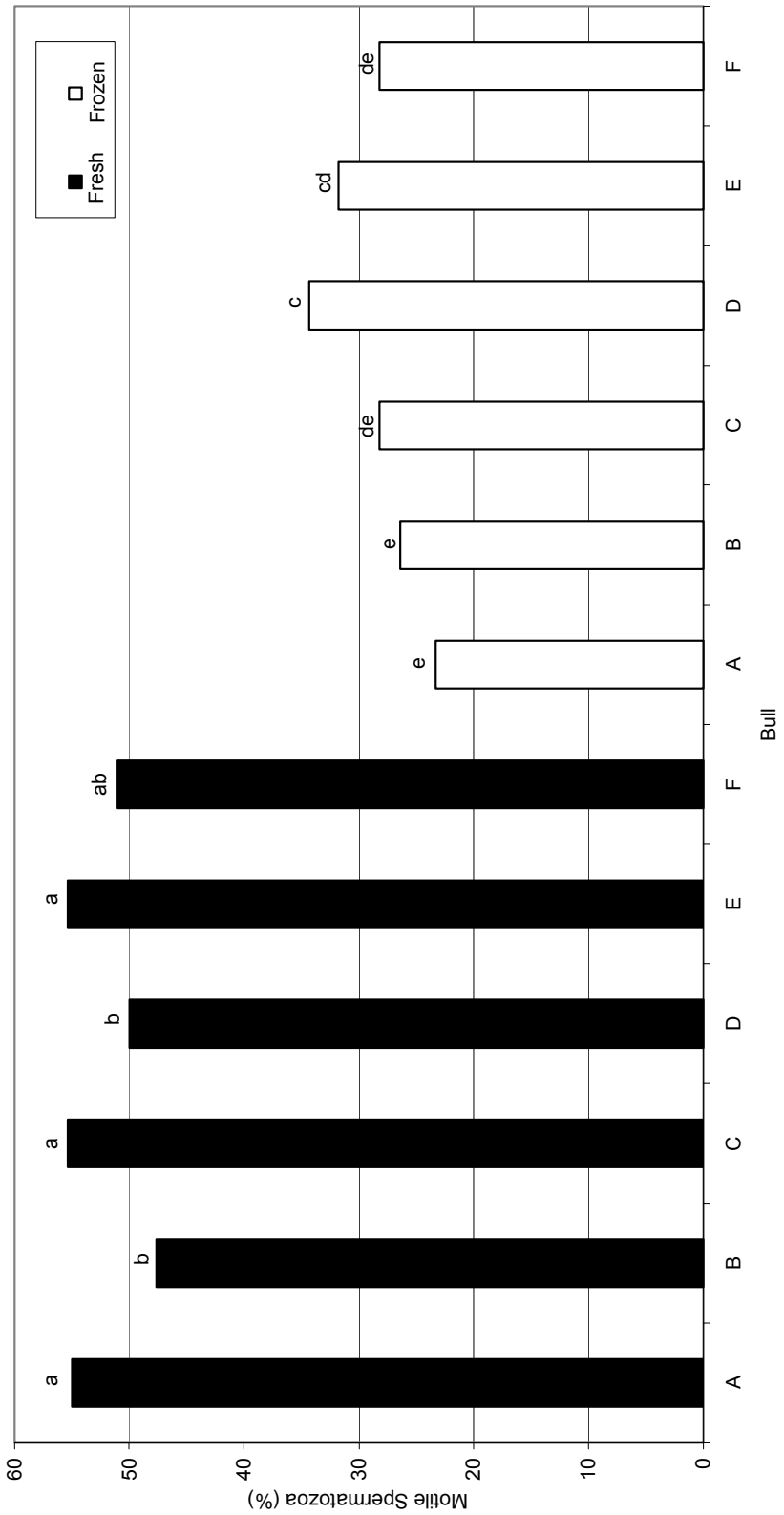

Fig. 19. Experiment 2: Mean percentage motile spermatozoa by bull (A to F) and semen storage type (fresh or frozen) at 0 hr of incubation. Pooled SEM is 1.78.
[a,b,c,d,e] LSMeans with different superscripts differ ($P < 0.05$).

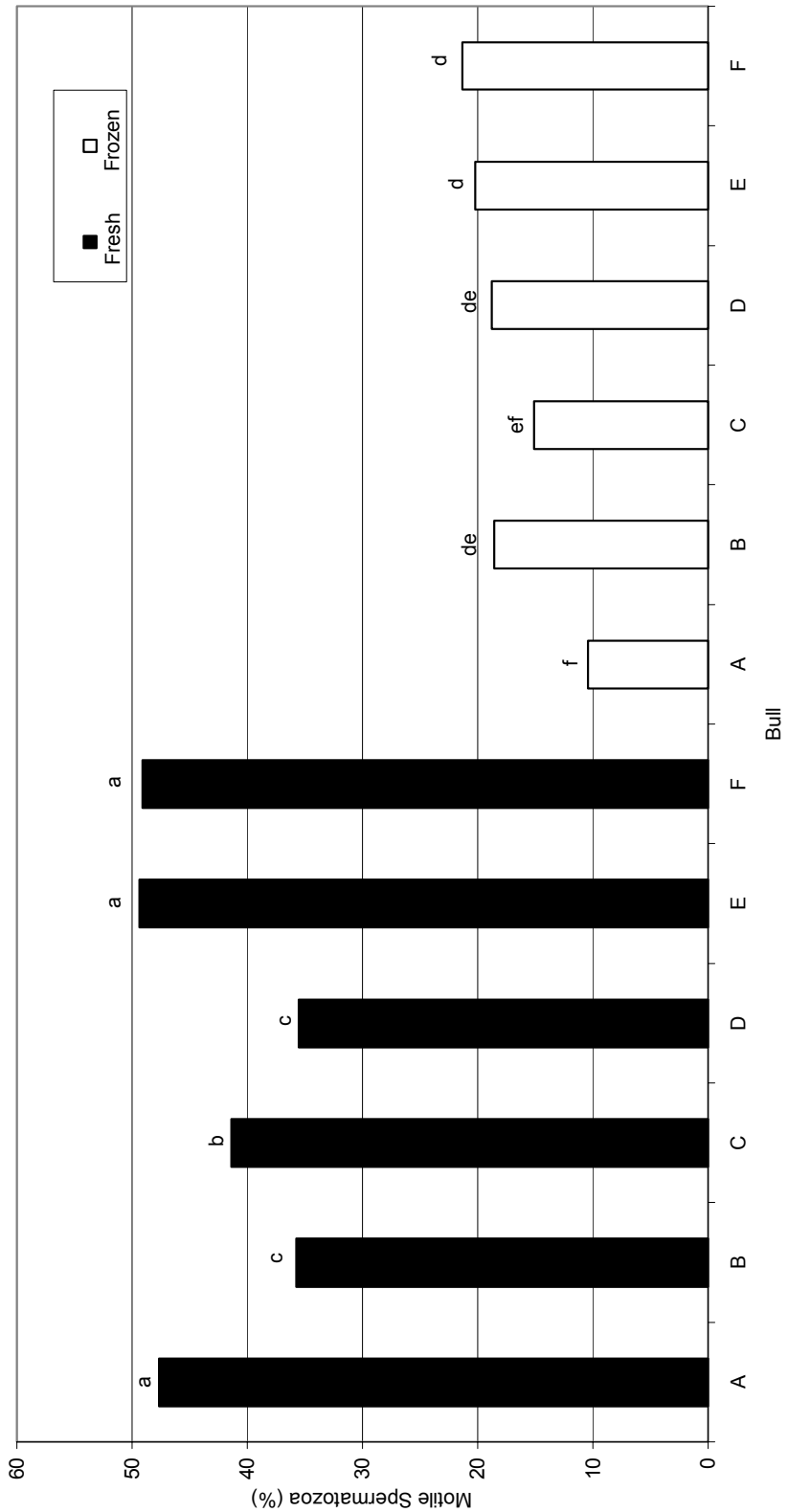

Fig. 20. Experiment 2: Mean percentage motile spermatozoa by bull (A to F) and semen storagte type (fresh or frozen) at 3 hr of incubation. Pooled SEM is 1.68.
[a,b,c,d,e,f] LSMeans with different superscripts differ ($P < 0.05$).

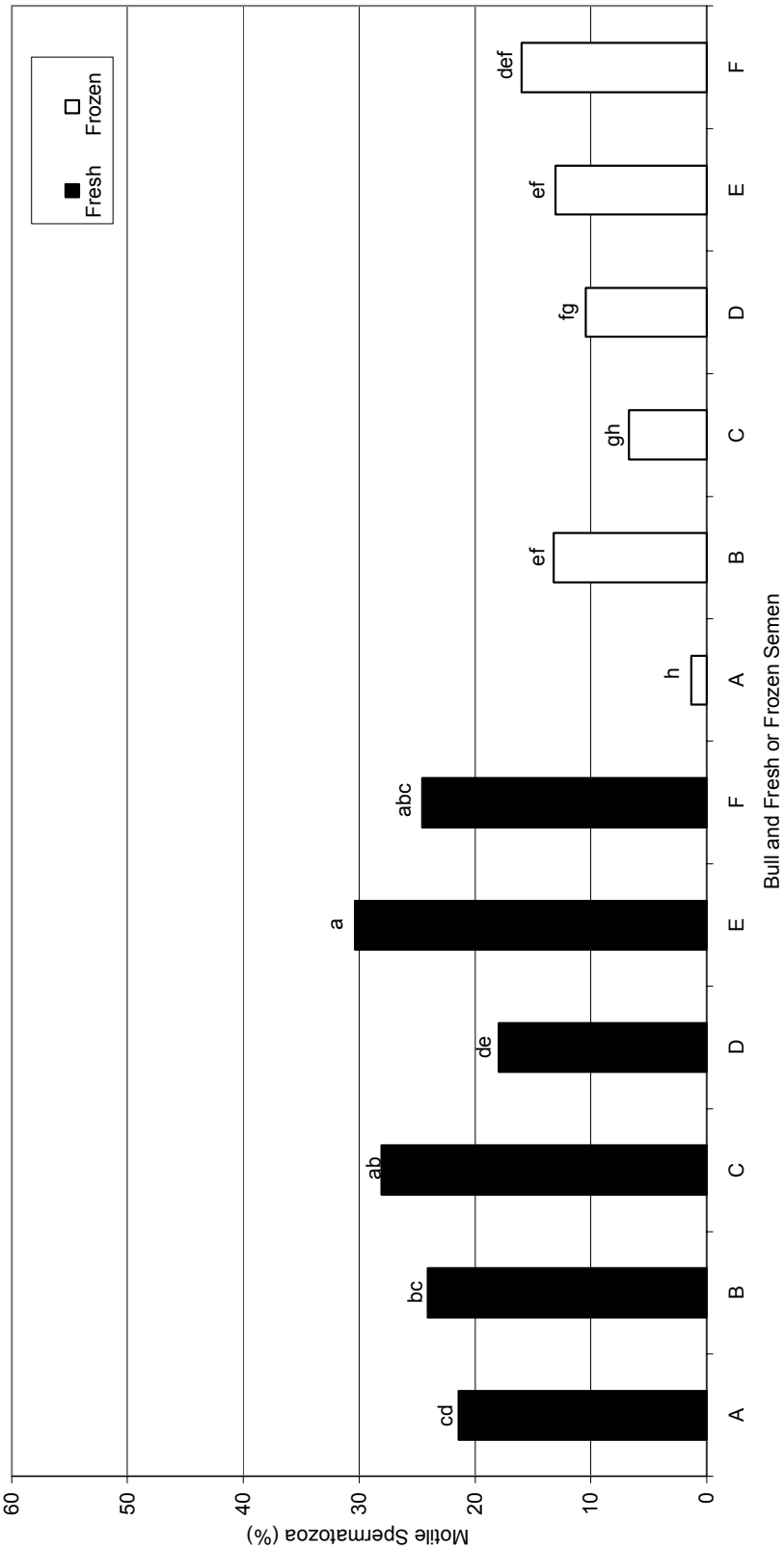

Fig. 21. Experiment 2: Mean percentage motile spermatozoa by bull (A to F) and semen storage type (fresh or frozen) at 6 hr of incubation. Pooled SEM is 2.15.
[a,b,c,d,e,f,g,h] LSMeans with different superscripts differ ($P < 0.05$).

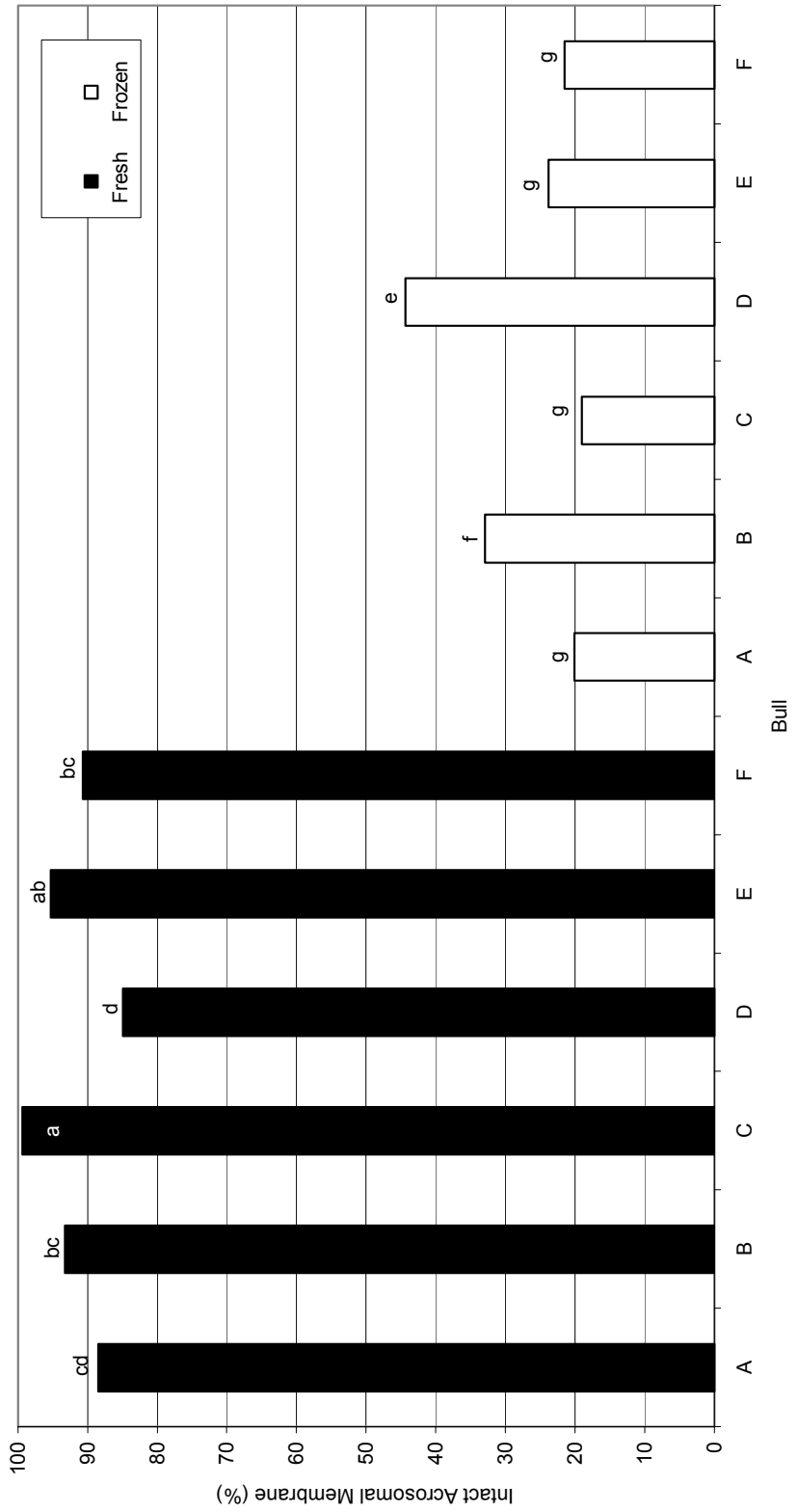

Fig. 22. Experiment 2: Mean percentage spermatozoa with an intact acrosomal membrane by bull (A to F) and semen storage type (fresh or frozen). Pooled SEM is 1.99. ^{a,b,c,d,e,f,g} LSMeans with different superscripts differ ($P < 0.05$).

3.9. Interaction of bull X extender type

Mean percentage of motile spermatozoa at 3 and 6 hr post-thaw differed ($P < 0.02$) due to an interaction between bull X extender (Table 8). The highest ($P < 0.05$) percentage of motile spermatozoa at 3 hr post-thaw was observed for bull E with the IMV extender and the lowest ($P < 0.05$) percentage of motile spermatozoa at 3 hr post-thaw was observed for bulls B and C with the milk extender (Table A-21; Figure A-33). The highest ($P < 0.05$) percentage of motile spermatozoa at 6 hr post-thaw was observed for bulls B, E, and F with the IMV extender and the lowest ($P < 0.05$) percentage of motile spermatozoa at 6 hr post-thaw was observed for bulls A and C with the milk extender (Table A-21; Figure A-34). Mean percentage of motile spermatozoa at 0 and 9 hr post-thaw did not differ ($P > 0.09$) for the interaction between bull X extender (Table 8). Mean percentage of intact acrosomal membranes, of primary, secondary, and tertiary morphological abnormalities, and of morphologically normal spermatozoa did not differ ($P > 0.07$) for the interaction between bull X extender (Table 9; Table A-22).

3.10. Interaction of Collection date X bull X semen storage type

Mean percentage of motile spermatozoa at 0 and 3 hr post-thaw differed ($P < 0.02$) due to an interaction among CDate X bull X semen (Table 8). Mean percentages for the interaction among CDate X bull X semen for 0 and 3 hr post-thaw are presented in Table A-23, Figure A-35 and Figure A-36. Mean percentage of motile spermatozoa at 6 and 9 hr post-thaw did not differ ($P > 0.07$) for the interaction among CDate X bull X semen (Table 8). Mean percentages for the interaction among CDate X bull X semen for 6 and 9 hr post-thaw are presented in Table A-23. Mean percentage of tertiary

morphological abnormalities and morphologically normal spermatozoa differed ($P < 0.05$) due to an interaction among CDate X bull X semen (Table 9). Mean percentages for the interaction among CDate X bull X semen for morphologically normal spermatozoa are presented in Table A-24 and Figure A-37. Mean percentage of intact acrosomal membranes and of primary and secondary morphological abnormalities did not differ ($P > 0.06$) for the interaction among CDate X bull X semen (Table 9). Mean percentages for the interaction among CDate X bull X semen for intact acrosomal membranes, primary, secondary, and tertiary morphological abnormalities are presented in Table A-24.

3.11. Interaction of collection date X semen storage type X extender type

Mean percentage of motile spermatozoa at 0 through 9 hr post-thaw did not differ ($P > 0.09$) for the interaction among CDate X semen X extender (Table 8). Mean percentages for the interaction among CDate X semen X extender for 0 through 9 hr post-thaw are presented in Table A-25. Mean percentage of intact acrosomal membranes differed ($P < 0.04$) due to an interaction among CDate X semen X extender (Table 9). Mean percentages for the interaction among CDate X semen X extender for intact acrosomal membranes are presented in Table A-26. Mean percentage of primary, secondary, and tertiary abnormalities and morphologically normal spermatozoa did not differ ($P > 0.08$) for the interaction among CDate X semen X extender (Table 9). Mean percentages for the interaction among CDate X semen X extender for primary, secondary, and tertiary morphological abnormalities and morphologically normal spermatozoa are presented in Table A-26.

3.12. Interaction of bull X semen storage type X extender type

Mean percentage of motile spermatozoa at 3 hr post-thaw differed ($P < 0.05$) due to an interaction among bull X semen X extender (Table 8). Mean percentages for the interaction among bull X semen X extender for 3 hr post-thaw are presented in Table A-27 and Figure A-38. Mean percentage of motile spermatozoa at 0, 6, and 9 hr post-thaw did not differ ($P > 0.09$) for the interaction among bull X semen X extender (Table 8). Mean percentages for the interaction among bull X semen X extender for 0, 6, and 9 hr post-thaw are represented in Table A-27. Mean percentage of intact acrosomal membranes, of primary, secondary, and tertiary abnormalities, and of morphologically normal spermatozoa did not differ ($P > 0.11$) for the interaction among bull X semen X extender (Table 9). Mean percentages for the interaction among bull X semen X extender for intact acrosomal membrane, primary, secondary, and tertiary morphological abnormalities, and morphologically normal spermatozoa are represented in Table A-28.

4. Discussion

4.1. Semen storage type

Extensive reviews regarding cryopreservation of mammalian spermatozoa have illustrated that spermatozoal viability is decreased upon the freezing and thawing process [1,48,74]. A reduction in viable spermatozoa of approximately 33 % is observed upon post-thaw evaluation of frozen-thawed semen [70]. Our study compared percentages of motility, intact acrosomes, and morphological characteristics of spermatozoa between fresh and frozen semen.

Fresh semen yielded higher percentages of motile spermatozoa at 0, 3, and 6 hr post-thaw and had higher percentages of intact acrosomes, tertiary abnormalities, and morphologically normal spermatozoa than frozen-thawed semen. We also observed higher percentages of secondary abnormalities in frozen-thawed semen than fresh. Bruemmer et al. [73] evaluated the effects of low temperature storage (-196 ºC) on the viability of spermatozoa based upon staining. The authors reported a decrease in spermatozoal viability of up to 45 % upon post-thaw examination. While Bruemmer et al. [73] did not evaluate percentages for spermatozoal motility, acrosomal integrity, or morphological characteristics, their research supports ours in that we both observed a decrease in spermatozoa viability upon post-thaw examination. In the current study, progressively motile sperm after 0, 3, an 6 hr of incubation were reduced by 44, 60, and 58 % for frozen-thawed compared with fresh semen.

Necrosis and apoptosis of spermatozoa are enhanced by cryopreservation. Peña et al. [75] evaluated boar ejaculates for percentage of spermatozoal motility and of live spermatozoa and the occurrence of apoptotic and necrotic spermatozoa and observed a decrease in spermatozoal motility between fresh (63 %) and frozen (40 %) semen. The authors evaluated the spermatozoa for the percentage of live, live early apoptotic, dead late apoptotic/early necrotic, and dead late necrotic cells in the sample based upon flow cytometry analysis. Cells were treated with fluorescein-labeled Annexin-V and propidium iodide and microscopically evaluated for the uptake of green and or red stain by the sperm cells. For fresh semen, Peña et al. [75] observed that 83 % of the cells were alive (no stain), 5 % were early apoptotic (green stain), 9 % were late

apoptotic/early necrotic (green and red stain), and 3 % were late necrotic (red). For frozen-thawed semen, Peña et al. [75] observed that 37 % of the cells were alive (no stain), 6 % were early apoptotic (green stain), 36 % were late apoptotic/early necrotic (green and red stain), and 21 % were late necrotic (red). The results of Peña et al. [75], clearly demonstrate a loss in spermatozoal motility and viability upon freezing and thawing. Peña et al. [75] speculated that the procedures surrounding the entire process for extending, cooling, freezing, and thawing semen induce cellular apoptosis and necrosis in frozen-thawed spermatozoa. In concurrence with the observations of Peña et al. [75], Lachaud et al. [76] also observed a decrease in spermatozoal motility, an increase in the percentage of dead cells, and an increased occurrence of necrotic and apoptotic cells after 24 hr of incubation and storage, but not cryopreservation of human sperm. The results of Lachaud et al. [76] lead the authors to speculate that the cause of increased spermatozoal death (loss of viability) and the decrease in motility are due to necrosis of the cell, but not apoptosis, because they did not observe an increase in DNA fragmentation or phosphatidylserine externalization, which would have occurred if the decreased motility and increased cell death were due to apoptosis. Furthermore, Anzar et al. [77] reported that bull semen contained 17 % apoptotic sperm in fresh semen and 31 % apoptotic sperm in frozen-thawed bovine semen. Interestingly, Anzar et al. [77] observed a decrease in the number of necrotic cells for frozen-thawed semen as compared to fresh. However, the authors reasoned that this decease in necrotic cell numbers is due to fragmentation of the spermatozoal membrane over the acrosomal region making it unable to be detected with fluorescent labeling. Anzar et al. [77] did

not evaluate spermatozoal motility in fresh or frozen semen in their study, however, it can be speculated that the motility and viability (percentage of live cells) of bull spermatozoa will also be decreased as seen in boar [75] and human [76] spermatozoa due to the increased number of apoptotic and necrotic cells post-thaw.

4.2. Extender type

Egg yolk-based extenders are utilized on a regular basis for semen processing in the beef cattle industry. However, milk-based extenders also yield acceptable post-thaw percentages for motility, intact acrosomes, morphological characteristics and non-return rates [41,78]. Our study evaluated the effect of three extenders, egg yolk-citrate (EC), IMV, and skim milk (milk) on pooled fresh and frozen-thawed percentages of spermatozoal motility, and the effect of two extenders (egg yolk-citrate and IMV) on pooled fresh and frozen-thawed percentages of morphology, and of acrosomal integrity.

Lower percentages for motility were observed for the milk extender than the EC or IMV extenders in our study. At 6 hr post-thaw, the IMV extender had the highest percentage of motile spermatozoa, however the IMV and EC extenders were not significantly different in the percentage of motile spermatozoa for the 0, 3, or 9 hr post-thaw examination. Thun et al. [13] reported that a soybean extract extender (Biociphos-Plus®) resulted in lower percentages for spermatozoal motility than the egg yolk-tris extenders packaged at 4 ºC and at room temperature. This trend follows our observation of decreased percentages for motility in the skim milk extender and increased percentages for motility in the egg yolk-based extender, even though the processing across all of our treatments was completed at 4 ºC.

Higher percentages for intact acrosomes and secondary morphological abnormalities and lower percentages of morphologically normal spermatozoa were observed for the IMV extender than the EC extender. However, no differences were observed between the EC and IMV extenders for primary and tertiary morphological abnormalities. Our observations are supported by Senger et al. [16], who reported that higher percentages of intact acrosomes were obtained with the egg yolk-tris-based extender than for egg yolk-citrate or skim milk (65, 54, and 48 %, respectively). In concurrence with our study, Thun et al. [13], evaluated the effects of Biociphos-Plus® and egg yolk-tris extenders packaged at 4 ºC and at room temperature on the percentage of morphologically normal spermatozoa and of primary and secondary morphological abnormalities. The authors observed no differences in the percentage of morphologically normal spermatozoa and of primary and secondary morphological abnormalities between the egg yolk-tris-based extenders. The Biociphos-Plus® had lower morphology percentages than the egg yolk-tris based extenders. While Thun et al. [13] evaluated spermatozoal morphological characteristics for all three extender treatments; their results demonstrate the effectiveness of utilizing an egg yolk-based extender over a soybean-based extender.

4.3. Interaction of semen storage type X extender type

Freezing and thawing cryopreserved spermatozoa for AI has a tremendous impact on spermatozoal motility and membrane integrity [75-77]. However, the type of extender also influences the viability of frozen-thawed spermatozoa upon the post-thaw evaluation [16,79]. It stands to reason, that an interaction between semen storage type

and extender type exists based on previous research that has demonstrated alterations in spermatozoa membrane integrity occur due to the freezing-thawing process. Damage to the plasma membrane may alter the means by which various extenders protect the spermatozoa, thus altering the extender effectiveness between semen that is stored at 4 ºC and semen that undergoes cryopreservation.

No differences ($P > 0.49$) were observed between fresh and frozen-thawed semen or among the extenders when the samples were evaluated at 0 hr for motility. The percentage of motile spermatozoa for the EC and IMV extenders did not differ for fresh semen; however, the IMV extender had a higher percentage of motile spermatozoa in frozen-thawed spermatozoa than the EC extender at the 3 hr post-thaw evaluation. At 6 hr of incubation at 37 ºC, both the IMV and EC extenders had a higher percentage of motile spermatozoa for both fresh and frozen-thawed spermatozoa than for the milk extender. Our findings are supported by Senger et al. [16], who demonstrated that egg yolk-tris based extenders resulted in higher percentages of motile spermatozoa upon post-thaw than egg yolk-citrate or skim milk based extenders. In concurrence with our results and those of Senger et al. [16], Schenk et al. [17] observed no significant differences among the three extenders for motility, with the exception of a higher percentage of motile spermatozoa for the egg yolk-citrate extender evaluated at 0 hr post-thaw. Previous research [80,81] on low density lipoproteins (LDL) from hen egg yolk or milk solids suggests that higher percentages of motile spermatozoa from frozen-thawed semen may be attained with the utilization of LDL in bovine seminal extenders as compared to the traditional egg yolk or milk extenders. Moussa et al. [29] reported

that higher percentages of motile spermatozoa were observed for extenders containing 5 and 10 % LDL (55 and 51 %, respectively) than for the commercial egg yolk-based TRILADYL® extender (27 %). Even though our study did not evaluate the effectiveness of substituting LDL for whole egg yolk, we speculate that extenders composed strictly of egg yolk and pH buffers, may not provide an adequate level of cryopreservation as compared to alternative extender components.

Acrosomal integrity, primary and tertiary morphological abnormalities, and morphologically normal spermatozoa, were not significant for the interaction between semen type and extender type. However, in the fresh and frozen-thawed semen, the IMV extender had a higher percentage of secondary morphological abnormalities than the EC extender. Our observation that acrosomal integrity is not influenced by extender, is supported by Schenk et al. [17], who also observed that the mean percentage of spermatozoa with an intact acrosome was not influenced by an egg yolk-citrate, egg yolk-tes-tris, or homogenized milk extender. In contrast to our study and that of Schenk et al. [17], Senger et al. [16] reported that the percentage of intact acrosomes was higher for spermatozoa frozen in an egg yolk-tris-based extender than for egg yolk-citrate or skim milk-based extenders (65, 54, and 48 %, respectively). Even though we did not analyze the frozen-thawed semen extended in skim milk for acrosomal integrity, we observed an increase of approximately 10 percentage points for intact acrosomes in the IMV-tris-based extender than in the egg yolk-citrate-based extenders as reported by Senger et al. [16].

The differences in spermatozoal post-thaw characteristics between the EC and IMV extenders could potentially be due to the effectiveness of the buffer and or the proprietary components of the IMV extender. Egg yolk-citrate and egg yolk-tris have been the primary extenders utilized for semen processing for the last 50 years. However, our results suggest that the use of a tris-based buffer may be advantageous over the citrate-based egg yolk buffer for enhancing motility and acrosomal integrity upon post-thaw evaluation. The proprietary components of the IMV extender may function to provide additional cryoprotection to the spermatozoa as the LDL fraction of egg yolk does. Semen collection facilities may wish to utilize an egg yolk based extender that contains ingredients for the enhancement of spermatozoal viability post-thaw, whether it is in the form of a pH buffer, such as tris, or alternative components to the whole egg yolk, such as the IMV concentrate or the LDL fraction of egg yolk.

Thacker and Almquist [15] demonstrated that the use of boiled skim milk and egg yolk-citrate extenders yield comparable percentages of motile spermatozoa when stored for 16 days. In contrast, our study did not obtain comparable percentages of motile spermatozoa among the extender types. This may be explained by our boiling technique or the 9 hr incubation at 37 ºC we utilized to determine motility. Thacker and Almquist [15] also observed that spermatozoa extended in the boiled skim milk moved faster than those extended in egg yolk-citrate, an observation that we also made. In our study, the egg yolk extenders appeared to be less viscous than the skim milk, opposite from what Thacker and Almquist [15] had reported, however, processing procedures for

pasteurizing skim milk may have been different than those of today, causing a discrepancy in our explanation of the faster movements in the milk extender.

Research conducted by Foote and Kaproth [41] evaluating the effects of whole milk extenders with and without the addition of fructose to the extender yielded post-thaw spermatozoal motility values approximately 10 percentage points higher than we observed at 0 hr post-thaw. Foote and Kaproth [41] reported 44 and 46 % motile spermatozoa for the whole milk and whole milk plus fructose extenders, respectively. Mitchell and Doak [44] stated that acceptable post-thaw motility ranges from 30 to 70 percent. This suggests, that milk-based extenders have the potential to attain acceptable percentages of post-thaw spermatozoal characteristics, beyond what was observed in our study. Further research is required on milk-based extenders in order to assess their value as bovine seminal extenders as compared to egg yolk-based extenders.

4.4. Collection date

Ejaculate characteristics vary from one ejaculate to the next depending on the collection frequency and the season of the year [59,82]. Environmental and management conditions surrounding the collection date have the potential to affect ejaculate characteristics, which in turn may alter the effectiveness of one seminal treatment over another. Graham et al. [4] evaluated the effect of three collection periods on the non-return rate of cows bred by AI and reported that variation existed among different collection periods and that the variation among collection periods affected the freezability and fertility. The effect of individual variation among the collection dates was significant ($P < 0.05$) for the percentage of motile spermatozoa at 0 hr incubation,

percentage of intact acrosomes, of primary, secondary, and tertiary morphological abnormalities, and of morphologically normal spermatozoa. Even though variation exists, we observed significant interactions between CDate X bull, CDate X extender, CDate X bull X semen, and CDate X semen X extender for select treatments only. It is difficult to interpret the interaction of CDate with bull, semen storage type, or seminal extender type since there were no consistent trends for any of the dependent variables.

If the collection date resulted in an ejaculate of poorer quality, the freezing-thawing process may be unduly stressful on the spermatozoa, thus reducing post-thaw viability. The interaction of the extender components with the plasma membrane may vary among collection dates as well, thus affecting the level of protection offered by the base component of egg yolk or milk and potentially enhancing or limiting the effectiveness of the base component among different collection dates. In chapter III we speculated that the variation among collection dates and collection date interactions were primarily due to the collection personnel, order of collection and seasonality. These same hypotheses hold true for this chapter.

4.5. Bull

Animal individuality influences the outcome of spermatozoal viability [70] as was demonstrated in our study. For all treatment groups, with the exception of motility at 9 hr incubation and tertiary morphological abnormalities, a high ($P < 0.008$) degree of variation among bulls was observed. However, even though a high degree of variation exists, we observed significant interactions only between bull X semen and bull X extender for motility at 0, 3, and 6 hr of incubation, and percentage of intact acrosomes

and for motility at 3 and 6 hr of incubation, respectively. The heritability of reproductive traits is one possible explanation for our observations.

The heritability of various traits, such as, ejaculate volume, spermatozoal concentration, motility and morphological characteristics may also influence the outcome of spermatozoal viability among bulls. While all of our bulls were from the same contemporary group, only two of the six were closely related (half brothers on the sire side). Mathevon et al. [24] determined of heritability estimates on volume, spermatozoal concentration, motility, total number of spermatozoa, as well as the total number of motile spermatozoa for each ejaculate. Bulls were divided into two groups, young and mature, based upon age and then heritability and repeatability were calculated from each group. Even though Mathevon et al. [24] evaluated groups of bulls, heritabilities for bulls in the young group were estimated to be 0.24, 0.52, 0.31, 0.38, and 0.49 for spermatozoal volume, concentration, motility, total number of sperm, and total motile sperm, respectively.

5. Conclusion

Freezing and thawing spermatozoa resulted in a lower percentage of motile spermatozoa and intact acrosome, and in a higher percentage of primary, secondary, and tertiary morphological abnormalities than fresh semen for all three extenders evaluated. The motility percentage in the skim milk extender was lower than for the EC and IMV extenders for incubation durations of 0 to 6 hr. Motility was not significantly different between the EC and IMV extenders until the 6 hr incubation, at which time, the IMV

extender yielded more motile spermatozoa than the EC extender. A higher percentage of intact acrosomes and secondary morphological abnormalities were observed in the IMV extender; however, the EC extender had a higher percentage of morphologically normal spermatozoa than the IMV extender. Collection date and the variation among bulls affected motility, intact acrosomes, and morphological characteristics of the spermatozoa. From our results, we conclude that the EC and IMV extenders are comparable in their effectiveness in promoting spermatozoal motility and livability post-thaw when incubated at 37 ºC for 9 hr in vitro and that the IMV extender is more conducive to acrosomal retention, while the EC extender is more efficacious in the promotion of morphologically normal spermatozoa. Compiling our observations, we recommend the use of the IMV extender over the EC or the skim milk extenders due to spermatozoal motility in combination with enhanced retention of the acrosomal membrane that is required for the completion of fertilization.

CHAPTER V

SUMMARY AND CONCLUSIONS

1. Experiment 1

The treatment combination of a 4 hr cooling duration and a 2 hr equilibration time with glycerol does not result in the optimum viability of spermatozoa after freezing and thawing. The results presented in this thesis, support the following conclusions:

1. A cooling duration of 4 hr before equilibration with glycerol resulted in the highest percentage of motile spermatozoa as assessed upon post-thaw evaluation,
2. An equilibration with glycerol of 4 or 6 hr resulted in comparable percentages of intact acrosomes and of morphologically normal spermatozoa, indicating that either 4 or 6 hr of glycerol equilibration is adequate for the retrieval of optimum acrosomal integrity and morphological characteristics as assessed upon post-thaw evaluation,
3. A cooling duration of 4 hr in combination with equilibration with glycerol for 4 hr resulted in comparable percentages of motile spermatozoa and of acrosomal integrity as compared to a cooling duration of 4 hr in combination with equilibration with glycerol for 6 hr, and
4. Significant differences exist among collection dates as well as individual bulls.

Future studies should evaluate the effects of cooling durations and equilibration times with glycerol that range between 2 and 4 hr on the percentage of spermatozoal motility, acrosomal integrity, and morphological characteristics. Future data may indicate that the duration of semen processing and freezing could be reduced from 8 hr to a total of 5 to 7 hr, which would more efficiently address the constraints of commercial semen processing.

We are not aware of any reports that specifically recommend a 4 hr cooling duration and a 4 hr equilibration time with glycerol. However, results of this study generally coincide with similar findings in regards to cooling duration and equilibration with glycerol. Upon review of the findings of the current experiment and consideration of the constraints imposed upon commercial semen collection facilities, we reject our hypothesis that the treatment combination of a 4 hr cooling duration and a 2 hr equilibration time with glycerol will result in the optimization of spermatozoal characteristics after freezing and thawing.

2. Experiment 2

The hypothesized rank of the three extenders as egg yolk-citrate, IMV, and skim milk in relevance to their beneficial effects on spermatozoal viability after freezing and thawing does not result in the correct order of rank for the optimum viability of spermatozoal characteristics after freezing and thawing. The results presented in this thesis, support the following conclusions:

1. Frozen-thawed spermatozoa had significantly lower percentages of spermatozoal motility, acrosomal integrity, and normal morphology than fresh semen for all three extender types,
2. Semen frozen in skim milk had lower percentages of spermatozoal motility than semen frozen in extenders composed of egg yolk-citrate or IMV,
3. Semen evaluated as fresh or frozen-thawed in the egg yolk-citrate and IMV extenders were comparable in their percentages for spermatozoal motility,
4. Semen evaluated as fresh in the IMV extender had higher percentages of intact acrosome than the egg yolk-citrate extender,
5. Semen evaluated as fresh in the egg yolk-citrate extender had higher percentages of morphologically normal spermatozoa than the IMV extender, and
6. Significant differences exist among collection dates as well as among bulls.

Future studies on the effects of semen type (fresh or frozen) and extender type on percentage of spermatozoal motility, acrosomal integrity, and morphological characteristics should be designed to optimize the effectiveness of egg yolk-citrate or egg yolk-tris extenders on post-thaw spermatozoa viability. The current study utilized a glycerol level of 7 % for all three extenders to eliminate the possibility of confounding the results with differing concentrations of glycerol for each of the extenders. Wiggin and Almquist [83] remarked that heated skim milk-based extenders yielded higher percentages of viable spermatozoa upon post-thaw when a 10 % level of glycerol was

employed. The effects of including low density lipoproteins with the IMV extender on post-thaw spermatozoal viability should also be evaluated.

The results of this study confirm the findings of Senger et al. [16] and Garcia and Graham [80], who reported that egg yolk-tris-based extenders, similar to the IMV extender utilized in the current study, had higher post-thaw viability than the egg yolk-citrate or skim milk extenders. We reject our hypothesis that the rank of the extenders to provide the optimal viability of spermatozoa after freezing and thawing would be egg yolk-citrate, IMV, and skim milk.

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APPENDIX A

TABLES

Table A-1
Experiment 1: Mean percentage of progressively motile, frozen-thawed spermatozoa at 0 (M0), 3 (M3), 6 (M6), and 9 (M9) hr of incubation by collection date and by bull.

Variable	M0	M3	M6	M9
CDate				
1	32	24[a]	10[b,c]	2[b]
2	31	23[a]	8[c]	2[b]
3	31	18[b]	12[a,b]	5[a]
4	32	25[a]	11[a,b]	.
5	31	24[a]	13[a,b]	5[a]
6	29	17[b]	8[c]	3[a]
Pooled SEM	0.91	1.04	0.92	0.51
Bull				
A	30[b]	16[d]	3[d]	0[d]
B	33[a]	25[b]	16[a]	5[b]
C	28[b]	21[c]	11[b]	3[c]
D	33[a]	24[b]	7[c]	0[d]
E	29[b]	18[d]	7[c]	2[c]
F	33[a]	28[a]	19[a]	10[a]
Pooled SEM	0.91	1.04	0.92	0.51

[a,b,c,d] LSMeans within a column by variable with different superscripts differ ($P < 0.05$).
CDate = date of ejaculate collection, where, 1 through 6 correspond to 5-13-2003, 5-16-2003, 5-19-2003, 5-22-2003, 5-27-2003, and 5-30-2003, respectively.
Any column containing "." represents no data entry in the data set.

Table A-2
Experiment 1: Mean percentage of intact acrosomal membrane (PIA), percentage primary (1º), secondary (2º), and tertiary (3º) abnormalities, and percentage morphologically normal (N) spermatozoa by collection date and by bull.

Variable	PIA	1º	2º	3º	N
CDate					
1	68 [a]	6 [a,b]	23 [c]	3 [a,b]	76 [b]
2	68 [a,b]	5 [c]	18 [d]	3 [a]	84 [a]
3	64 [b]	6 [b,c]	27 [b]	1 [c]	73 [b,c]
4	67 [a,b]	7 [a]	27 [b]	2 [b,c]	71 [c]
5	66 [a,b]	7 [a,b]	29 [b]	1 [c]	69 [c,d]
6	54 [c]	6 [a,b,c]	34 [a]	0 [d]	66 [d]
Pooled SEM	1.37	0.48	1.28	0.29	1.67
Bull					
A	61 [c]	4 [c]	28 [a,b]	2 [a,b]	72 [b]
B	76 [a]	4 [c]	19 [d]	2 [b,c]	86 [a]
C	61 [c]	3 [c]	31 [a]	1 [d]	73 [b]
D	70 [b]	7 [b]	24 [c]	1 [c,d]	75 [b]
E	62 [c]	10 [a]	27 [b,c]	3 [a]	66 [c]
F	57 [d]	8 [b]	29 [a,b]	1 [b,c,d]	67 [c]
Pooled SEM	1.37	0.48	1.28	0.29	1.67

[a,b,c,d] LSMeans within a column by variable with different superscripts differ ($P < 0.05$).
CDate = date of ejaculate collection, where, 1 through 6 correspond to 5-13-2003, 5-16-2003, 5-19-2003, 5-22-2003, 5-27-2003, and 5-30-2003, respectively.

Table A-3
Experiment 1: Mean percentage of progressively motile, frozen-thawed spermatozoa at 0 (M0), 3 (M3), 6 (M6), and 9 (M9) hr of incubation for collection date X bull.

CDate x Bull	M0	M3	M6	M9
1 x A	31	13	2	0
1 x B	33	30	21	9
1 x C	29	26	11	0
1 x D	31	24	9	0
1 x E	33	18	1	0
1 x F	36	33	19	2
2 x A	34	17	2	0
2 x B	31	26	18	5
2 x C	29	24	5	1
2 x D	33	31	5	1
2 x E	33	19	8	1
2 x F	25	23	13	6
3 x A	33	9	4	0
3 x B	27	18	11	5
3 x C	30	18	16	10
3 x D	36	20	8	0
3 x E	28	21	16	8
3 x F	31	23	18	7
4 x A	31	23	2	.
4 x B	40	32	17	.
4 x C	26	24	16	.
4 x D	31	24	6	.
4 x E	31	18	5	.
4 x F	31	30	20	.
5 x A	27	24	10	2
5 x B	32	23	19	3
5 x C	30	19	10	5
5 x D	38	28	7	0
5 x E	22	13	8	0
5 x F	38	34	25	18

Table A-3
Continued,

CDate x Bull	M0	M3	M6	M9
6 x A	22	11	0	0
6 x B	33	19	12	5
6 x C	27	13	7	0
6 x D	28	17	6	0
6 x E	27	17	8	0
6 x F	36	28	18	15
Pooled SEM	2.24	2.54	2.26	1.25

CDate = date of ejaculate collection, where, 1 through 6 correspond to 5-13-2003, 5-16-2003, 5-19-2003, 5-22-2003, 5-27-2003, and 5-30-2003, respectively.
Any column containing "." represents no data entry in the data set.

Table A-4
Experiment 1: Mean percentage of intact acrosomal membrane (PIA), percentage primary (1º), secondary (2º), and tertiary (3º) abnormalities, and percentage morphologically normal (N) spermatozoa for collection date X bull.

CDate x Bull	PIA	1º	2º	3º	N
1 x A	71	3	30	5	68
1 x B	89	5	20	3	81
1 x C	59	3	28	2	75
1 x D	73	8	24	2	73
1 x E	67	8	19	2	83
1 x F	49	10	18	2	77
2 x A	62	2	19	3	87
2 x B	87	4	9	3	99
2 x C	65	3	21	1	88
2 x D	65	8	16	2	85
2 x E	77	2	21	7	77
2 x F	49	11	23	2	70
3 x A	58	5	30	1	69
3 x B	70	3	19	1	89
3 x C	62	2	32	1	72
3 x D	67	5	21	1	83
3 x E	65	12	28	1	63
3 x F	61	7	34	2	61
4 x A	71	5	27	2	73
4 x B	75	4	17	3	88
4 x C	61	4	33	1	70
4 x D	64	7	26	1	74
4 x E	67	18	23	4	60
4 x F	64	7	36	1	62
5 x A	65	7	24	1	74
5 x B	67	4	24	2	79
5 x C	65	3	38	0	64
5 x D	76	9	23	0	76
5 x E	61	12	30	4	58
5 x F	61	7	35	1	61

Table A-4
Continued,

CDate x Bull	PIA	1º	2º	3º	N
6 x A	41	4	38	1	63
6 x B	68	6	23	0	79
6 x C	55	4	36	0	66
6 x D	72	9	34	1	61
6 x E	34	6	43	0	55
6 x F	56	7	30	0	69
Pooled SEM	3.35	1.17	3.14	0.71	4.08

CDate = date of ejaculate collection, where, 1 through 6 correspond to 5-13-2003, 5-16-2003, 5-19-2003, 5-22-2003, 5-27-2003, and 5-30-2003, respectively.

Table A-5
Experiment 1: Percentage of progressively motile, frozen-thawed spermatozoa at 0 (M0), 3 (M3), 6 (M6), and 9 (M9) hr of incubation for collection date X cooling duration.

CDate x CD	M0	M3	M6	M9
1 x 2	31	23	9	2[b]
1 x 4	33	24	12	2[b]
2 x 2	31	23	7	2[b]
2 x 4	31	24	10	2[b]
3 x 2	30	18	10	3[b]
3 x 4	31	19	14	7[a]
4 x 2	29	22	9	.
4 x 4	34	28	13	.
5 x 2	29	22	11	3[b]
5 x 4	33	25	15	6[a]
6 x 2	28	15	8	3[b]
6 x 4	29	20	9	3[b]
Pooled SEM	1.29	1.47	1.30	0.72

[a,b] LSMeans within a column by variable with different superscripts differ ($P < 0.05$).
CDate = date of ejaculate collection, where, 1 through 6 correspond to 5-13-2003, 5-16-2003, 5-19-2003, 5-22-2003, 5-27-2003, and 5-30-2003, respectively.
CD = cooling duration at 4ºC, with 2 representing a 2 hr cooling duration and 4 representing a 4 hr cooling duration.
Any column containing "." represents no data entry in the data set.

Table A-6
Experiment 1: Mean percentage of intact acrosomal membrane (PIA), percentage primary (1º), secondary (2º), and tertiary (3º) abnormalities, and percentage morphologically normal (N) spermatozoa for collection date X cooling duration.

CDate x CD	PIA	1º	2º	3º	N
1 x 2	68	6	24	3	75
1 x 4	68	6	22	2	78
2 x 2	64	5	18	3	83
2 x 4	71	5	18	3	86
3 x 2	63	5	28	2	73
3 x 4	64	6	27	1	73
4 x 2	68	6	29	2	70
4 x 4	67	8	25	2	72
5 x 2	67	7	29	1	69
5 x 4	65	7	30	1	68
6 x 2	55	6	34	0	66
6 x 4	53	6	34	0	65
Pooled SEM	1.94	0.67	1.81	0.41	2.36

CDate = date of ejaculate collection, where, 1 through 6 correspond to 5-13-2003, 5-16-2003, 5-19-2003, 5-22-2003, 5-27-2003, and 5-30-2003, respectively.
CD = cooling duration at 4ºC, with 2 representing a 2 hr cooling duration and 4 representing a 4 hr cooling duration.

Table A-7
Experiment 1: Mean percentage of progressively motile, frozen-thawed spermatozoa at 0 (M0), 3 (M3), 6 (M6), and 9 (M9) hr of incubation for collection date X equilibration with glycerol.

CDate x EG	M0	M3	M6	M9
1 x 2	31	23	12	1
1 x 4	32	24	8	2
1 x 6	34	25	11	2
2 x 2	28	22	6	2
2 x 4	33	22	9	3
2 x 6	31	25	10	2
3 x 2	30	17	10	3
3 x 4	31	20	14	6
3 x 6	31	18	12	6
4 x 2	31	24	10	.
4 x 4	29	25	13	.
4 x 6	35	27	10	.
5 x 2	31	24	12	5
5 x 4	29	22	12	4
5 x 6	33	25	16	5
6 x 2	29	17	7	3
6 x 4	29	19	7	3
6 x 6	28	16	10	4
Pooled SEM	1.58	1.80	1.60	0.88

CDate = date of ejaculate collection, where, 1 through 6 correspond to 5-13-2003, 5-16-2003, 5-19-2003, 5-22-2003, 5-27-2003, and 5-30-2003, respectively.
EG = equilibration with glycerol at 4ºC, with 2, 4, and 6 representing 2 hr, 4hr, and 6hr equilibration times with glycerol, respectively.
Any column containing "." represents no data entry in the data set.

Table A-8
Experiment 1: Mean percentage of intact acrosomal membrane (PIA), percentage primary (1º), secondary (2º), and tertiary (3º) abnormalities, and percentage morphologically normal (N) spermatozoa for collection date X equilibration with glycerol.

CDate x EG	PIA	1º	2º	3º	N
1 x 2	69	6	23	2	77
1 x 4	69	6	23	3	77
1 x 6	65	7	24	3	75
2 x 2	64	4	17	3	85
2 x 4	67	5	21	3	81
2 x 6	72	5	16	3	87
3 x 2	59	6	26	1	74
3 x 4	63	6	29	1	71
3 x 6	69	5	27	1	74
4 x 2	64	7	28	2	70
4 x 4	67	7	30	2	68
4 x 6	71	7	23	2	75
5 x 2	65	7	29	1	69
5 x 4	65	7	30	1	67
5 x 6	68	6	28	1	70
6 x 2	51	8	33	0	65
6 x 4	56	5	37	0	63
6 x 6	55	5	32	1	68
Pooled SEM	2.37	0.83	2.22	0.50	2.88

CDate = date of ejaculate collection, where, 1 through 6 correspond to 5-13-2003, 5-16-2003, 5-19-2003, 5-22-2003, 5-27-2003, and 5-30-2003, respectively.
EG = equilibration with glycerol at 4ºC, with 2, 4, and 6 representing 2 hr, 4hr, and 6hr equilibration times with glycerol, respectively.

Table A-9
Experiment 1: Mean percentage of progressively motile, frozen-thawed spermatozoa at 0 (M0), 3 (M3), 6 (M6), and 9 (M9) hr of incubation for bull X cooling duration.

Bull x CD	M0	M3	M6	M9
A x 2	28	14	2	0[e]
A x 4	31	18	4	0[e]
B x 2	32	24	16	3[c]
B x 4	33	26	17	7[b]
C x 2	27	19	8	3[c,d]
C x 4	30	22	14	4[c]
D x 2	33	24	6	0[e]
D x 4	33	23	8	0[e]
E x 2	26	15	5	1[d,e]
E x 4	31	20	9	3[c]
F x 2	32	28	16	10[a]
F x 4	33	29	21	10[a]
Pooled SEM	1.29	1.47	1.30	0.72

[a,b,c,d,e] LSMeans within a column by variable with different superscripts differ ($P < 0.05$).
CD = cooling duration at 4ºC, with 2 representing a 2 hr cooling duration and 4 representing a 4 hr cooling duration.

Table A-10
Experiment 1: Mean percentage of intact acrosomal membrane (PIA), percentage primary (1º), secondary (2º), and tertiary (3º) abnormalities, and percentage morphologically normal (N) spermatozoa for bull X cooling duration.

Bull x CD	PIA	1º	2º	3º	N
A x 2	63	4	29	3	69
A x 4	60	4	27	2	75
B x 2	75	4	20	2	86
B x 4	77	5	18	2	86
C x 2	60	3	30	1	73
C x 4	62	3	33	1	72
D x 2	70	7	26	1	72
D x 4	69	7	21	1	79
E x 2	62	9	28	3	66
E x 4	62	11	27	3	66
F x 2	55	8	29	1	68
F x 4	58	8	30	2	66
Pooled SEM	1.94	0.67	1.81	0.41	2.36

CD = cooling duration at 4ºC, with 2 representing a 2 hr cooling duration and 4 representing a 4 hr cooling duration.

Table A-11
Experiment 1: Mean percentage of progressively motile, frozen-thawed Spermatozoa at 0 (M0), 3 (M3), 6 (M6), and 9 (M9) hr of incubation for bull X equilibration with glycerol.

Bull x EG	M0	M3	M6	M9
A x 2	28	14	3	0
A x 4	32	18	4	0
A x 6	29	16	3	1
B x 2	32	25	14	3
B x 4	33	23	16	6
B x 6	33	27	18	6
C x 2	27	18	11	3
C x 4	28	21	10	3
C x 6	30	23	11	4
D x 2	32	23	5	0
D x 4	33	24	7	0
D x 6	34	24	8	0
E x 2	26	17	6	2
E x 4	29	19	7	3
E x 6	32	16	9	2
F x 2	34	28	17	8
F x 4	30	26	18	9
F x 6	35	31	20	11
Pooled SEM	1.58	1.80	1.60	0.88

EG = equilibration with glycerol at 4ºC, with 2, 4, and 6 representing 2 hr, 4hr, and 6hr equilibration times with glycerol, respectively.

Table A-12
Experiment 1: Mean percentage of intact acrosomal membrane (PIA), percentage primary (1º), secondary (2º), and tertiary (3º) abnormalities, and percentage morphologically normal (N) spermatozoa for bull X equilibration with glycerol.

Bull x EG	PIA	1º	2º	3º	N
A x 2	58	4	28	2	74
A x 4	63	5	29	2	70
A x 6	63	5	27	3	73
B x 2	76	4	19	2	86
B x 4	74	4	19	1	87
B x 6	78	5	18	2	85
C x 2	59	3	30	0	75
C x 4	61	3	35	1	68
C x 6	63	3	30	1	75
D x 2	70	8	24	1	74
D x 4	66	6	24	1	77
D x 6	73	7	24	1	75
E x 2	58	10	26	2	66
E x 4	64	10	29	3	63
E x 6	64	8	27	3	69
F x 2	52	9	29	2	66
F x 4	59	8	35	1	61
F x 6	59	8	24	2	72
Pooled SEM	2.37	0.83	2.22	0.50	2.88

EG = equilibration with glycerol at 4ºC, with 2, 4, and 6 representing 2 hr, 4hr, and 6hr equilibration times with glycerol, respectively.

Table A-13
Experiment 2: Mean percentage of progressively motile, frozen-thawed spermatozoa at 0 (M0), 3 (M3), 6 (M6), and 9 (M9) hr of incubation by collection date and by bull.

Variable	M0	M3	M6	M9
CDate				
1	42[a]	30	16	3
2	43[a]	32	17	4
3	37[b]	31	18	5
4	40[a,b]	30	18	6
5	41[a]	31	19	7
6	41[a]	28	16	2
Pooled SEM	1.26	1.19	1.52	1.31
Bull				
A	39[b,c]	29[b]	11[d]	1
B	37[c]	27[b]	19[a,b]	4
C	42[a,b]	28[b]	17[b,c]	6
D	42[a,b]	27[b]	14[c,d]	2
E	44[a]	35[a]	22[a]	9
F	40[b,c]	35[a]	20[a,b]	6
Pooled SEM	1.26	1.19	1.52	1.31

[a,b,c,d] LSMeans within a column by variable with different superscripts differ ($P < 0.05$).
CDate = date of ejaculate collection, where, 1 through 6 correspond to 6-13-2003, 6-17-2003, 6-20-2003, 6-24-2003, 6-27-2003, and 7-1-2003, respectively.

Table A-14
Experiment 2: Mean percentage of intact acrosomal membrane (PIA), percentage primary (1º), secondary (2º), and tertiary (3º) morphological abnormalities, and percentage of morphologically normal (N) spermatozoa by collection date and by bull.

Variable	PIA	1º	2º	3º	N
CDate					
1	63[a,b]	7	27[a]	2[a]	71[c]
2	66[a]	7	17[d]	1[b]	85[a]
3	60[b]	7	23[b]	0[c]	78[b]
4	62[a,b]	8	21[b,c]	1[b]	78[b]
5	64[a]	10	18[d]	0[b,c]	81[a,b]
6	43[c]	9	20[c,d]	1[b]	80[b]
Pooled SEM	1.40	0.52	1.07	0.23	1.64
Bull					
A	54[d]	7[a,b,c]	23[a]	1	77[b]
B	63[a,b]	5[c]	20[b]	1	86[a]
C	59[b,c]	6[c]	25[a]	1	77[b]
D	65[a]	11[a,b]	19[b,c]	1	77[b]
E	60[b,c]	7[b,c]	19[c]	1	85[a]
F	56[c,d]	12[a]	22[a,b]	1	73[b]
Pooled SEM	1.40	0.52	1.07	0.23	1.64

[a,b,c,d] LSMeans within a column by variable with different superscripts differ ($P < 0.05$).
CDate = date of ejaculate collection, where, 1 through 6 correspond to 6-13-2003, 6-17-2003, 6-24-2003, 6-27-2003, and 7-1-2003, respectively.

Table A-15
Experiment 2: Mean percentage of progressively motile, frozen-thawed spermatozoa at 0 (M0), 3 (M3), 6 (M6), and 9 (M9) hr of incubation for collection date X bull.

CDate x Bull	M0	M3	M6	M9
1 x A	45	33	8	1
1 x B	34	21	9	2
1 x C	46	31	19	6
1 x D	47	25	17	0
1 x E	43	31	20	5
1 x F	39	36	20	6
2 x A	42	29	7	1
2 x B	43	33	21	2
2 x C	43	31	16	6
2 x D	42	25	15	1
2 x E	44	35	24	9
2 x F	42	40	20	8
3 x A	36	30	13	1
3 x B	28	27	25	3
3 x C	39	34	19	9
3 x D	44	32	9	1
3 x E	44	36	21	10
3 x F	29	28	25	3
4 x A	41	23	9	1
4 x B	39	31	20	4
4 x C	42	27	19	6
4 x D	36	25	21	8
4 x E	36	37	18	9
4 x F	44	37	19	8
5 x A	37	31	12	3
5 x B	44	30	26	11
5 x C	39	21	15	5
5 x D	40	33	18	2
5 x E	45	37	26	11
5 x F	40	31	17	8

Table A-15
Continued,

CDate x Bull	M0	M3	M6	M9
6 x A	33	27	19	1
6 x B	33	21	12	0
6 x C	41	26	16	1
6 x D	43	24	5	0
6 x E	49	32	22	9
6 x F	45	39	20	2
Pooled SEM	3.07	2.91	3.73	3.22

CDate = date of ejaculate collection, where, 1 through 6 correspond to 6-13-2003, 6-17-2003, 6-20-2003, 6-24-2003, 6-27-2003, and 7-1-2003, respectively.

Table A-16
Experiment 2: Mean percentage of intact acrosomal membrane (PIA), percentage primary (1º), secondary (2º), and tertiary (3º) morphological abnormalities, and percentage of morphologically normal (N) spermatozoa for collection date X bull.

CDate x Bull	PIA	1º	2º	3º	N
1 x A	48	8	35	3	58
1 x B	68	2	26	0	86
1 x C	63	5	30	4	66
1 x D	72	9	18	1	83
1 x E	63	7	30	1	70
1 x F	62	13	25	3	64
2 x A	64	5	21	1	83
2 x B	75	7	13	1	93
2 x C	66	6	21	0	83
2 x D	70	9	21	1	76
2 x E	64	7	13	1	94
2 x F	55	11	15	2	84
3 x A	61	6	25	0	78
3 x B	65	4	22	0	84
3 x C	58	5	24	0	81
3 x D	55	11	25	1	70
3 x E	62	6	17	0	90
3 x F	56	11	29	0	67
4 x A	61	5	20	2	82
4 x B	72	10	14	2	84
4 x C	59	5	34	1	68
4 x D	66	11	20	1	73
4 x E	66	4	19	0	89
4 x F	49	12	22	0	74
5 x A	60	8	16	1	85
5 x B	61	5	17	1	90
5 x C	59	11	20	0	77
5 x D	78	13	19	1	75
5 x E	64	11	19	1	79
5 x F	64	11	16	0	82

Table A-16
Continued,

CDate x Bull	PIA	1º	2º	3º	N
6 x A	32	11	21	1	75
6 x B	38	4	26	1	79
6 x C	51	5	20	0	87
6 x D	47	15	12	0	85
6 x E	39	7	15	1	90
6 x F	51	13	24	4	65
Pooled SEM	3.44	1.28	2.62	0.56	4.01

CDate = date of ejaculate collection, where, 1 through 6 correspond to 6-13-2003, 6-17-2003, 6-24-2003, 6-27-2003, and 7-1-2003, respectively.

Table A-17
Experiment 2: Mean percentage of progressively motile, frozen-thawed spermatozoa at 0 (M0), 3 (M3), 6 (M6), and 9 (M9) hr of incubation for collection date X extender.

CDate x Extender	M0	M3	M6	M9
1 x 1	44	36	14	1
1 x 2	40	23	9	4
1 x 3	43	30	24	4
2 x 1	43	31	17	2
2 x 2	43	27	8	5
2 x 3	43	38	26	6
3 x 1	41	33	21	5
3 x 2	28	23	8	2
3 x 3	40	38	26	6
4 x 1	40	36	15	2
4 x 2	38	18	11	7
4 x 3	42	36	27	9
5 x 1	46	34	20	5
5 x 2	34	22	10	4
5 x 3	42	36	26	11
6 x 1	45	33	16	4
6 x 2	32	15	7	1
6 x 3	45	36	24	2
Pooled SEM	2.17	2.06	2.63	2.28

CDate = date of ejaculate collection, where, 1 through 6 correspond to 6-13-2003, 6-17-2003, 6-20-2003, 6-24-2003, 6-27-2003, and 7-1-2003, respectively.
Extender = seminal extender type, where, 1 through 3 correspond to egg yolk-citrate, skim milk, and IMV, respectively.

Table A-18
Experiment 2: Mean percentage of intact acrosomal membrane (PIA), percentage primary (1º), secondary (2º), and tertiary (3º) morphological abnormalities, and percentage of morphologically normal (N) spermatozoa for collection date X extender.

CDate x Extender	PIA	1º	2º	3º	N
1 x 1	55	8	22	2	78
1 x 3	70	7	33	2	64
2 x 1	60	7	16	1	89
2 x 3	71	8	19	1	82
3 x 1	55	7	21	0	82
3 x 3	65	7	26	0	74
4 x 1	59	10	16	1	85
4 x 3	64	7	27	1	72
5 x 1	61	10	16	1	83
5 x 3	67	9	20	0	80
6 x 1	39	8	19	1	82
6 x 3	46	10	21	0	77
Pooled SEM	1.99	0.74	1.51	0.32	2.32

CDate = date of ejaculate collection, where, 1 through 6 correspond to 6-13-2003, 6-17-2003, 6-24-2003, 6-27-2003, and 7-1-2003, respectively.
Extender = seminal extender type, where, 1 and 3 correspond to egg yolk-citrate and IMV, respectively.

Table A-19
Experiment 2: Mean percentage of progressively motile, frozen-thawed spermatozoa at 0 (M0), 3 (M3), 6 (M6), and 9 (M9) hr of incubation for bull X semen storage type.

Bull x Semen	M0	M3	M6	M9
A x 1	55[a]	48[a]	21[c,d]	2
A x 2	23[e]	10[f]	1[h]	0
B x 1	48[b]	36[c]	24[b,c]	4
B x 2	26[e]	19[d,e]	13[e,f]	3
C x 1	55[a]	41[b]	28[a,b]	9
C x 2	28[d,e]	15[e,f]	7[g,h]	2
D x 1	50[b]	36[c]	18[d,e]	1
D x 2	34[c]	19[d,e]	10[f,g]	2
E x 1	55[a]	49[a]	30[a]	11
E x 2	32[c,d]	20[d]	13[e,f]	7
F x 1	51[a,b]	49[a]	25[a,b,c]	5
F x 2	28[d,e]	21[d]	16[d,e,f]	7
Pooled SEM	1.78	1.68	2.15	1.86

[a,b,c,d,e,f,g,h] LSMeans within a column with different superscripts differ ($P < 0.05$). Semen = semen storage type, where, 1 denotes fresh semen and 2 denotes frozen-thawed semen.

Table A-20
Experiment 2: Mean percentage of intact acrosomal membrane (PIA), percentage primary (1º), secondary (2º), and tertiary (3º) morphological abnormalities, and percentage of morphologically normal (N) spermatozoa for bull X semen storage type.

Bull x Semen	PIA	1º	2º	3º	N
A x 1	88[c,d]	6	17	2	85
A x 2	20[g]	8	29	0	68
B x 1	93[b,c]	4	13	2	97
B x 2	33[f]	7	26	0	75
C x 1	99[a]	6	18	2	86
C x 2	19[g]	6	31	0	68
D x 1	85[d]	11	13	1	84
D x 2	44[e]	12	25	0	69
E x 1	95[a,b]	6	10	1	98
E x 2	24[g]	8	27	0	72
F x 1	91[b,c]	11	13	3	84
F x 2	21[g]	13	31	0	61
Pooled SEM	1.99	0.74	1.51	0.32	2.32

[a,b,c,d,e,f,g] LSMeans within a column by variable with different superscripts differ ($P < 0.05$).
Semen = semen storage type, where, 1 denotes fresh semen and 2 denotes frozen-thawed semen.

Table A-21
Experiment 1: Mean percentage of progressively motile, frozen-thawed spermatozoa at 0 (M0), 3 (M3), 6 (M6), and 9 (M9) hr of incubation for bull X extender.

Bull x Extender	M0	M3	M6	M9
A x 1	41	31 [c,d,e]	11 [e,f,g]	0
A x 2	35	22 [f]	5 [g]	3
A x 3	42	34 [a,b,c,d]	18 [c,d,e]	1
B x 1	41	31 [c,d,e]	19 [c]	2
B x 2	30	15 [g]	8 [f,g]	3
B x 3	40	35 [a,b,c,d]	29 [a]	7
C x 1	45	35 [a,b,c,d]	19 [c]	7
C x 2	37	15 [g]	6 [g]	3
C x 3	43	35 [a,b,c,d]	27 [a,b]	7
D x 1	45	30 [d,e]	18 [c,d]	1
D x 2	39	16 [f,g]	10 [f,g]	2
D x 3	42	35 [a,b,c,d]	15 [c,d,e,f]	3
E x 1	43	38 [a,b]	20 [b,c]	8
E x 2	40	28 [e]	14 [c,d,e,f]	7
E x 3	47	39 [a]	31 [a]	11
F x 1	43	36 [a,b,c]	17 [c,d,e]	2
F x 2	35	32 [b,c,d,e]	11 [d,e,f,g]	6
F x 3	41	37 [a,b,c]	33 [a]	10
Pooled SEM	2.17	2.06	2.63	2.28

[a,b,c,d,e,f,g] LSMeans within a column with different superscripts differ ($P < 0.05$). Extender = seminal extender type, where, 1 through 3 correspond to egg yolk-citrate, skim milk, and IMV, respectively.

Table A-22
Experiment 2: Mean percentage of intact acrosomal membrane (PIA), percentage primary (1º), secondary (2º), and tertiary (3º) morphological abnormalities, and percentage of morphologically normal (N) spermatozoa for bull X extender.

Bull x Extender	PIA	1º	2º	3º	N
A x 1	51	7	22	1	79
A x 3	57	8	24	1	75
B x 1	58	5	18	1	88
B x 3	68	6	21	1	84
C x 1	54	7	19	0	85
C x 3	64	5	31	1	69
D x 1	59	10	17	1	82
D x 3	70	13	21	1	72
E x 1	55	7	15	1	89
E x 3	64	7	22	0	81
F x 1	52	12	18	2	75
F x 3	60	11	26	1	70
Pooled SEM	1.99	0.74	1.51	0.32	2.32

Extender = seminal extender type, where, 1 and 3 correspond to egg yolk-citrate and IMV, respectively.

Table A-23
Experiment 2: Mean percentage of progressively motile, frozen-thawed spermatozoa at 0 (M0), 3 (M3), 6 (M6), and 9 (M9) hr of incubation for collection date X bull X semen storage type.

CDate x Bull x Semen	M0	M3	M6	M9
1 x A x 1	60	54	14	1
1 x A x 2	30	12	2	0
1 x B x 1	56	33	12	2
1 x B x 2	12	10	7	2
1 x C x 1	64	42	25	1
1 x C x 2	27	20	13	10
1 x D x 1	60	36	26	1
1 x D x 2	34	13	8	0
1 x E x 1	58	45	26	3
1 x E x 2	27	17	13	7
1 x F x 1	52	52	22	1
1 x F x 2	25	20	18	12
2 x A x 1	62	54	13	2
2 x A x 2	22	4	0	0
2 x B x 1	58	47	33	2
2 x B x 2	29	19	8	2
2 x C x 1	58	51	27	12
2 x C x 2	29	12	5	0
2 x D x 1	52	36	26	1
2 x D x 2	32	13	5	0
2 x E x 1	56	50	28	1
2 x E x 2	32	20	20	17
2 x F x 1	56	52	26	10
2 x F x 2	27	27	15	5
3 x A x 1	52	52	24	1
3 x A x 2	20	8	2	0
3 x B x 1	26	33	29	1
3 x B x 2	31	22	20	5
3 x C x 1	53	53	35	19
3 x C x 2	25	15	2	0
3 x D x 1	52	43	5	2
3 x D x 2	36	20	12	0

Table A-23
Continued,

CDate x Bull x Semen	M0	M3	M6	M9
3 x E x 1	58	52	28	17
3 x E x 2	29	20	15	4
3 x F x 1	33	34	31	1
3 x F x 2	25	22	18	5
4 x A x 1	62	31	15	1
4 x A x 2	20	15	4	0
4 x B x 1	47	34	16	0
4 x B x 2	32	27	24	8
4 x C x 1	52	33	26	11
4 x C x 2	32	20	12	1
4 x D x 1	41	27	22	2
4 x D x 2	31	22	20	13
4 x E x 1	39	52	27	12
4 x E x 2	34	22	8	7
4 x F x 1	56	50	19	4
4 x F x 2	32	24	20	12
5 x A x 1	54	52	24	7
5 x A x 2	20	10	0	0
5 x B x 1	58	42	36	19
5 x B x 2	29	19	15	3
5 x C x 1	52	34	29	10
5 x C x 2	25	8	1	0
5 x D x 1	47	38	24	2
5 x D x 2	34	29	12	1
5 x E x 1	54	52	32	14
5 x E x 2	36	22	19	7
5 x F x 1	49	47	21	12
5 x F x 2	31	15	13	4
6 x A x 1	38	41	38	2
6 x A x 2	27	13	0	0
6 x B x 1	40	26	19	1
6 x B x 2	26	16	5	0
6 x C x 1	52	36	26	2
6 x C x 2	30	15	7	0
6 x D x 1	47	33	5	0
6 x D x 2	39	15	5	0

Table A-23
Continued,

CDate x Bull x Semen	M0	M3	M6	M9
6 x E x 1	66	44	42	17
6 x E x 2	32	20	2	0
6 x F x 1	60	58	29	2
6 x F x 2	29	20	10	2
Pooled SEM	4.35	4.12	5.27	4.55

CDate = date of ejaculate collection, where, 1 through 6 correspond to 6-13-2003, 6-17-2003, 6-20-2003, 6-24-2003, 6-27-2003, and 7-1-2003, respectively.
Semen = semen storage type, where, 1 denotes fresh semen and 2 denotes frozen-thawed semen.

Table A-24
Experiment 2: Mean percentage of intact acrosomal membrane (PIA), percentage primary (1º), secondary (2º), and tertiary (3º) morphological abnormalities, and percentage of morphologically normal (N) spermatozoa for collection date X bull X semen storage type.

CDate x Bull x Semen	PIA	1º	2º	3º	N
1 x A x 1	76	9	32	5	58
1 x A x 2	19	8	39	0	59
1 x B x 1	93	2	6	1	116
1 x B x 2	42	3	46	0	57
1 x C x 1	90	8	20	7	74
1 x C x 2	36	3	40	0	58
1 x D x 1	83	10	10	2	93
1 x D x 2	61	9	25	0	73
1 x E x 1	90	11	16	2	81
1 x E x 2	36	3	44	0	58
1 x F x 1	89	15	17	5	68
1 x F x 2	35	12	34	0	59
2 x A x 1	108	4	14	1	95
2 x A x 2	20	7	29	0	70
2 x B x 1	107	4	6	3	107
2 x B x 2	44	11	19	0	78
2 x C x 1	105	6	13	1	95
2 x C x 2	26	6	29	0	72
2 x D x 1	95	9	14	2	86
2 x D x 2	44	10	28	0	67
2 x E x 1	100	6	7	2	103
2 x E x 2	28	8	18	0	85
2 x F x 1	90	9	6	3	97
2 x F x 2	20	12	24	0	70
3 x A x 1	103	5	17	0	90
3 x A x 2	19	7	33	0	65
3 x B x 1	88	3	27	0	78
3 x B x 2	42	5	17	0	90
3 x C x 1	103	4	24	0	82
3 x C x 2	14	6	24	0	79
3 x D x 1	75	14	22	2	69
3 x D x 2	36	8	27	0	71

Table A-24
Continued,

CDate x Bull x Semen	PIA	1º	2º	3º	N
3 x E x 1	105	5	12	0	101
3 x E x 2	19	8	22	0	78
3 x F x 1	93	9	19	0	80
3 x F x 2	20	13	39	0	53
4 x A x 1	101	5	7	5	100
4 x A x 2	21	6	34	0	65
4 x B x 1	114	7	12	5	90
4 x B x 2	30	14	16	0	78
4 x C x 1	102	5	25	1	80
4 x C x 2	16	6	42	0	56
4 x D x 1	93	11	12	1	79
4 x D x 2	38	12	27	0	66
4 x E x 1	104	3	10	1	107
4 x E x 2	27	6	29	0	71
4 x F x 1	84	10	12	1	90
4 x F x 2	14	15	33	0	57
5 x A x 1	97	5	16	2	89
5 x A x 2	24	11	17	0	81
5 x B x 1	99	3	12	1	101
5 x B x 2	22	7	22	0	80
5 x C x 1	105	11	14	1	84
5 x C x 2	13	11	26	0	69
5 x D x 1	103	14	13	1	81
5 x D x 2	53	11	26	0	69
5 x E x 1	101	6	11	2	97
5 x E x 2	27	16	27	0	61
5 x F x 1	106	11	10	0	92
5 x F x 2	23	11	23	0	73
6 x A x 1	47	9	19	1	80
6 x A x 2	17	13	24	0	69
6 x B x 1	59	4	17	1	90
6 x B x 2	17	5	34	0	67
6 x C x 1	92	5	12	0	99
6 x C x 2	9	5	28	0	75
6 x D x 1	61	9	8	0	99
6 x D x 2	33	21	16	0	71

Table A-24
Continued,

CDate x Bull x Semen	PIA	1º	2º	3º	N
6 x E x 1	72	6	8	1	103
6 x E x 2	6	8	22	0	78
6 x F x 1	83	11	14	8	75
6 x F x 2	18	15	35	0	54
Pooled SEM	4.87	1.81	3.70	0.79	5.68

CDate = date of ejaculate collection, where, 1 through 6 correspond to 6-13-2003, 6-17-2003, 6-24-2003, 6-27-2003, and 7-1-2003, respectively.
Semen = semen storage type, where, 1 denotes fresh semen and 2 denotes frozen-thawed semen.

Table A-25
Experiment 2: Mean percentage of progressively motile, frozen-thawed spermatozoa at 0 (M0), 3 (M3), 6 (M6), and 9 (M9) hr of incubation for collection date X semen storage type X extender.

CDate x Semen x Extender	M0	M3	M6	M9
1 x 1 x 1	60	54	18	1
1 x 1 x 2	58	33	9	3
1 x 1 x 3	57	44	35	1
1 x 2 x 1	28	17	10	2
1 x 2 x 2	22	13	8	6
1 x 2 x 3	29	17	13	8
2 x 1 x 1	56	52	28	1
2 x 1 x 2	58	39	9	4
2 x 1 x 3	57	54	40	10
2 x 2 x 1	30	10	7	3
2 x 2 x 2	27	15	8	6
2 x 2 x 3	29	22	13	3
3 x 1 x 1	52	47	33	7
3 x 1 x 2	34	34	5	2
3 x 1 x 3	52	53	39	11
3 x 2 x 1	31	18	10	3
3 x 2 x 2	23	13	12	3
3 x 2 x 3	29	22	13	1
4 x 1 x 1	47	44	14	1
4 x 1 x 2	49	22	11	6
4 x 1 x 3	53	49	37	9
4 x 2 x 1	33	28	16	4
4 x 2 x 2	28	14	11	8
4 x 2 x 3	30	23	17	9
5 x 1 x 1	54	50	27	6
5 x 1 x 2	49	33	15	7
5 x 1 x 3	54	50	40	20
5 x 2 x 1	37	18	13	4
5 x 2 x 2	20	11	6	1
5 x 2 x 3	31	23	11	2

Table A-25
Continued,

CDate x Semen x Extender	M0	M3	M6	M9
6 x 1 x 1	56	47	27	8
6 x 1 x 2	41	20	12	2
6 x 1 x 3	55	52	40	3
6 x 2 x 1	34	18	4	0
6 x 2 x 2	23	10	3	0
6 x 2 x 3	35	21	9	1
Pooled SEM	3.07	2.91	3.73	3.22

CDate = date of ejaculate collection, where, 1 through 6 correspond to 6-13-2003, 6-17-2003, 6-20-2003, 6-24-2003, 6-27-2003, and 7-1-2003, respectively.
Semen = semen storage type, where, 1 denotes fresh semen and 2 denotes frozen-thawed semen.
Extender = seminal extender type, where, 1 through 3 correspond to egg yolk-citrate, skim milk, and IMV, respectively.

Table A-26
Experiment 2: Mean percentage of intact acrosomal membrane (PIA), percentage primary (1º), secondary (2º), and tertiary (3º) morphological abnormalities, and percentage of morphologically normal (N) spermatozoa for collection date X semen X extender.

CDate x Semen x Extender	PIA	1º	2º	3º	N
1 x 1 x 1	82	8	14	3	87
1 x 1 x 3	92	10	19	4	77
1 x 2 x 1	28	7	29	0	69
1 x 2 x 3	49	5	46	0	52
2 x 1 x 1	100	6	9	2	98
2 x 1 x 3	101	6	11	2	96
2 x 2 x 1	21	7	23	0	79
2 x 2 x 3	40	10	27	0	69
3 x 1 x 1	92	6	19	0	85
3 x 1 x 3	97	7	21	0	82
3 x 2 x 1	18	7	22	0	79
3 x 2 x 3	32	8	32	0	66
4 x 1 x 1	94	6	8	2	100
4 x 1 x 3	105	7	17	2	82
4 x 2 x 1	25	13	23	0	70
4 x 2 x 3	24	6	37	0	62
5 x 1 x 1	98	9	13	1	89
5 x 1 x 3	105	7	12	1	93
5 x 2 x 1	24	11	20	0	77
5 x 2 x 3	30	11	27	0	67
6 x 1 x 1	64	6	13	3	92
6 x 1 x 3	75	8	13	1	90
6 x 2 x 1	15	10	25	0	73
6 x 2 x 3	18	12	28	0	65
Pooled SEM	2.81	1.04	2.14	0.45	3.28

CDate = date of ejaculate collection, where, 1 through 6 correspond to 6-13-2003, 6-17-2003, 6-24-2003, 6-27-2003, and 7-1-2003, respectively.
Semen = semen storage type, where, 1 denotes fresh semen and 2 denotes frozen-thawed semen.
Extender = seminal extender type, where, 1 and 3 correspond to egg yolk-citrate and IMV, respectively.

Table A-27
Experiment 2: Mean percentage of progressively motile, frozen-thawed spermatozoa at 0 (M0), 3 (M3), 6 (M6), and 9 (M9) hr of incubation for bull X semen X extender.

Bull x Semen x Extender	M0	M3	M6	M9
A x 1 x 1	57	52	21	0
A x 1 x 2	49	37	8	5
A x 1 x 3	58	53	35	2
A x 2 x 1	24	10	0	0
A x 2 x 2	20	7	3	0
A x 2 x 3	25	14	1	0
B x 1 x 1	52	45	25	1
B x 1 x 2	40	16	6	2
B x 1 x 3	51	46	41	10
B x 2 x 1	29	18	13	2
B x 2 x 2	21	14	9	4
B x 2 x 3	29	24	18	4
C x 1 x 1	57	52	31	12
C x 1 x 2	53	21	9	5
C x 1 x 3	56	51	44	10
C x 2 x 1	32	19	7	2
C x 2 x 2	22	9	3	1
C x 2 x 3	31	18	10	3
D x 1 x 1	52	41	25	1
D x 1 x 2	46	19	9	2
D x 1 x 3	52	47	20	2
D x 2 x 1	39	19	12	1
D x 2 x 2	31	14	10	3
D x 2 x 3	32	23	9	3
E x 1 x 1	50	53	26	7
E x 1 x 2	57	42	21	8
E x 1 x 3	58	52	44	16
E x 2 x 1	36	22	14	9
E x 2 x 2	23	13	8	6
E x 2 x 3	37	25	18	6

Table A-27
Continued,

Bull x Semen x Extender	M0	M3	M6	M9
F x 1 x 1	55	51	19	1
F x 1 x 2	45	45	8	2
F x 1 x 3	53	52	47	13
F x 2 x 1	31	22	15	3
F x 2 x 2	25	19	14	10
F x 2 x 3	28	23	18	7
Pooled SEM	3.07	2.91	3.73	3.22

Semen = semen storage type, where, 1 denotes fresh semen and 2 denotes frozen-thawed semen.
Extender = seminal extender type, where, 1 through 3 correspond to egg yolk-citrate, skim milk, and IMV, respectively.

Table A-28
Experiment 2: Mean percentage of intact acrosomal membrane (PIA), percentage primary (1º), secondary (2º), and tertiary (3º) morphological abnormalities, and percentage of morphologically normal (N) spermatozoa for collection date X bull X extender.

Bull x Semen x Extender	PIA	1º	2º	3º	N
A x 1 x 1	84	6	17	3	86
A x 1 x 3	92	6	18	2	85
A x 2 x 1	18	8	27	0	71
A x 2 x 3	22	9	31	0	65
B x 1 x 1	86	2	14	2	98
B x 1 x 3	100	5	13	2	96
B x 2 x 1	30	8	23	0	78
B x 2 x 3	36	6	29	0	72
C x 1 x 1	97	7	12	1	95
C x 1 x 3	102	6	24	2	77
C x 2 x 1	12	7	26	0	75
C x 2 x 3	26	5	37	0	61
D x 1 x 1	82	8	13	1	91
D x 1 x 3	88	14	14	1	78
D x 2 x 1	36	12	22	0	73
D x 2 x 3	52	12	28	0	66
E x 1 x 1	93	7	8	2	100
E x 1 x 3	97	5	13	1	97
E x 2 x 1	17	8	22	0	79
E x 2 x 3	30	9	32	0	65
F x 1 x 1	87	12	14	3	81
F x 1 x 3	95	10	12	2	87
F x 2 x 1	17	13	23	0	70
F x 2 x 3	26	12	40	0	52
Pooled SEM	2.81	1.04	2.14	0.45	3.28

Semen = semen storage type, where, 1 denotes fresh semen and 2 denotes frozen-thawed semen.
Extender = seminal extender type, where, 1 and 3 correspond to egg yolk-citrate and IMV, respectively.

APPENDIX B

FIGURES

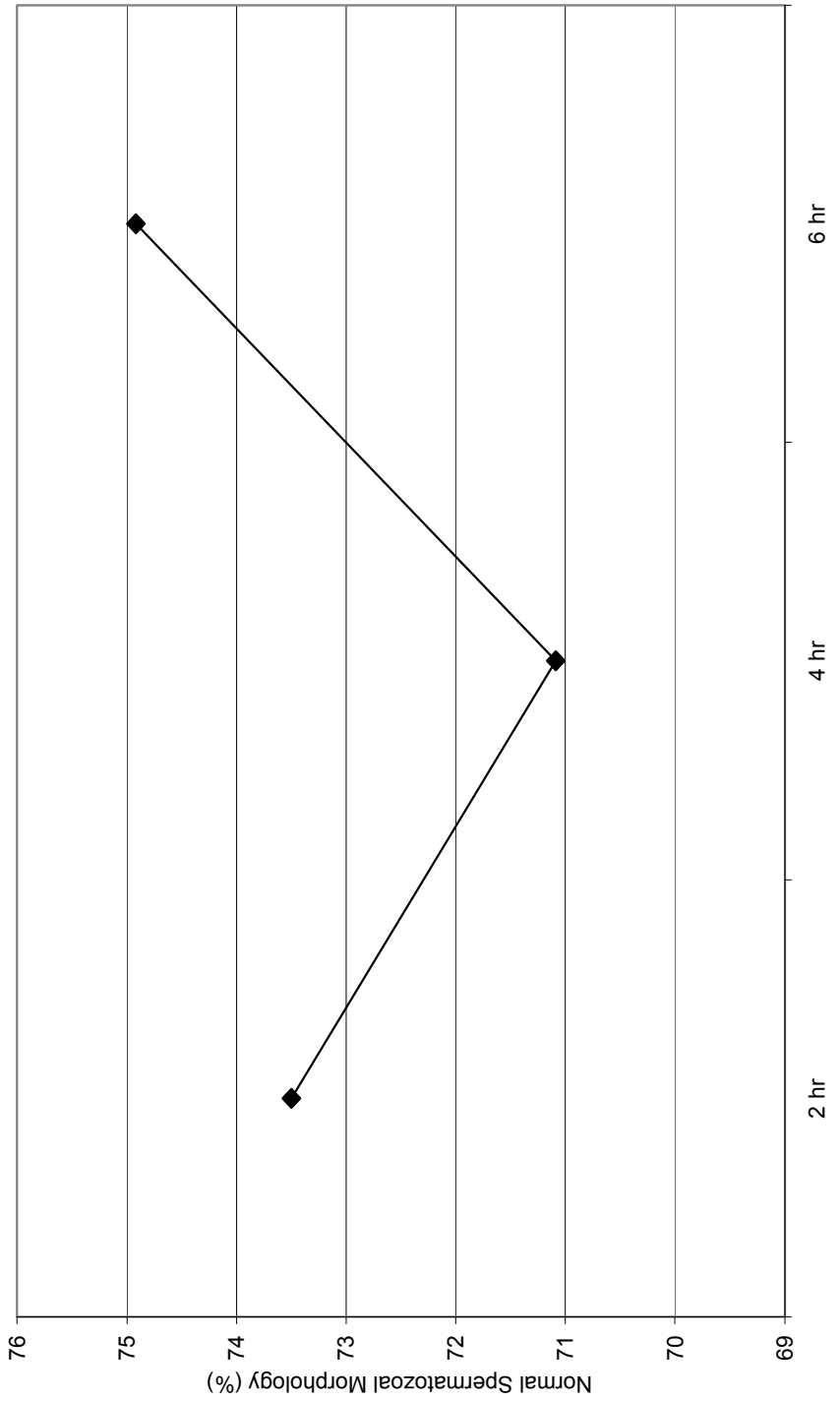

Fig. A-1. Experiment 1: Mean percentage morphologically normal spermatozoa by duration of equilibration with glycerol (2, 4, or 6 hr). Pooled SEM is 1.18.

Fig. A-2. Experiment 1: Mean percentage of spermatozoa with an intact acrosomal membrane by collection date (1 to 6). Pooled SEM is 1.37. ^{a,b,c}LSMeans with different superscripts differ ($P < 0.05$).

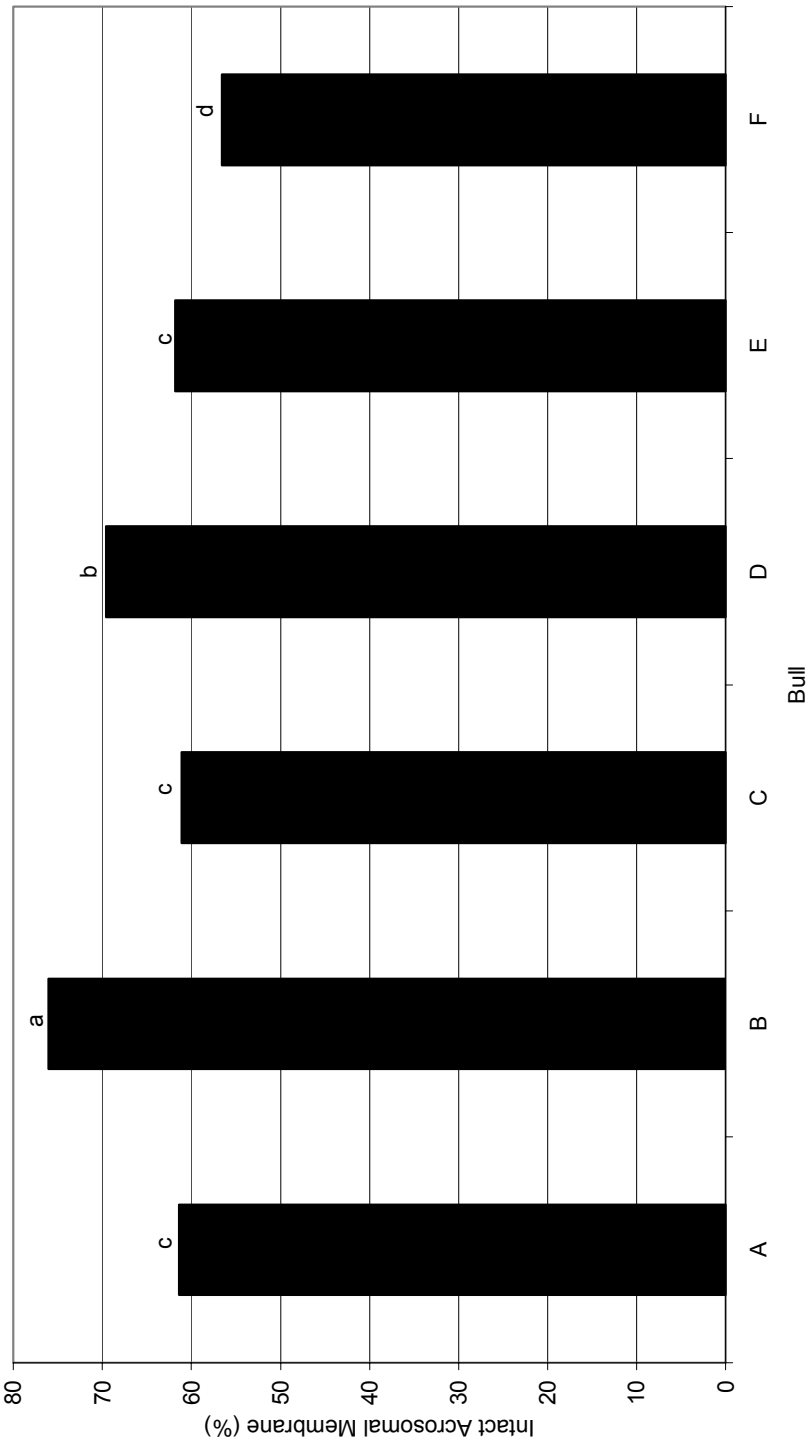

Fig. A-3. Experiment 1: Mean percentage of spermatozoa with an intact acrosomal membrane by bull (A to F). Pooled SEM is 1.37. ^{a,b,c,d} LSMeans with different superscripts differ ($P < 0.05$).

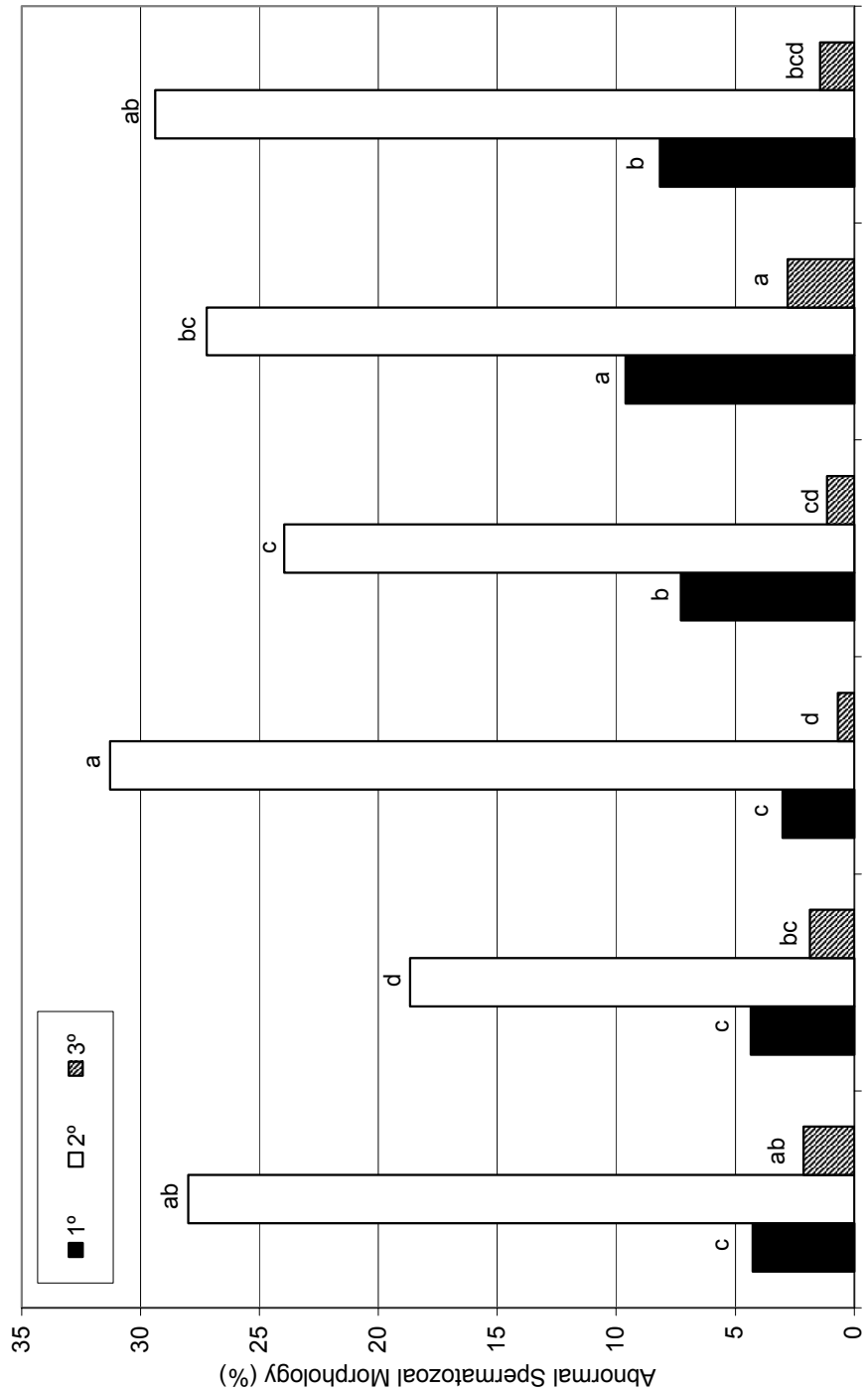

Fig. A-4. Experiment 1: Mean percentage morphologically abnormal spermatozoa (1° = primary, 2° = secondary, or 3° = tertiary abnormalities) by bull (A to F). Pooled SEM is 0.48, 1.28, and 0.29 for 1°, 2°, and 3°, respectively. ^{a,b,c,d} LSMeans with different superscripts within abnormality classification differ ($P < 0.05$).

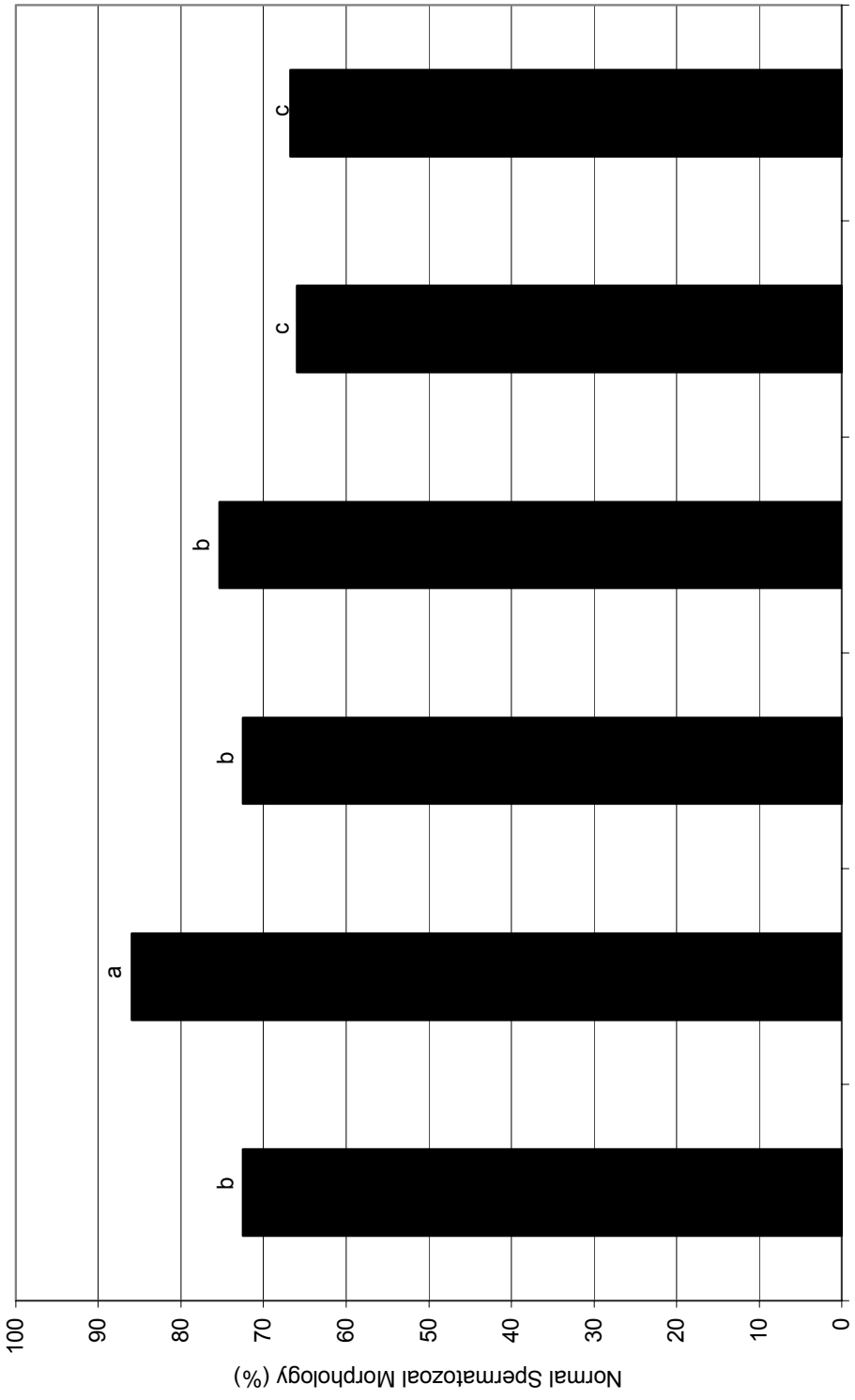

Fig. A-5. Experiment 1: Mean percentage morphologically normal spermatozoa by bull (A to F). Pooled SEM is 1.67.
[a,b,c] LSMeans with different superscripts differ (P < 0.05).

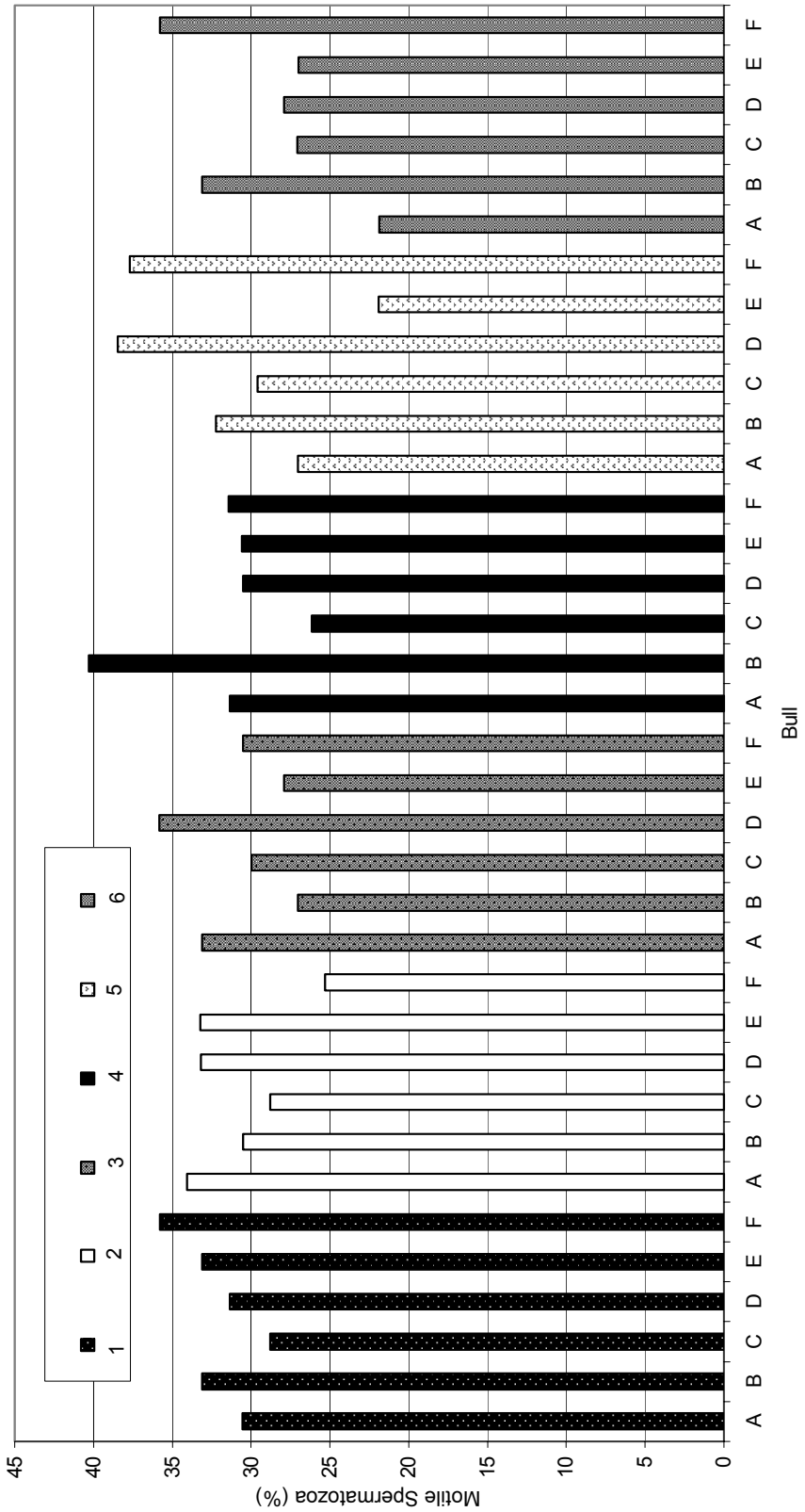

Fig. A-6. Experiment 1: Mean percentage motile spermatozoa by bull (A to F) and collection date (1 to 6) at 0 hr of incubation. Pooled SEM is 2.24.

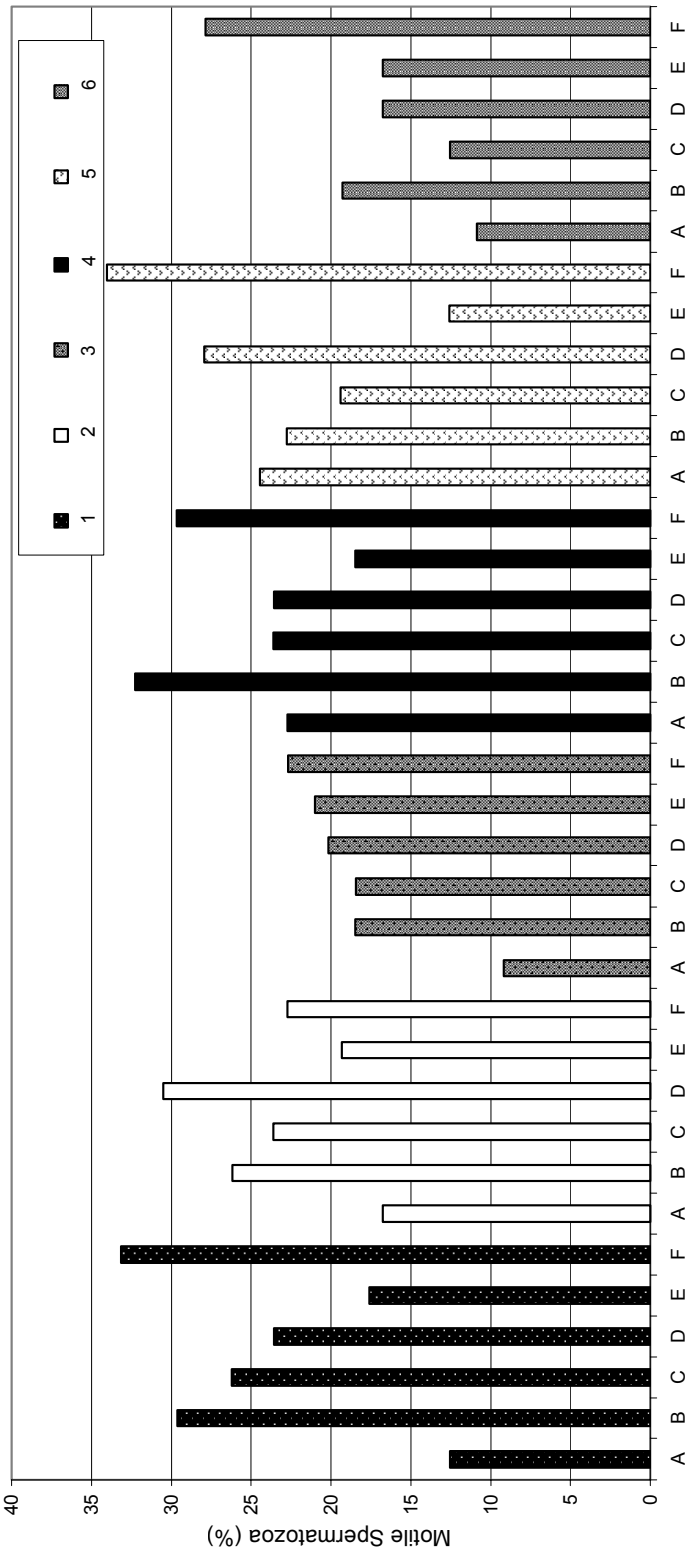

Fig. A-7. Experiment 1: Mean percentage motile spermatozoa by bull (A to F) and collection date (1 to 6) at 3 hr of incubation. Pooled SEM is 2.54.

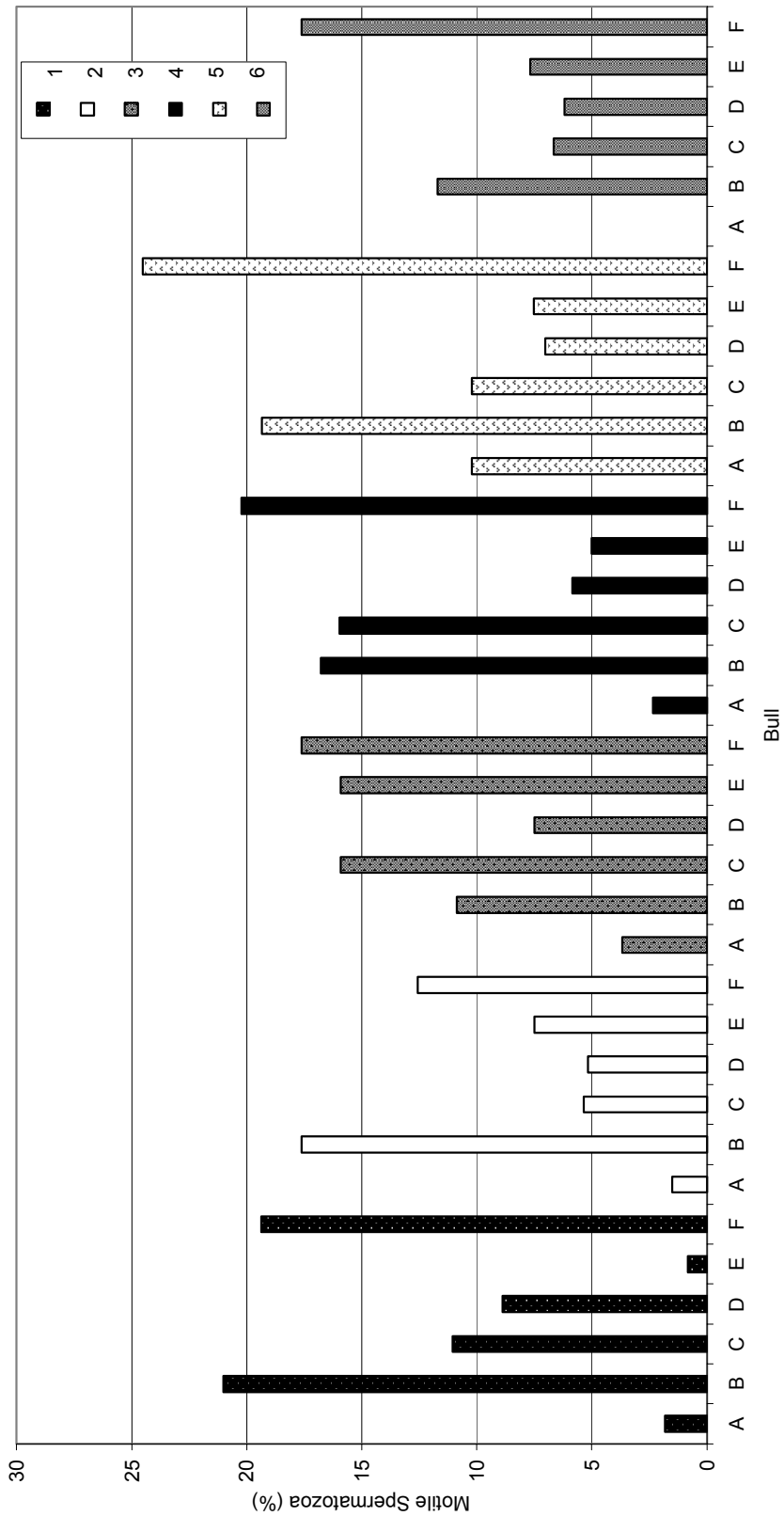

Fig. A-8. Experiment 1: Mean percentage motile spermatozoa by bull (A to F) and collection date (1-6) at 6 hr of incubation. Pooled SEM is 2.26.

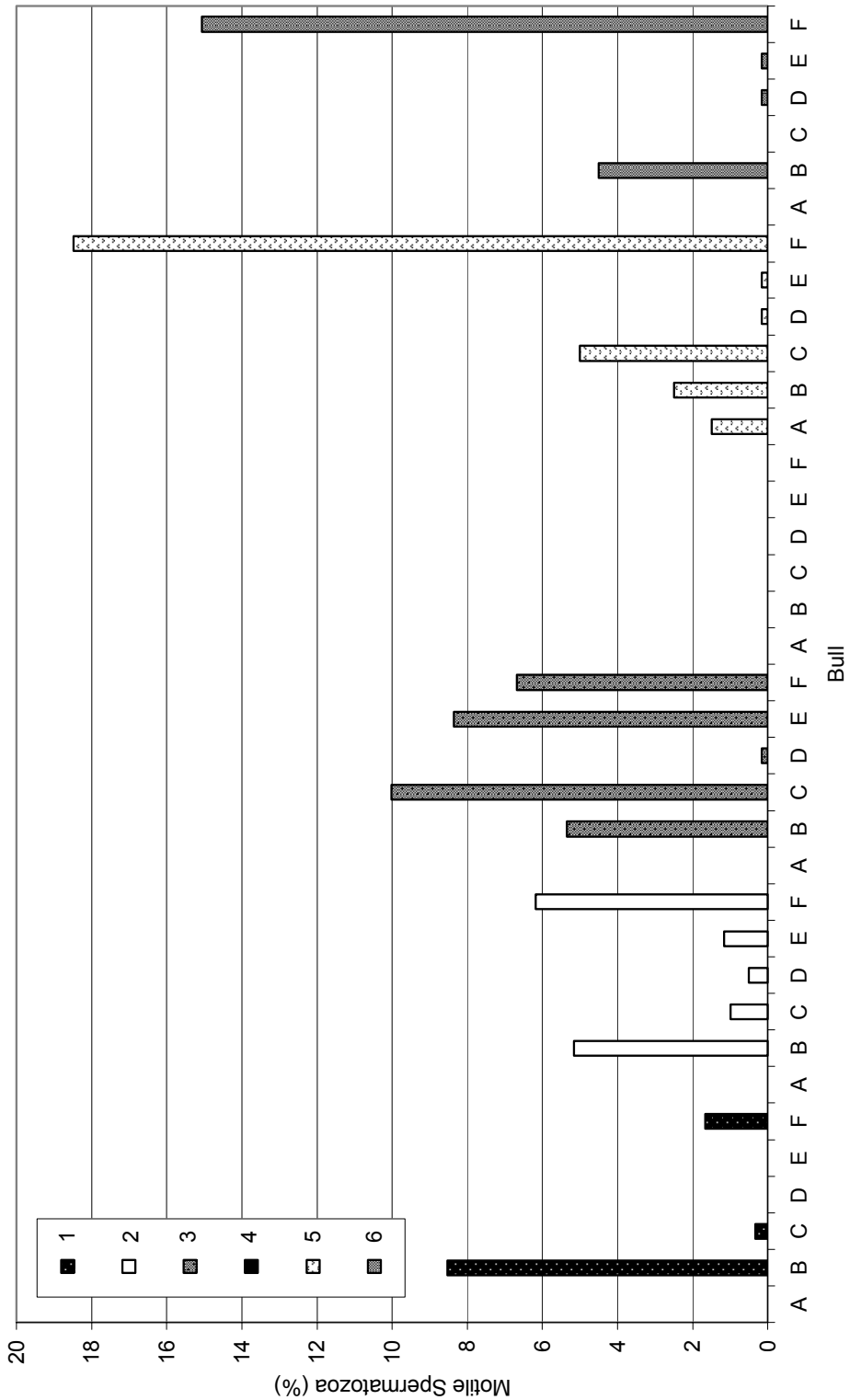

Fig. A-9. Experiment 1: Mean percentage motile spermatozoa by bull (A to F) and collection date (1 to 6) at 9 hr of incubation. Pooled SEM is 1.25.

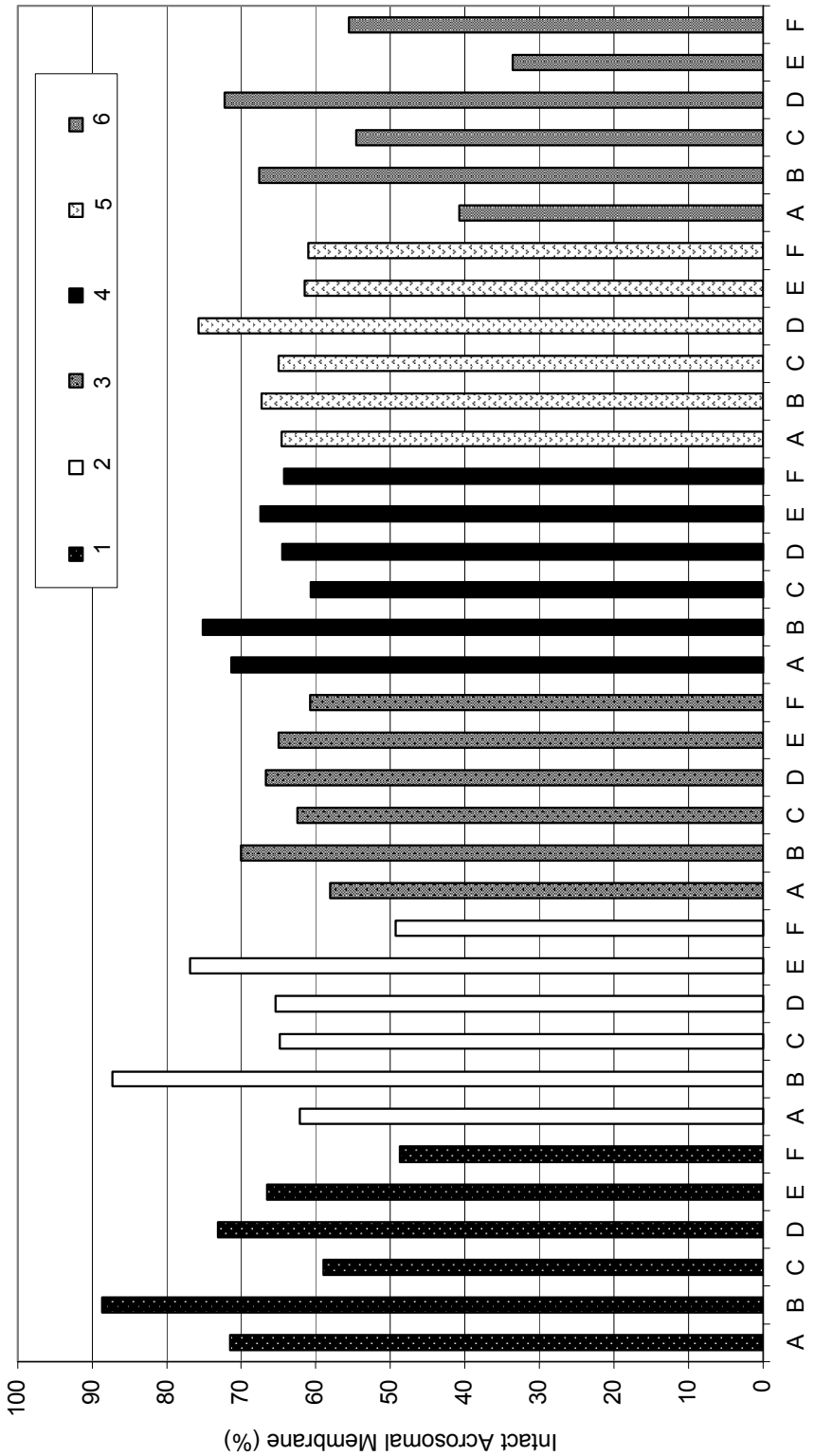

Fig. A-10. Experiment 1: Mean percentage of spermatozoa with an intact acrosomal membrane by bull (A to F) and collection date (1 to 6). Pooled SEM is 3.35.

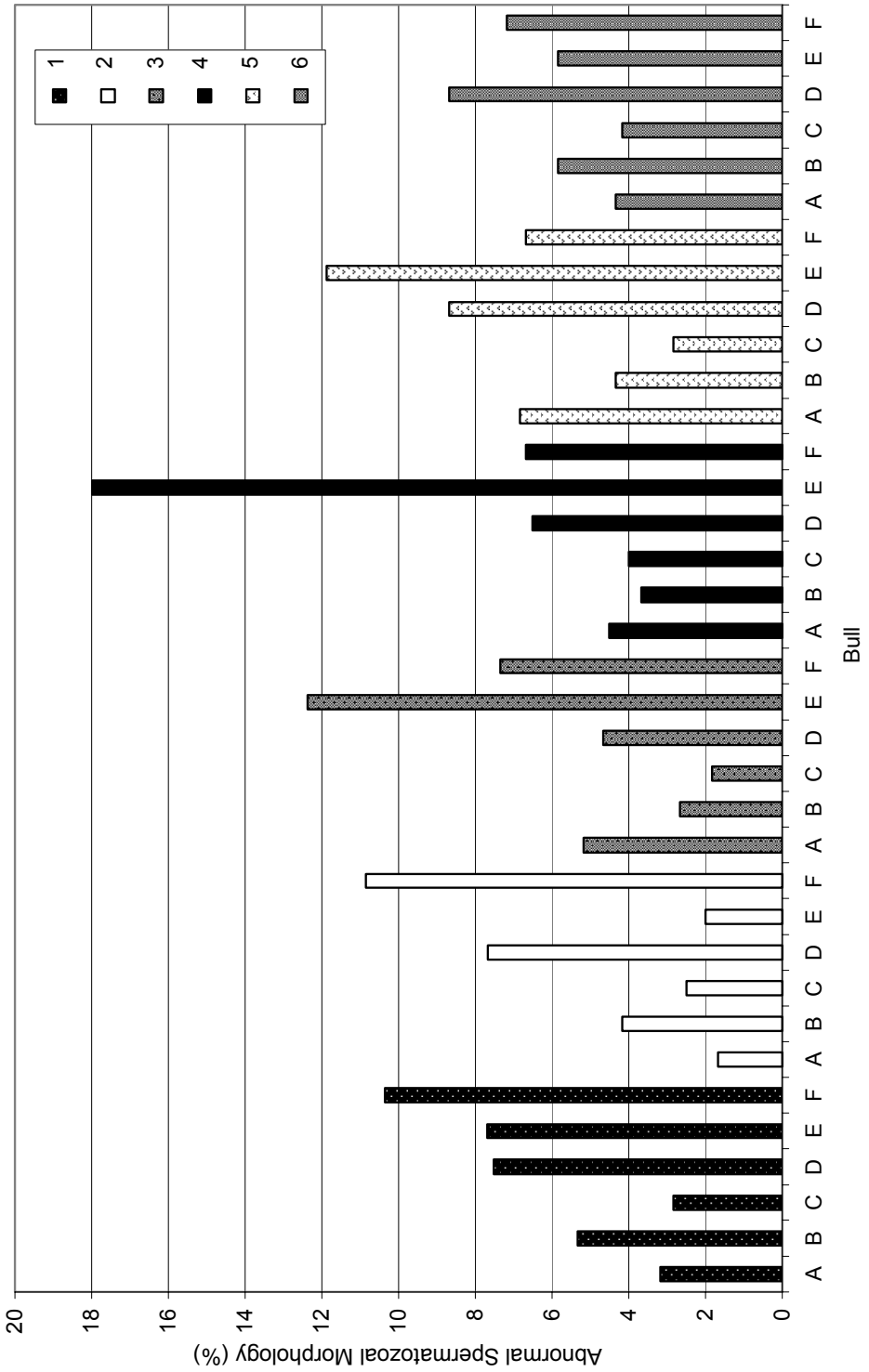

Fig. A-11. Experiment 1: Mean percentage primary morphologically abnormal spermatozoa by bull (A to F) and collection date (1 to 6). Pooled SEM is 1.17.

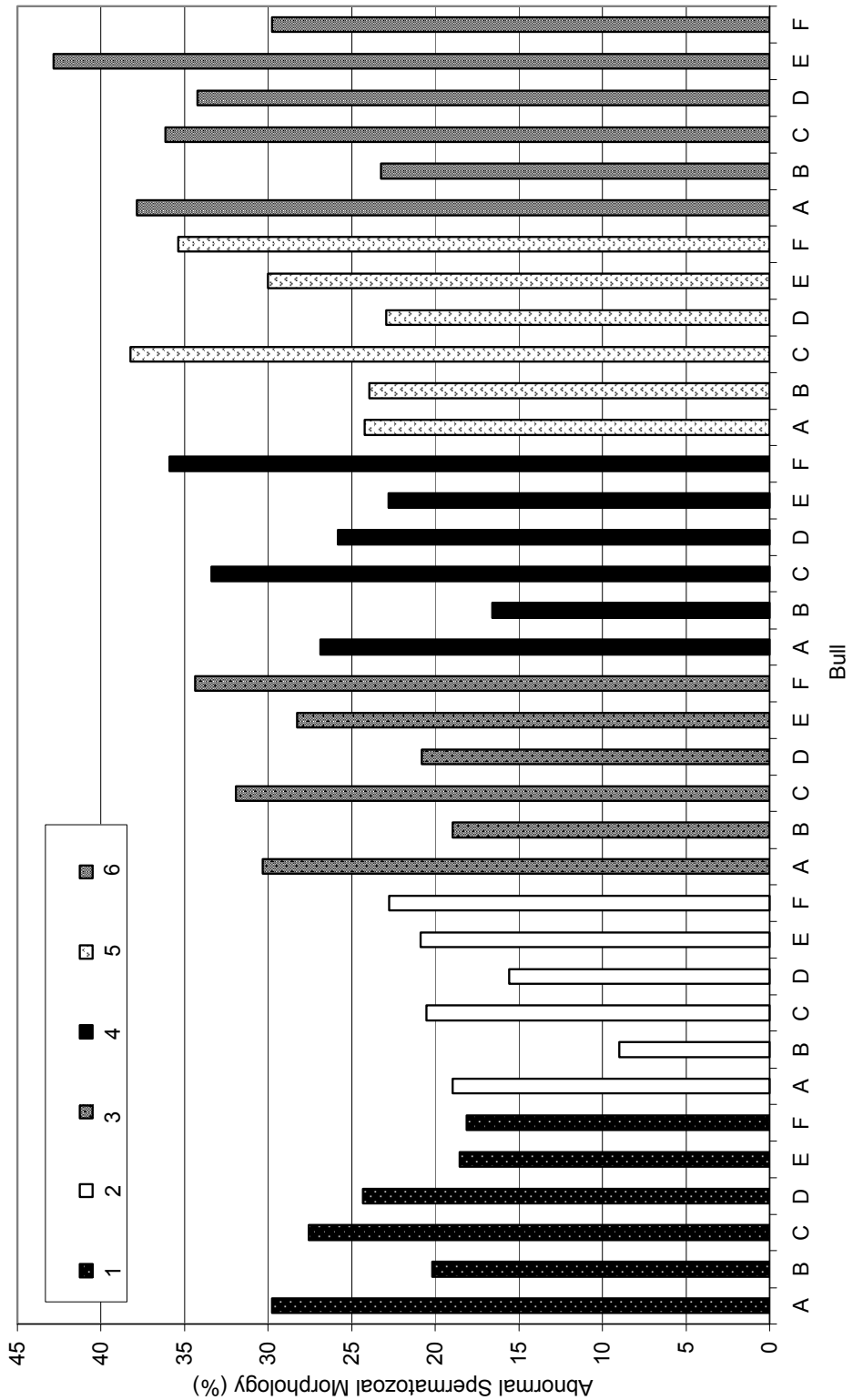

Fig. A-12. Experiment 1: Mean percentage secondary morphologically abnormal spermatozoa by bull (A to F) and collection date (1 to 6). Pooled SEM is 3.14.

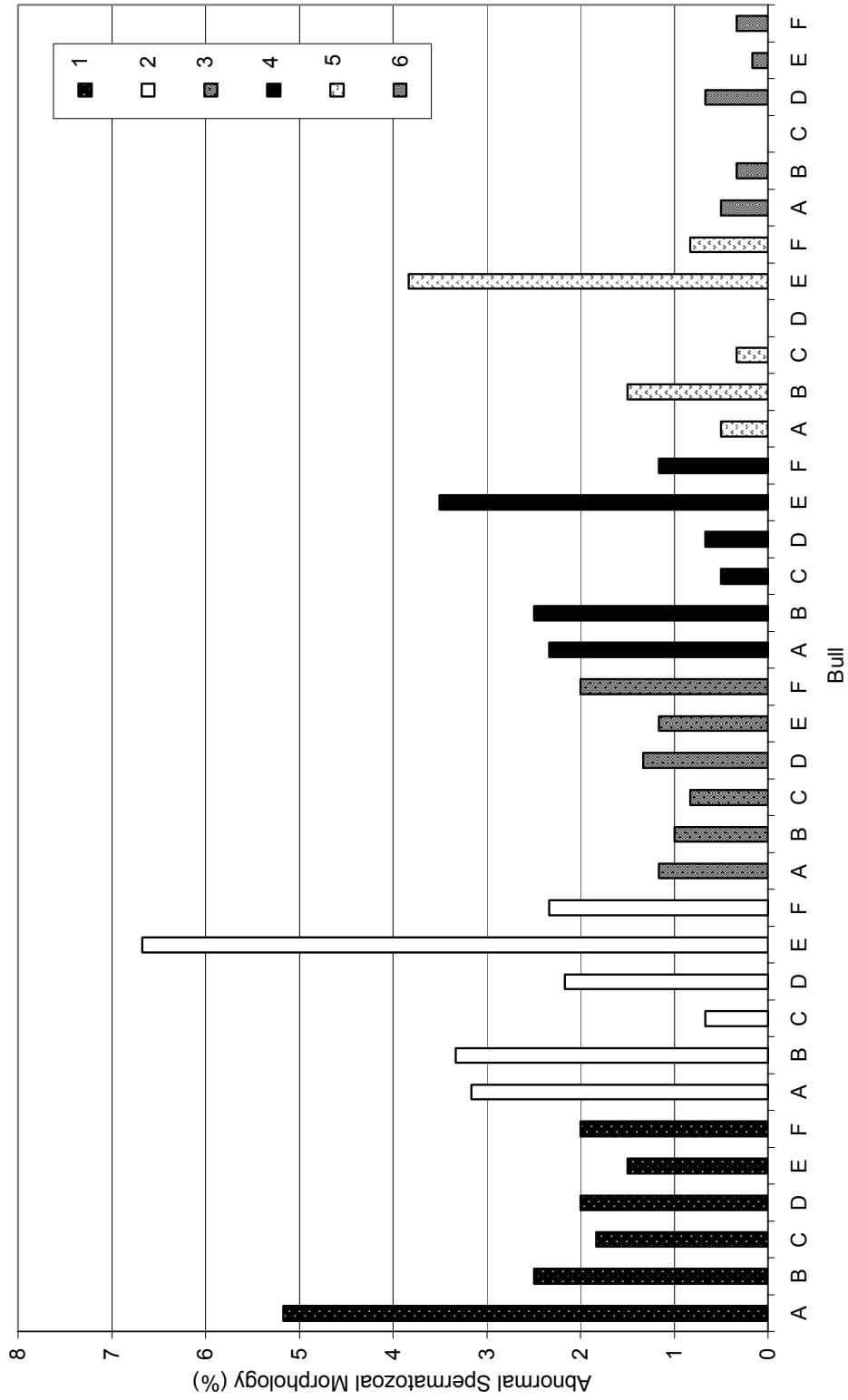

Fig. A-13. Experiment 1: Mean percentage tertiary morphologically abnormal spermatozoa bull (A to F) and collection date (1 to 6). Pooled SEM is 0.71.

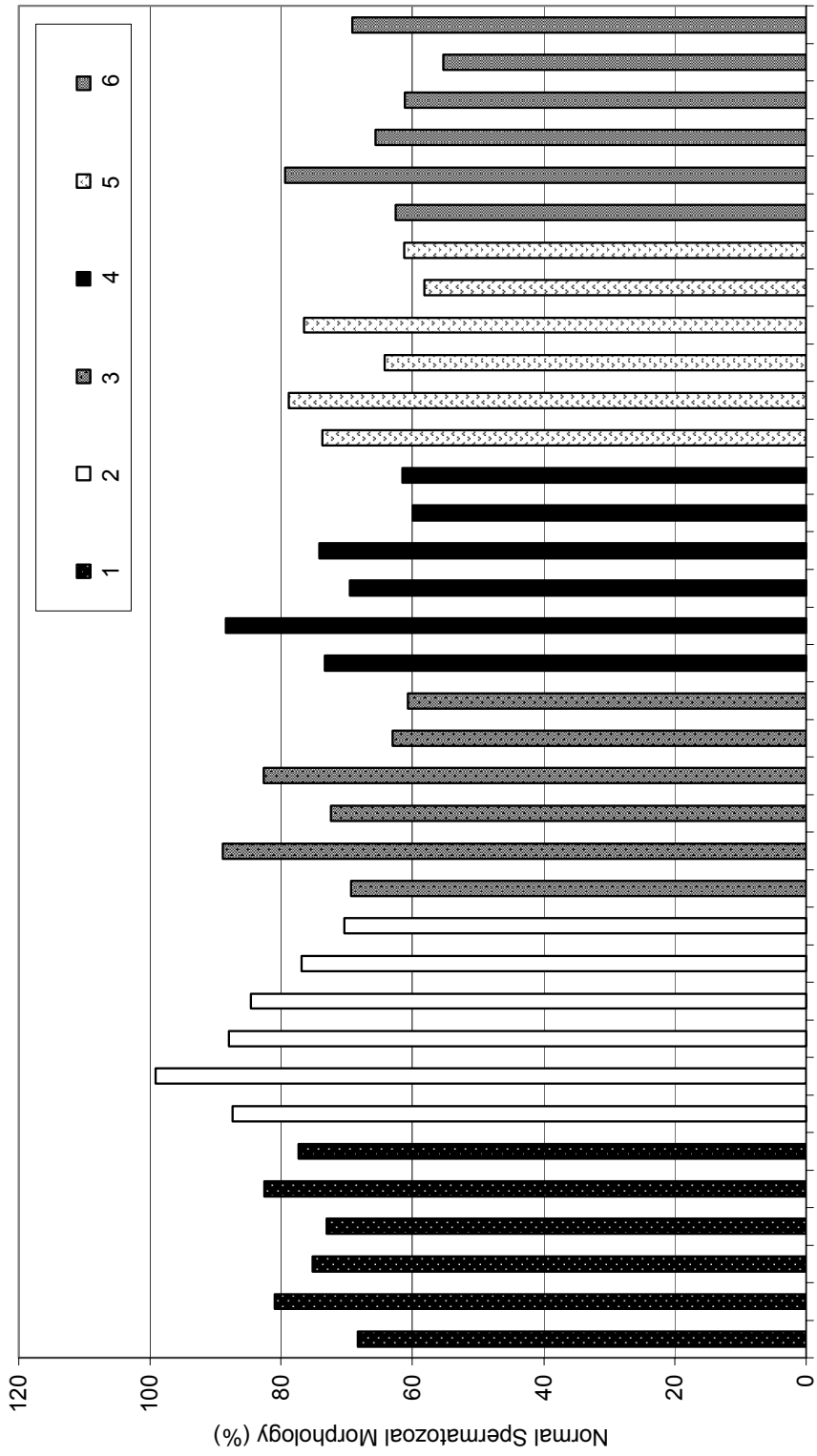

Fig. A-14. Experiment 1: Mean percentage morphologically normal spermatozoa by bull (A to F) and collection date (1 to 6). Pooled SEM is 4.08.

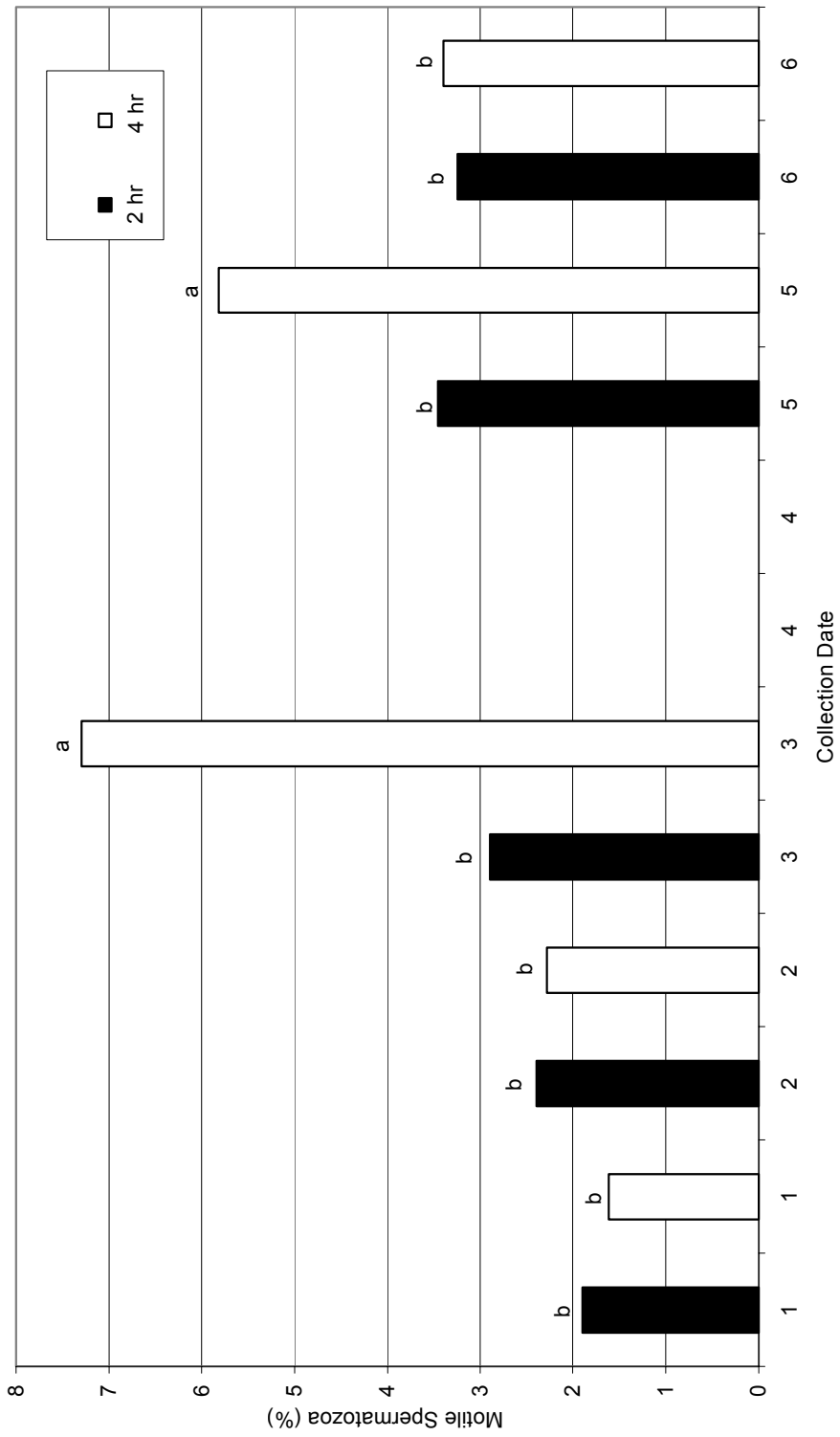

Fig. A-15. Experiment 1: Mean percentage motile spermatozoa by collection date (1 to 6) and cooling duration (2 or 4 hr) at 9 hr of incubation. Pooled SEM is 0.72. [a,b] LSMeans with different superscripts differ ($P < 0.05$).

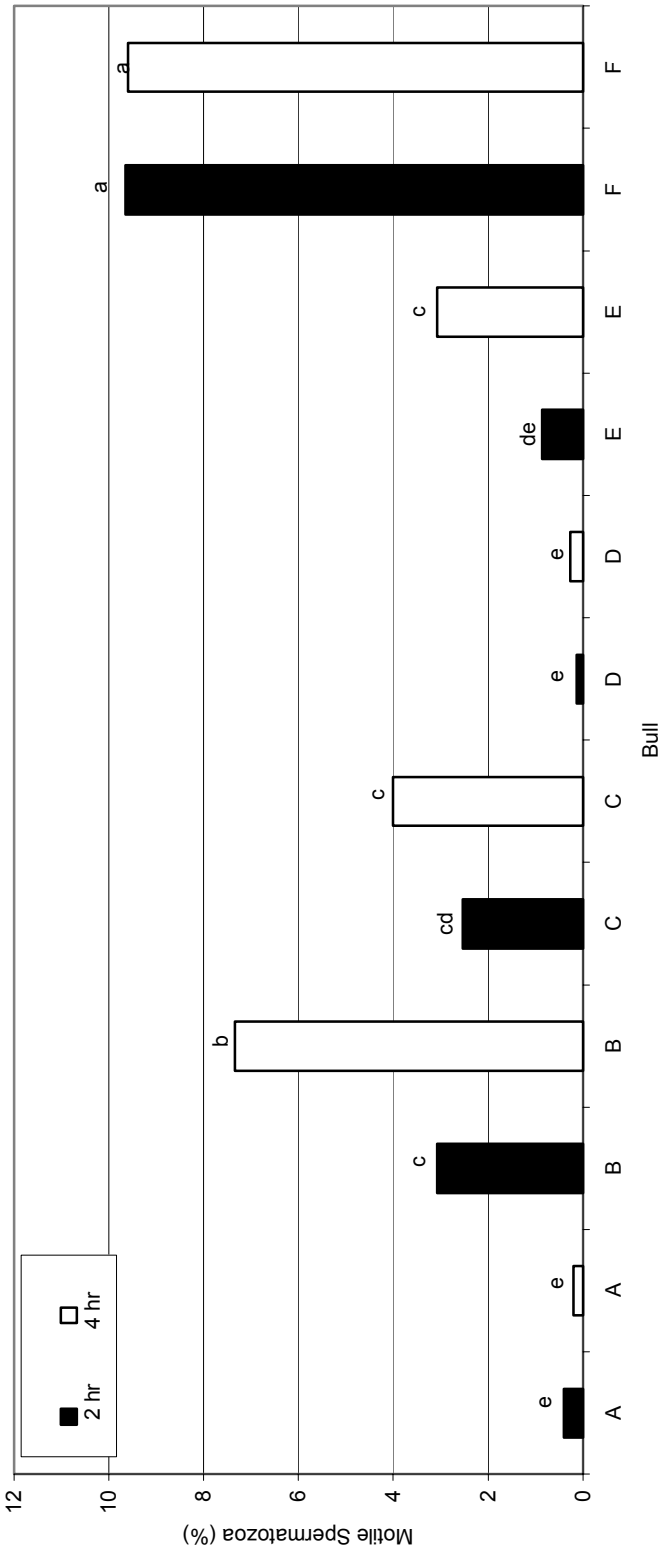

Fig. A-16. Experiment 1: Mean percentage motile spermatozoa by bull (A to F) and cooling duration (2 or 4 hr) at 9 hr of incubation. Pooled SEM is 0.72.
^{a,b,c,d,e} LSMeans with different superscripts differ ($P < 0.05$).

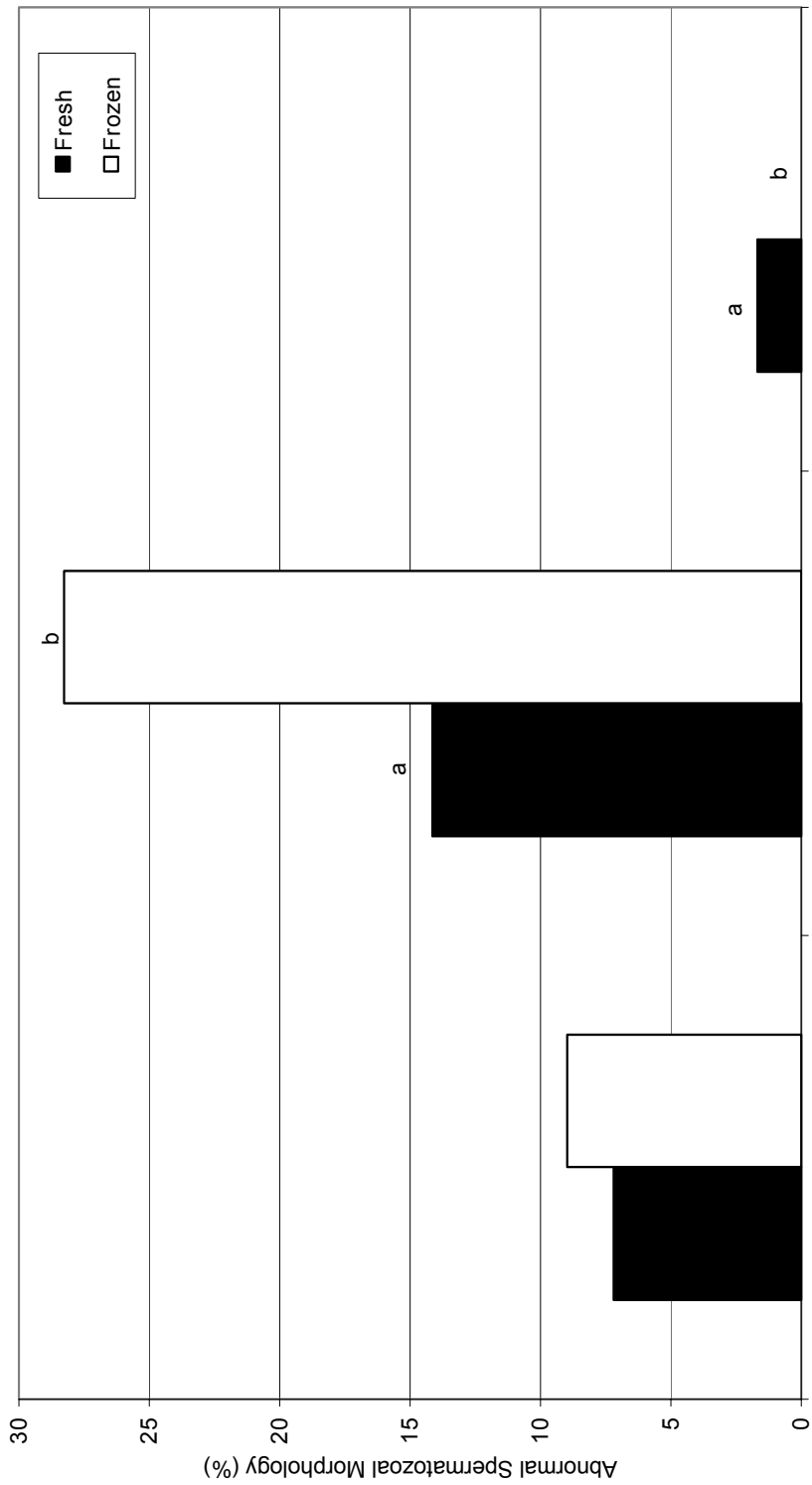

Fig. A-17. Experiment 2: Mean percentage morphologically abnormal spermatozoa (1° = primary, 2° = secondary, or 3° = tertiary abnormalities) by semen storage type (fresh or frozen). Pooled SEM is 0.30, 0.62, and 0.13 for 1°, 2°, and 3°, respectively.
[a,b] LSMeans with different superscripts within abnormality classification differ ($P < 0.05$).

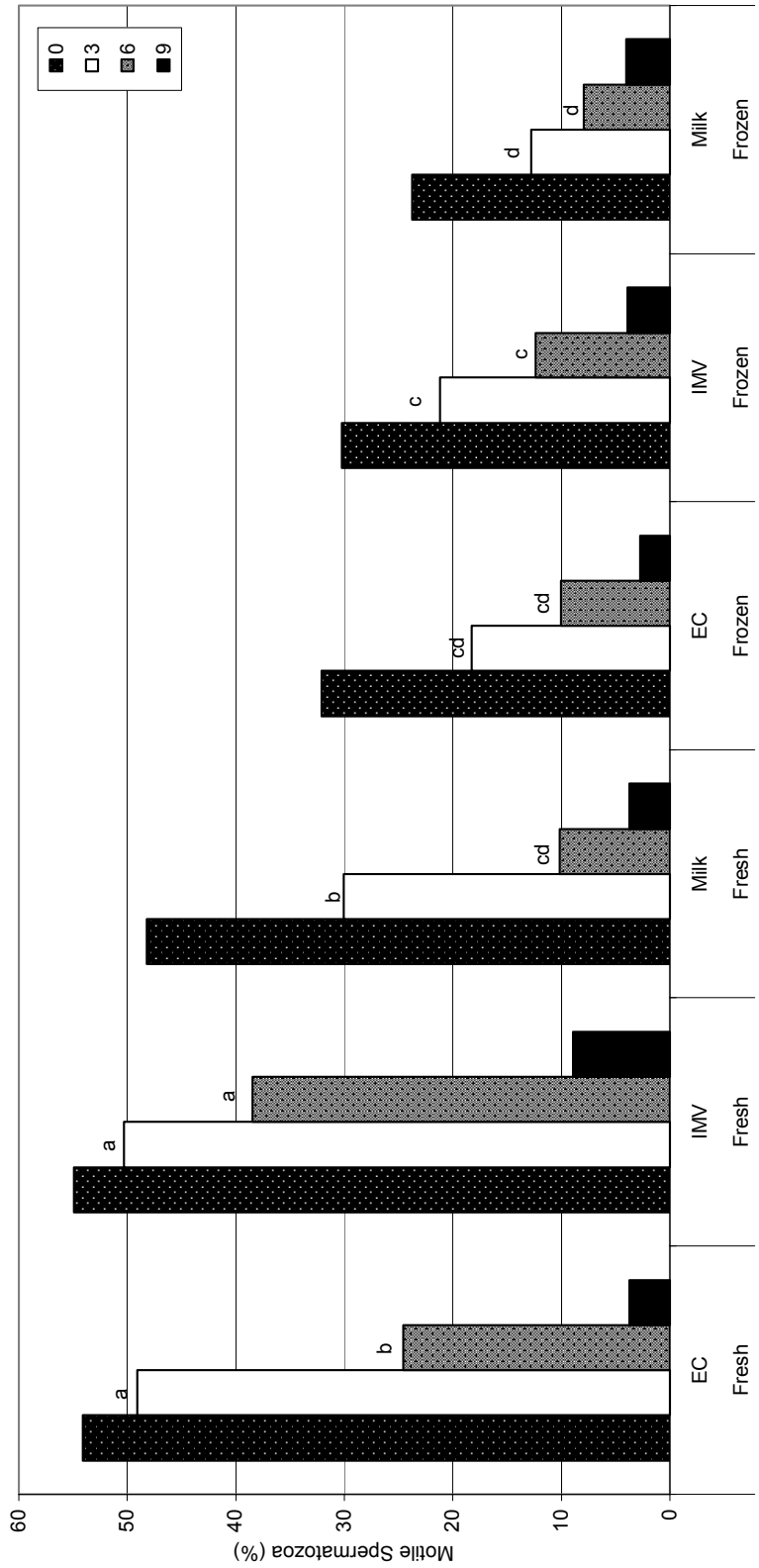

Fig. A-18. Experiment 2: Mean percentage motile spermatozoa by semen storage type (fresh or frozen) and extender (EC = egg yolk-citrate, IMV, or Milk = skim milk) at 0, 3, 6, and 9 hr of incubation. Pooled SEM is 1.78, 1.68, 2.15, and 1.86 for 0, 3, 6, and 9 hr of incubation, respectively.
[a,b,c,d] LSMeans with different superscripts within incubation time differ ($P < 0.05$).

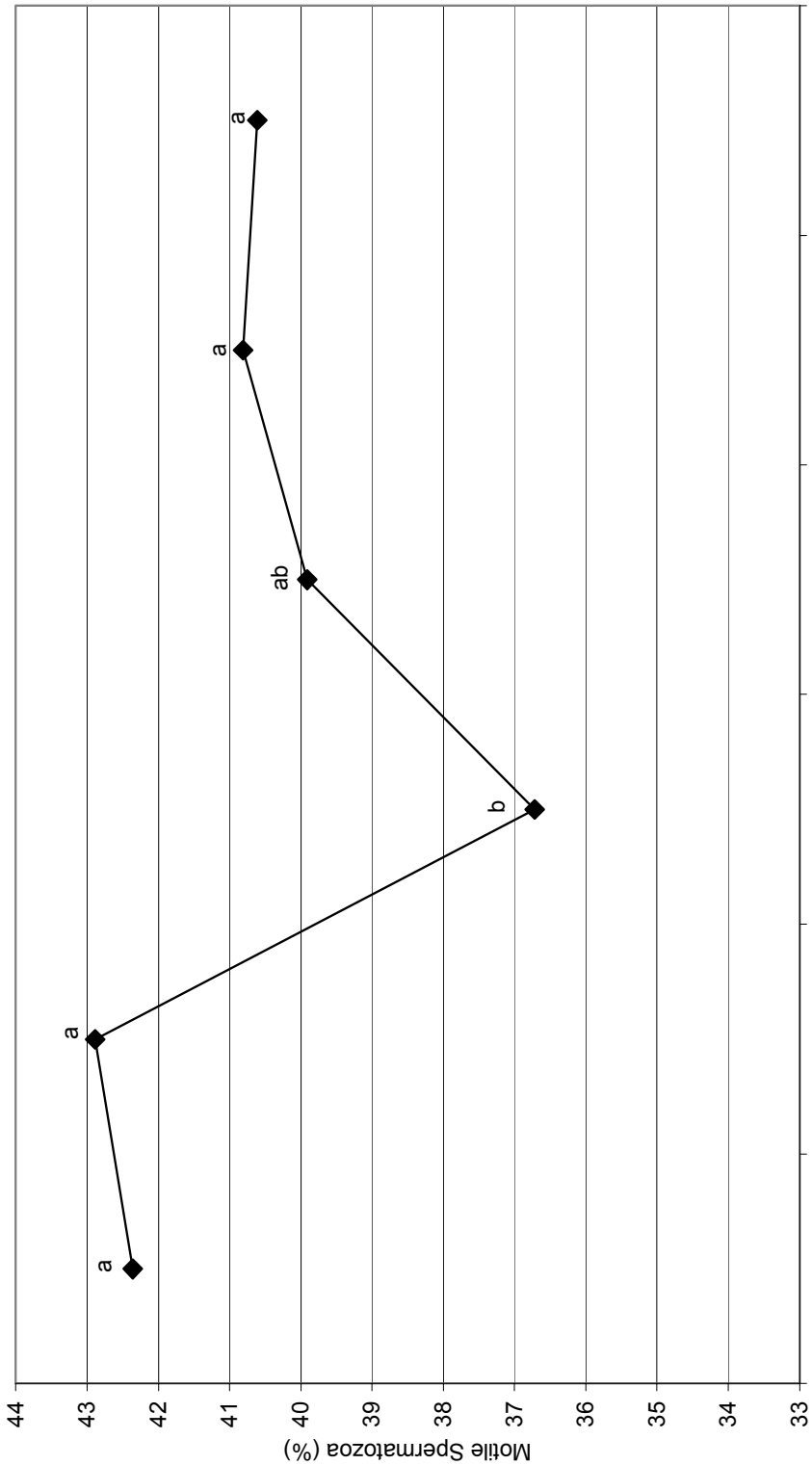

Fig. A-19. Experiment 2: Mean percentage motile spermatozoa by collection date (1 to 6) at 0 hr of incubation. Pooled SEM is 1.26.
[a,b] LSMeans with different superscripts differ ($P < 0.05$).

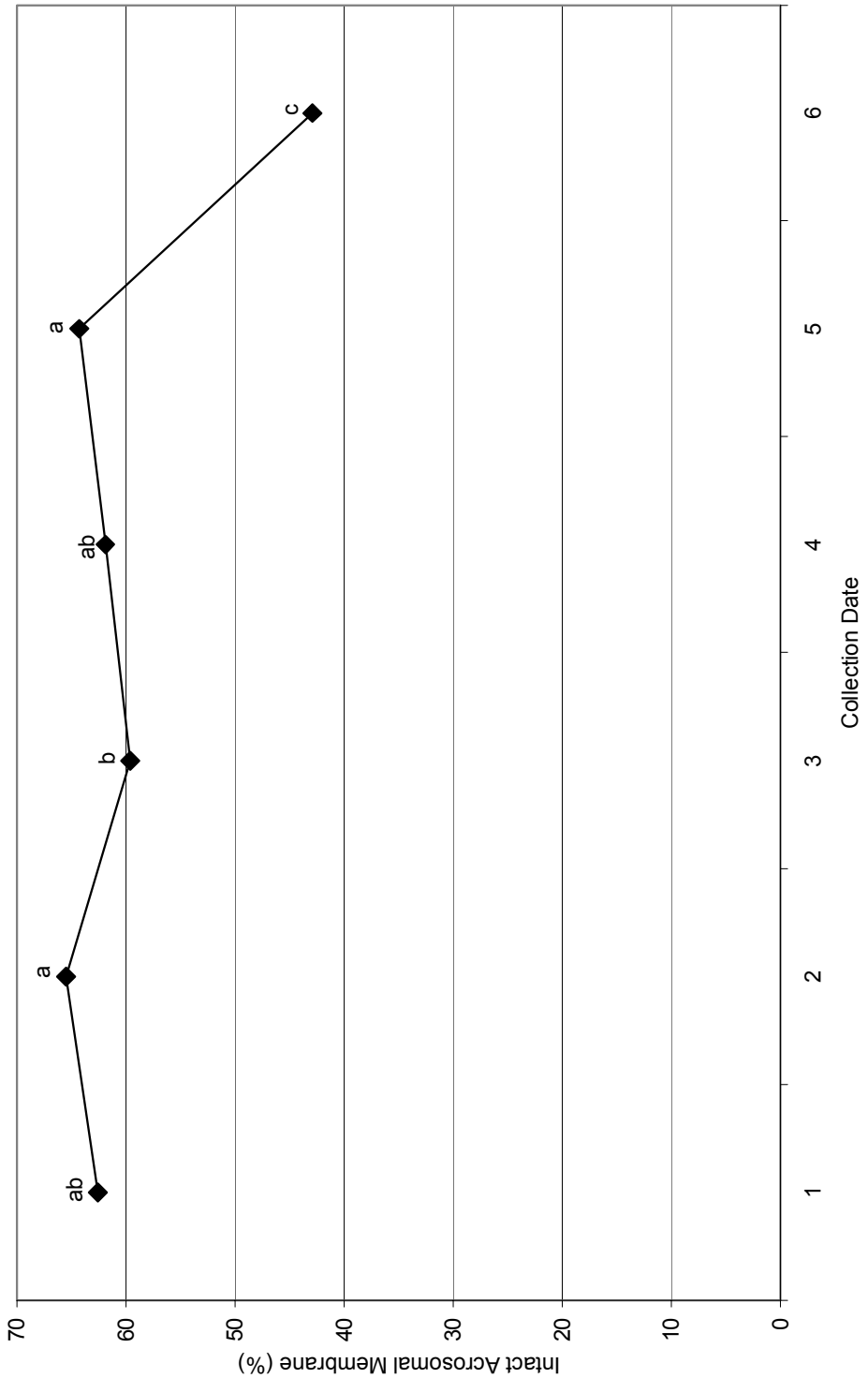

Fig. A-20. Experiment 2: Mean percentage of spermatozoa with an intact acrosomal membrane by collection date (1 to 6). Pooled SEM is 1.40. ^{a,b,c} LSMeans with different superscripts differ ($P < 0.05$).

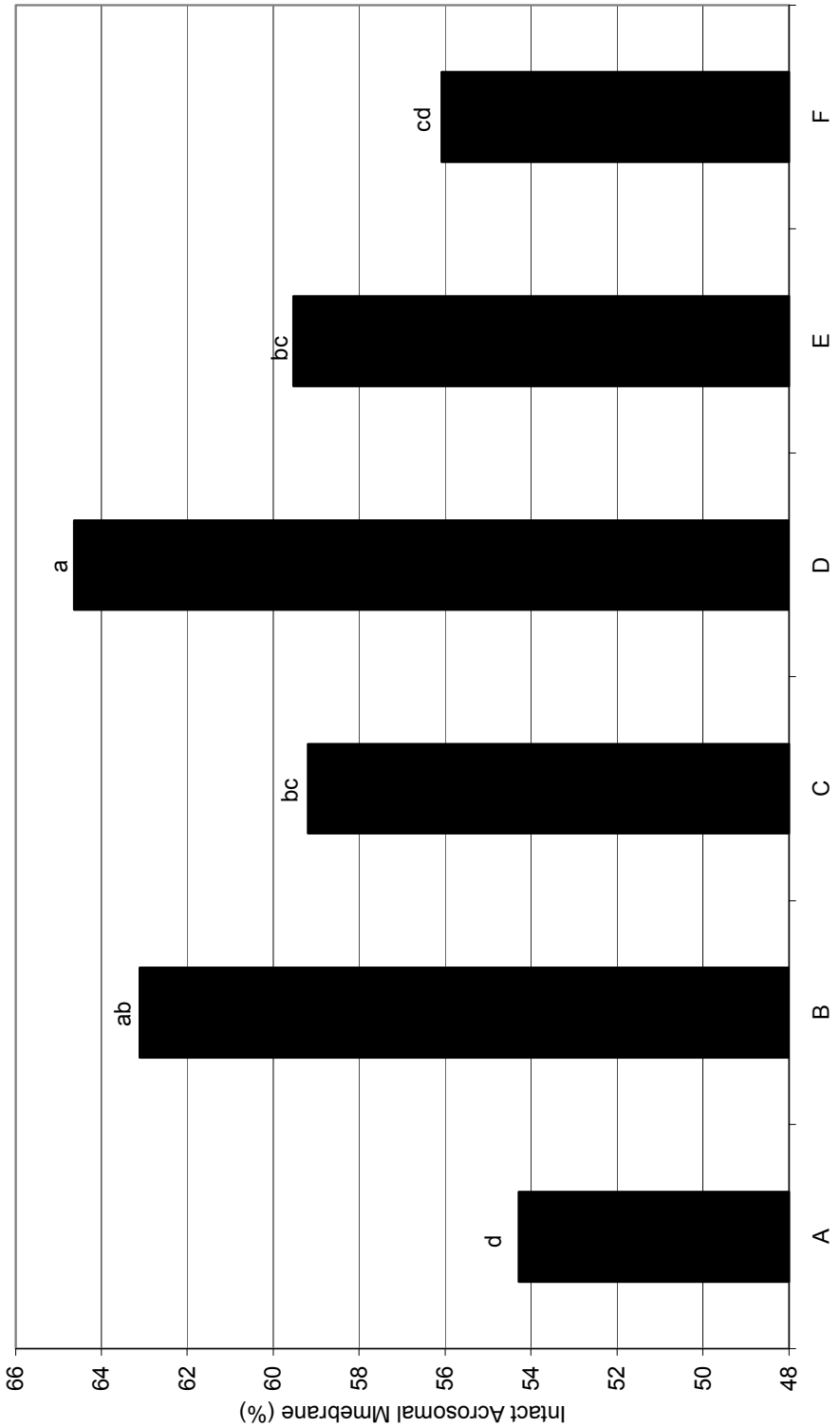

Fig. A-21. Experiment 2: Mean percentage of spermatozoa with an intact acrosomal membrane by bull (A to F). Pooled SEM is 1.40. ^{a,b,c,d} LSMeans with different superscripts differ ($P < 0.05$).

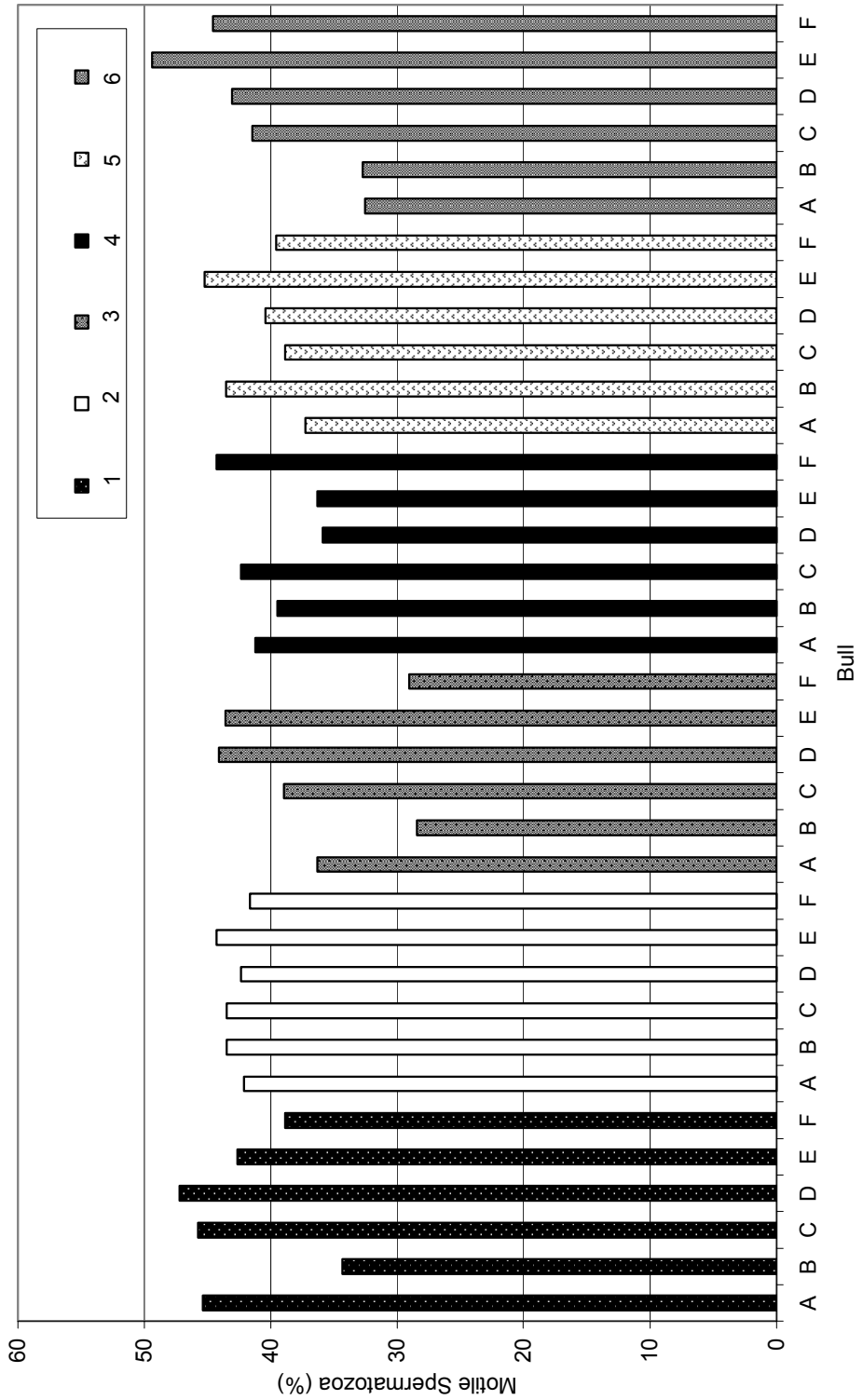

Fig. A-22. Experiment 2: Mean percentage motile spermatozoa by bull (A to F) and collection date (1 to 6) at 0 hr of incubation. Pooled SEM is 3.07.

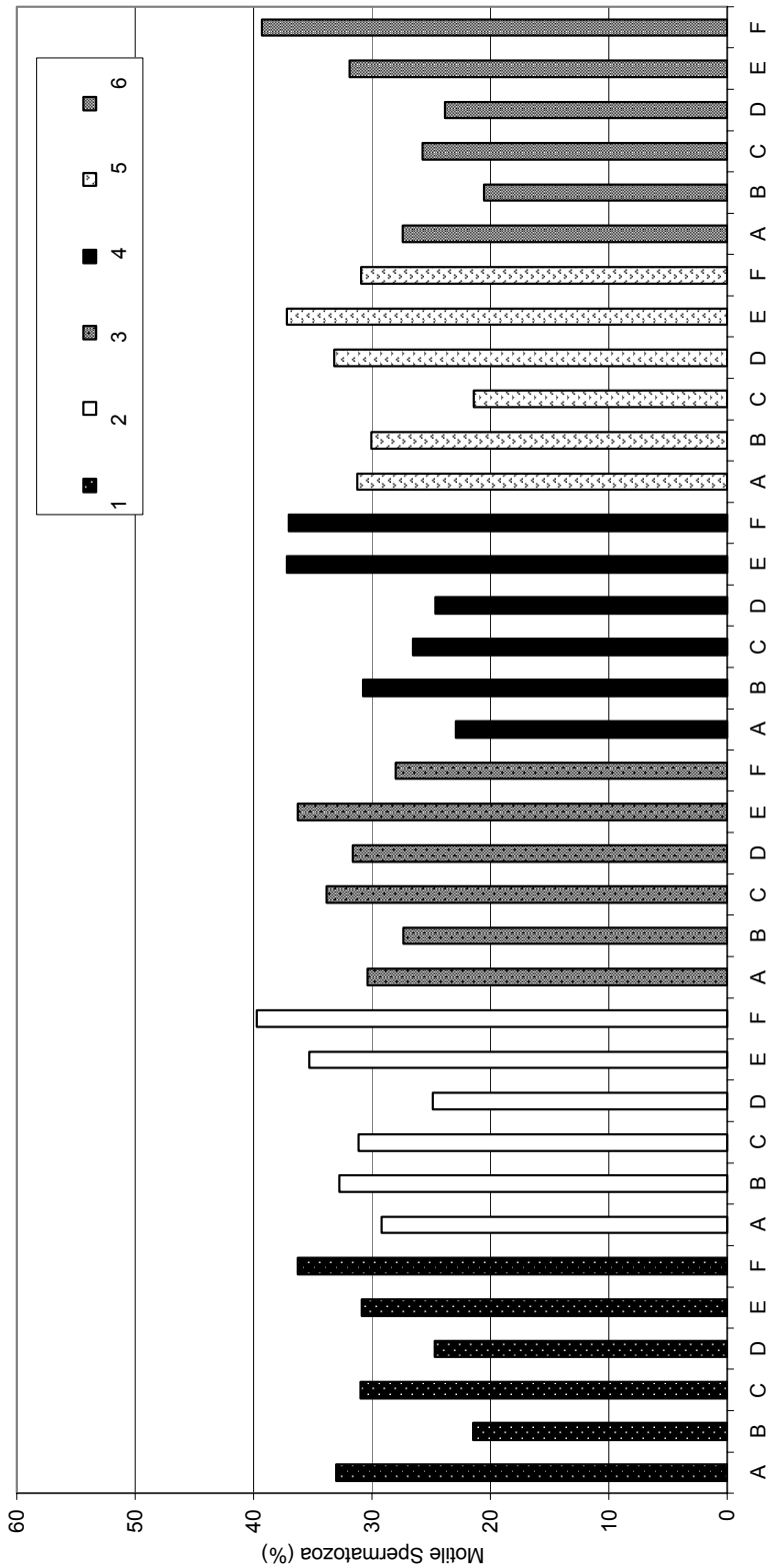

Fig. A-23. Experiment 2: Mean percentage motile spermatozoa by bull (A to F) and collection date (1 to 6) at 3 hr of incubation. Pooled SEM is 2.91.

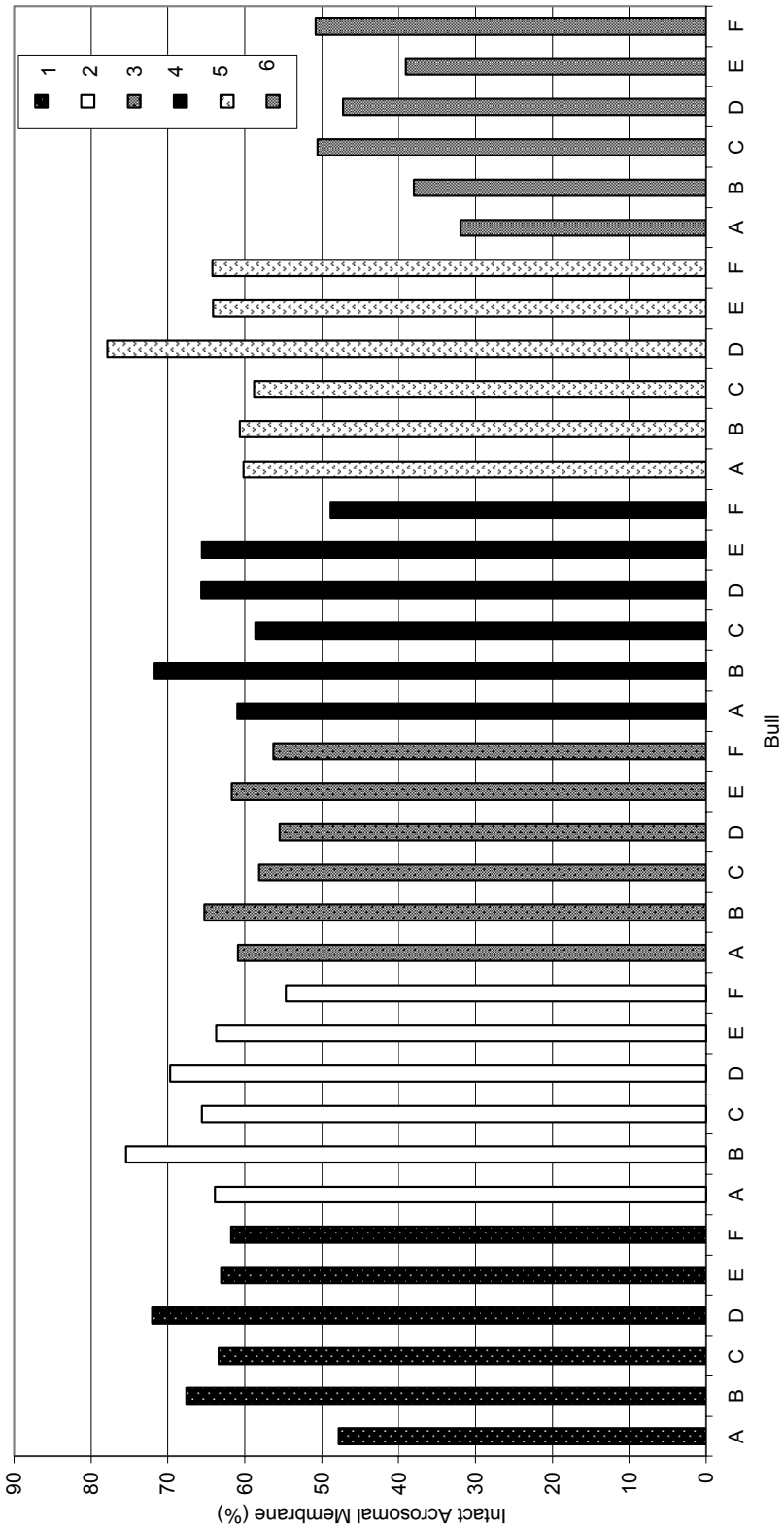

Fig. A-24. Experiment 2: Mean percentage of spermatozoa with an intact acrosomal membrane by bull (A to F) and collection date (1 to 6). Pooled SEM is 3.44.

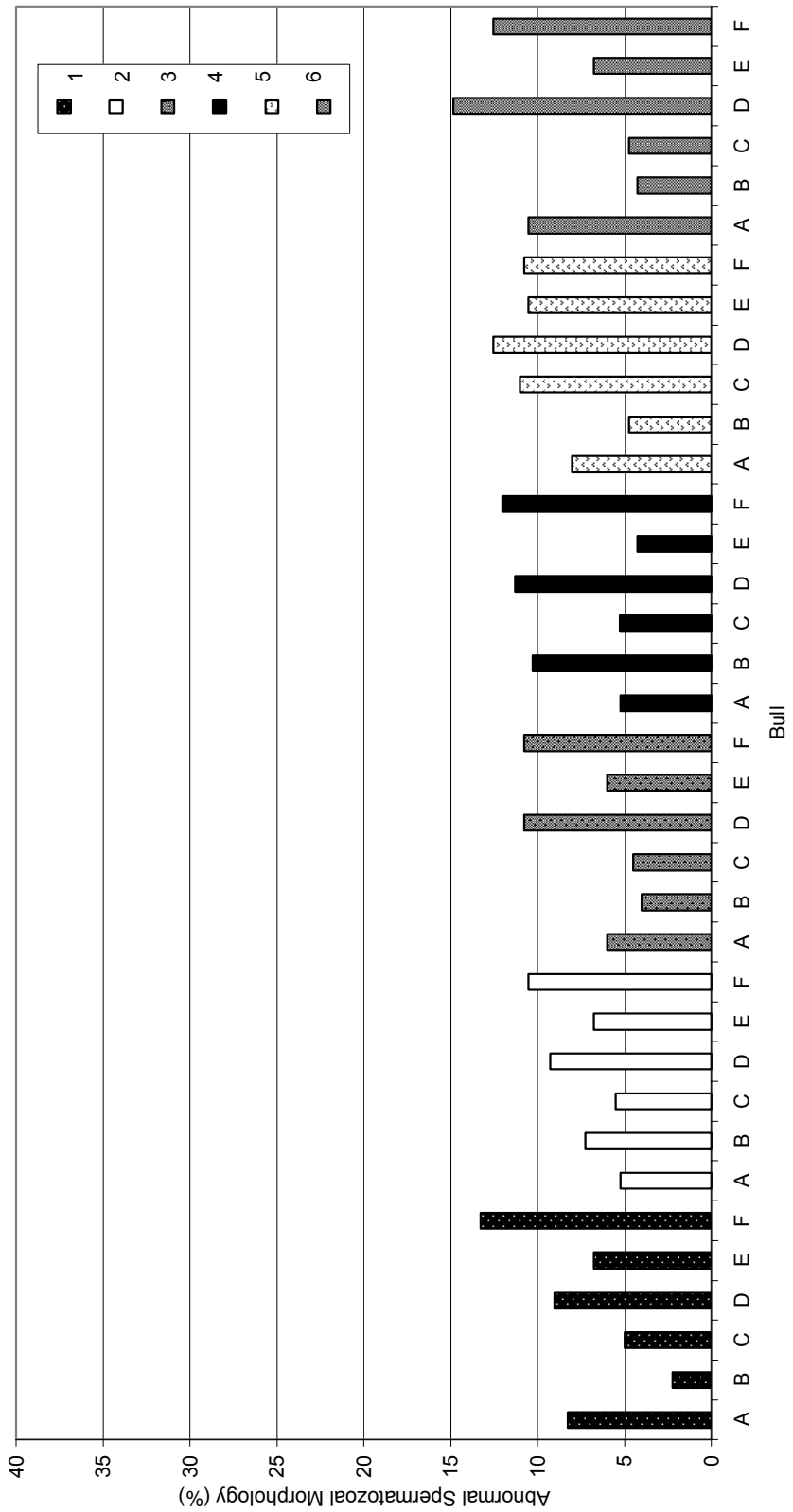

Fig. A-25. Experiment 2: Mean percentage primary morphologically abnormal spermatozoa by bull (A to F) and collection date (1 to 6). Pooled SEM is 1.28.

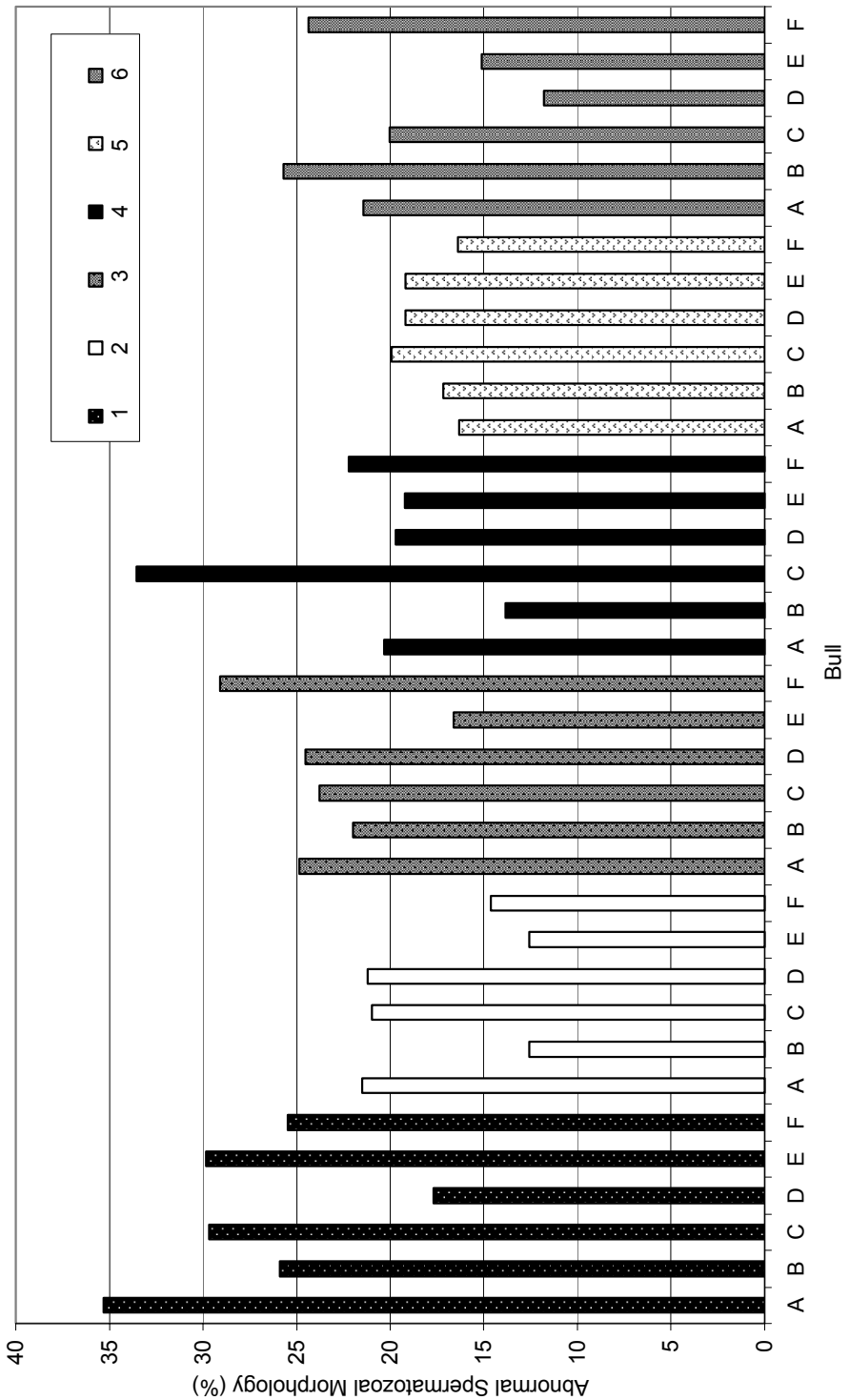

Fig. A-26. Experiment 2: Mean percentage secondary morphologically abnormal spermatozoa by bull (A to F) and collection date (1 to 6). Pooled SEM is 2.62.

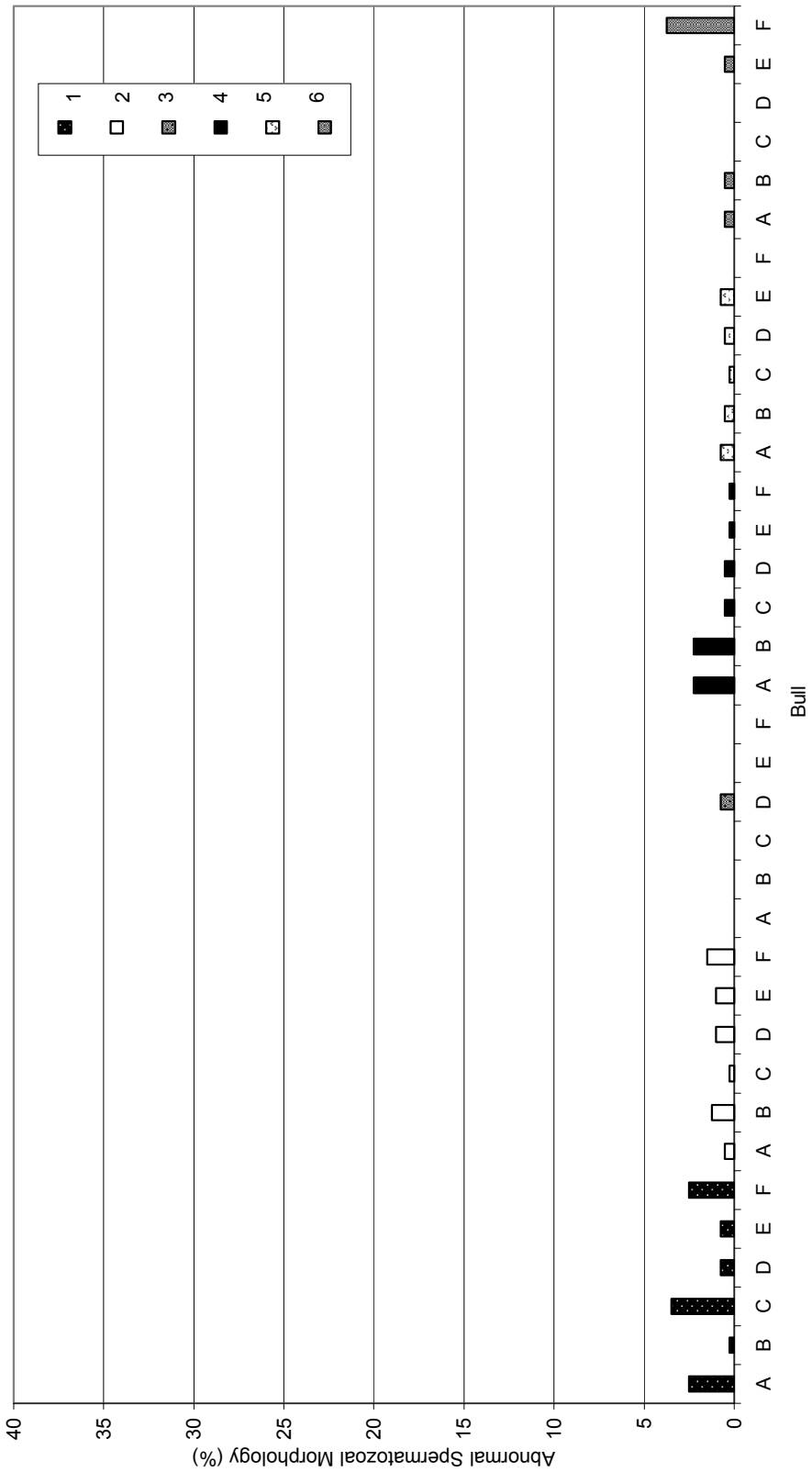

Fig. A-27. Experiment 2: Mean percentage tertiary morphologically abnormal spermatozoa by bull (A to F) and collection date (1 to 6). Pooled SEM is 0.56.

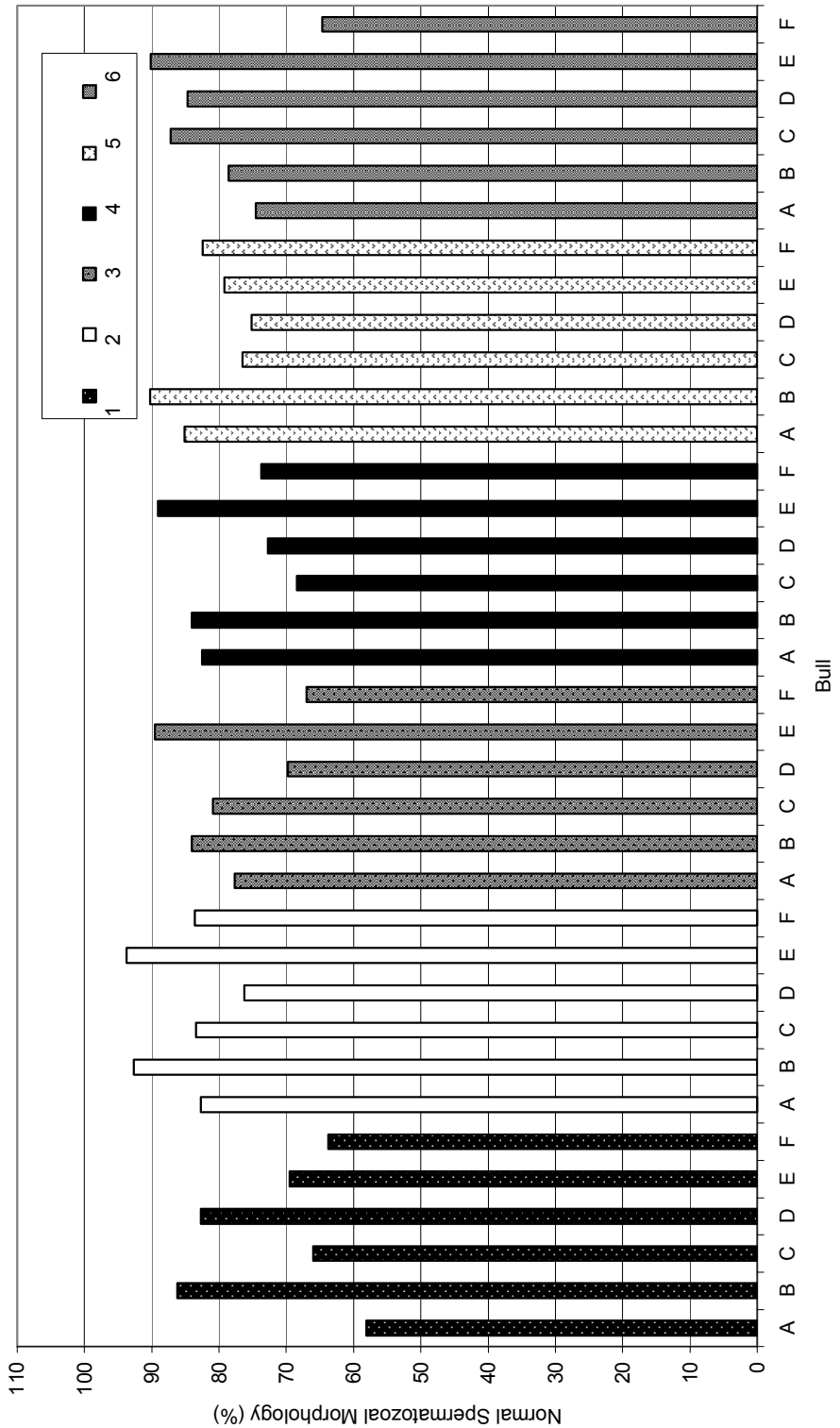

Fig. A-28. Experiment 2: Mean percentage morphologically normal spermatozoa by bull (A to F) and collection date (1 to 6). Pooled SEM is 4.01.

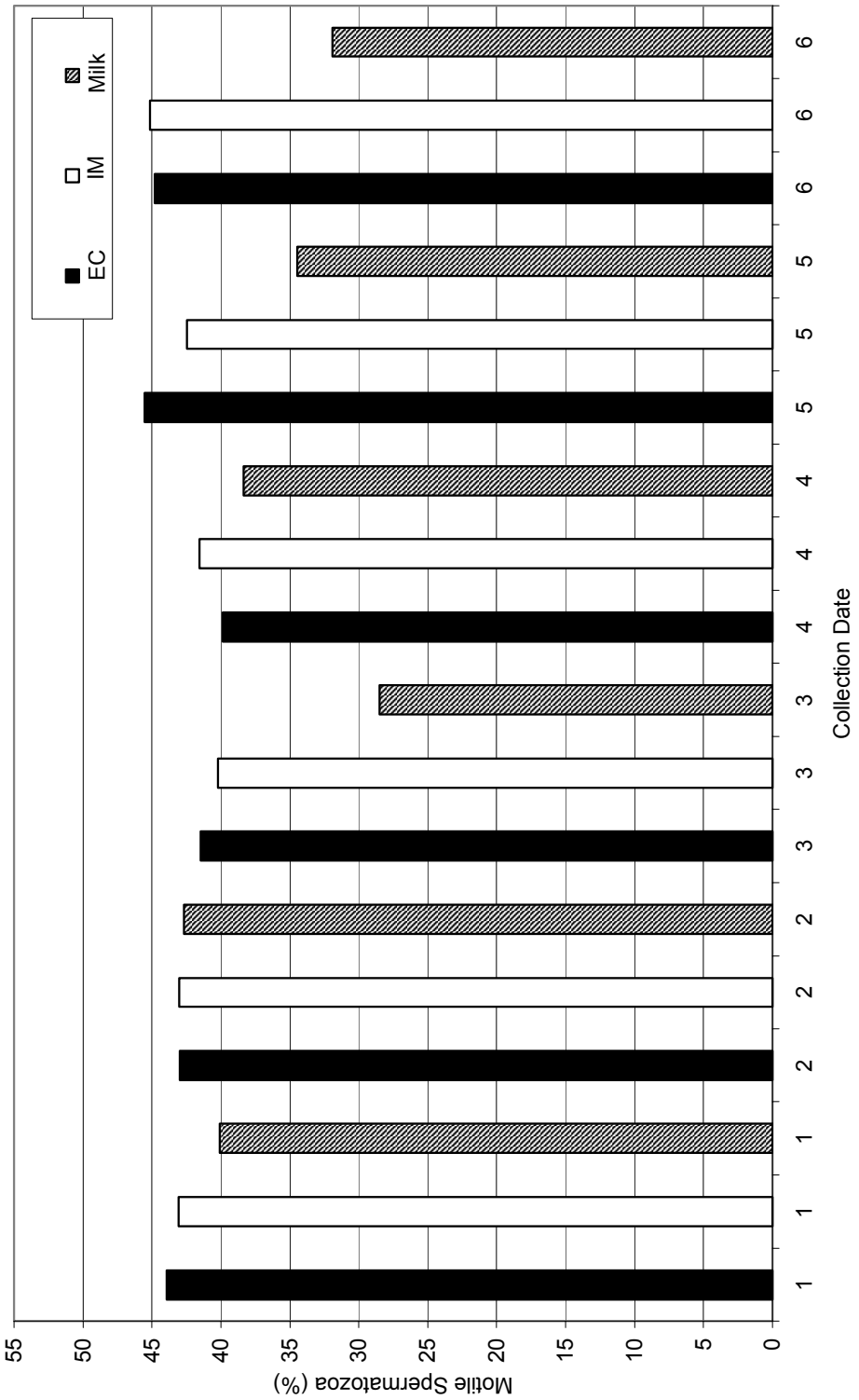

Fig. A-29. Experiment 2: Mean percentage motile spermatozoa by collection date (1 to 6) and extender (EC = egg yolk-citrate, Milk = skim milk, or IMV) at 0 hr of incubation. Pooled SEM is 2.17.

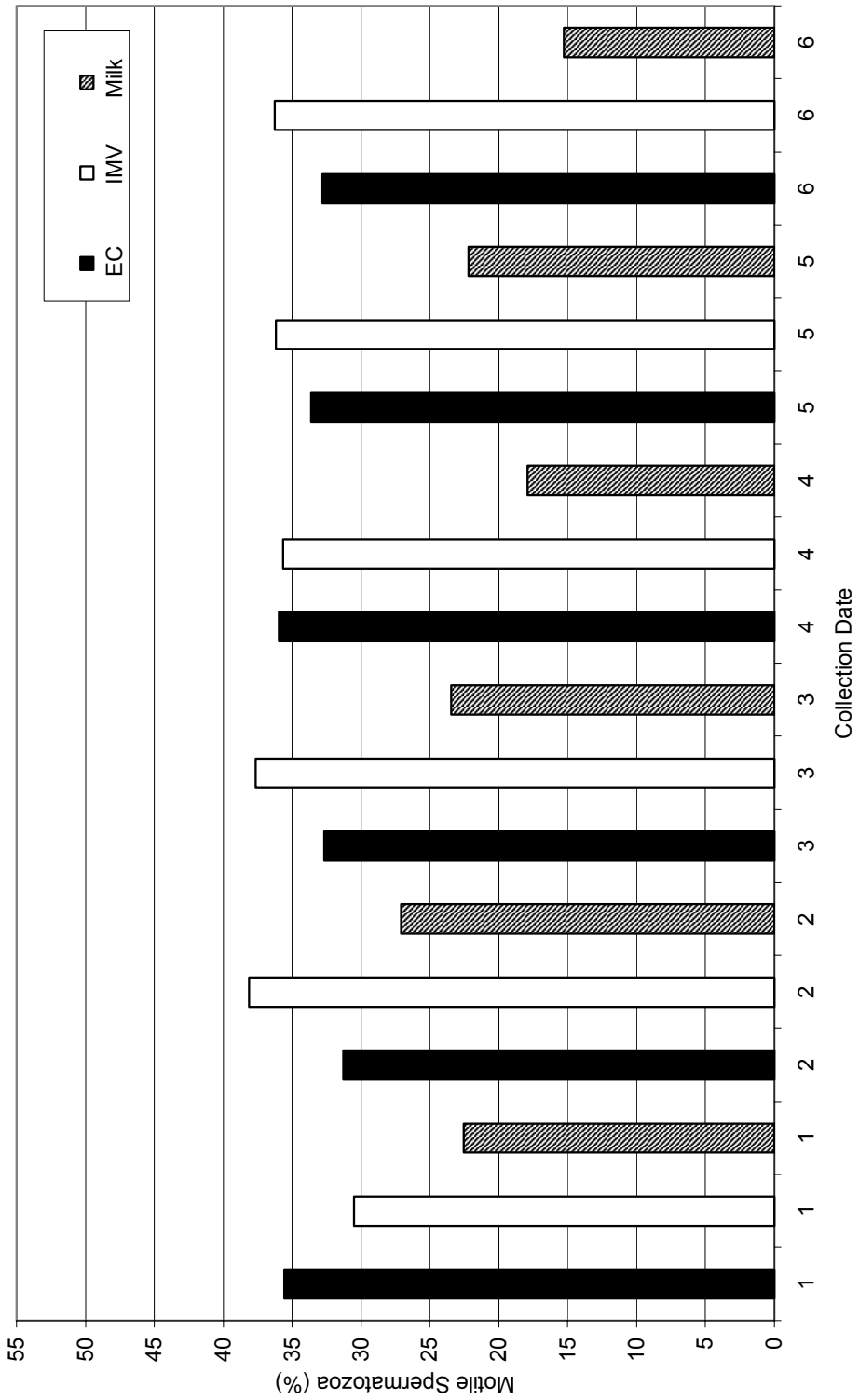

Fig. A-30. Experiment 2: Mean percentage motile spermatozoa by collection date (1 to 6) and extender (EC = egg yolk-citrate, Milk = skim milk, or IMV) at 3 hr of incubation. Pooled SEM is 2.06.

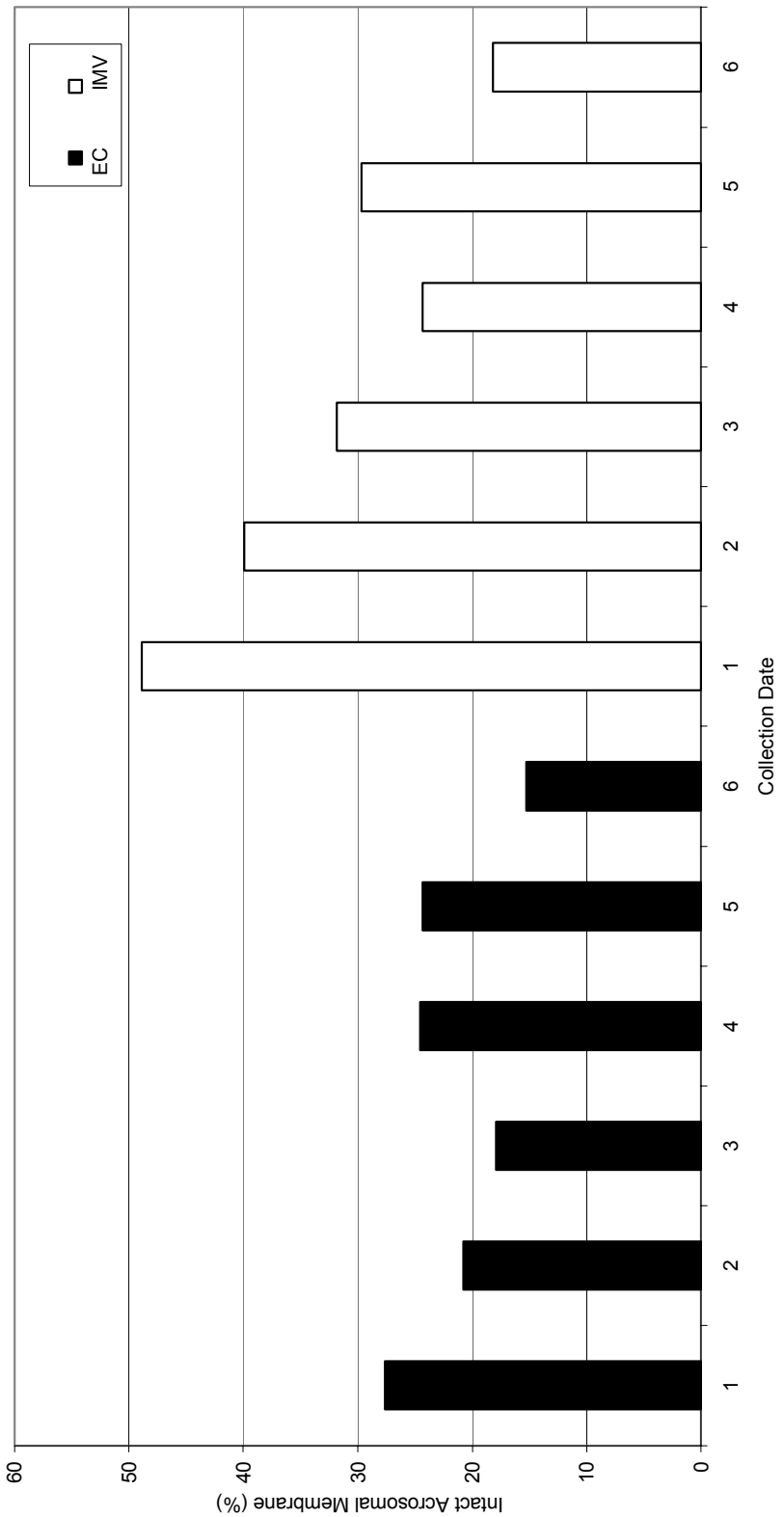

Fig. A-31. Experiment 2: Mean percentage semen with an intact acrosomal membrane by collection date (1 to 6) and extender (EC = egg yolk-citrate or IMV). Pooled SEM is 1.99.

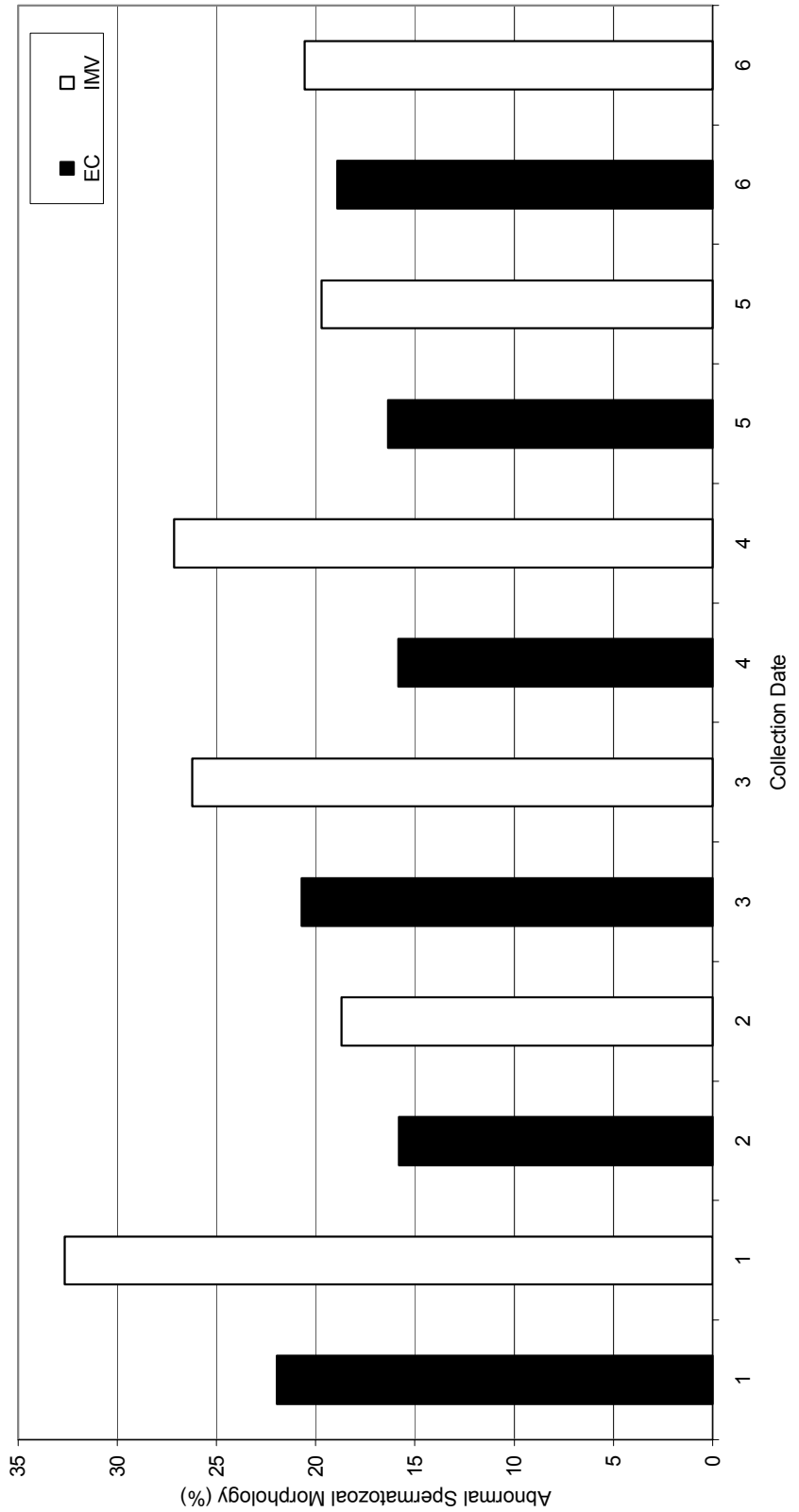

Fig. A-32. Mean percentage secondary morphologically abnormal spermatozoa by collection date (1 to 6) and extender (EC = egg yolk-citrate and IMV). Pooled SEM is 1.51.

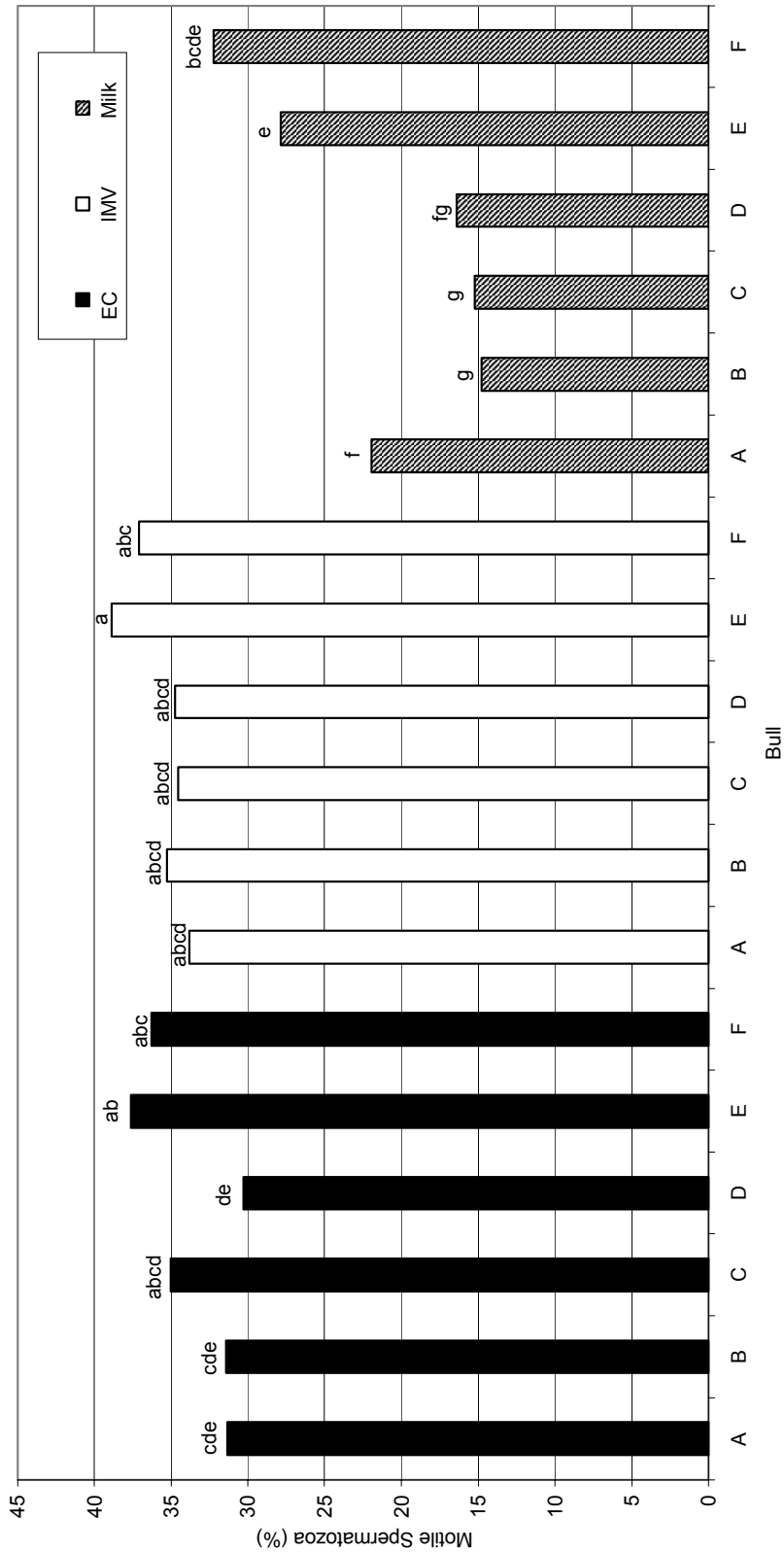

Fig. A-33. Experiment 2: Mean percentage motile spermatozoa by bull (A to F) and extender (EC = egg yolk-citrate, Milk = skim milk, or IMV) at 3 hr of incubation. Pooled SEM is2.06. ^{a,b,c,d,e,f,g} LSMeans with different superscripts differ ($P < 0.05$).

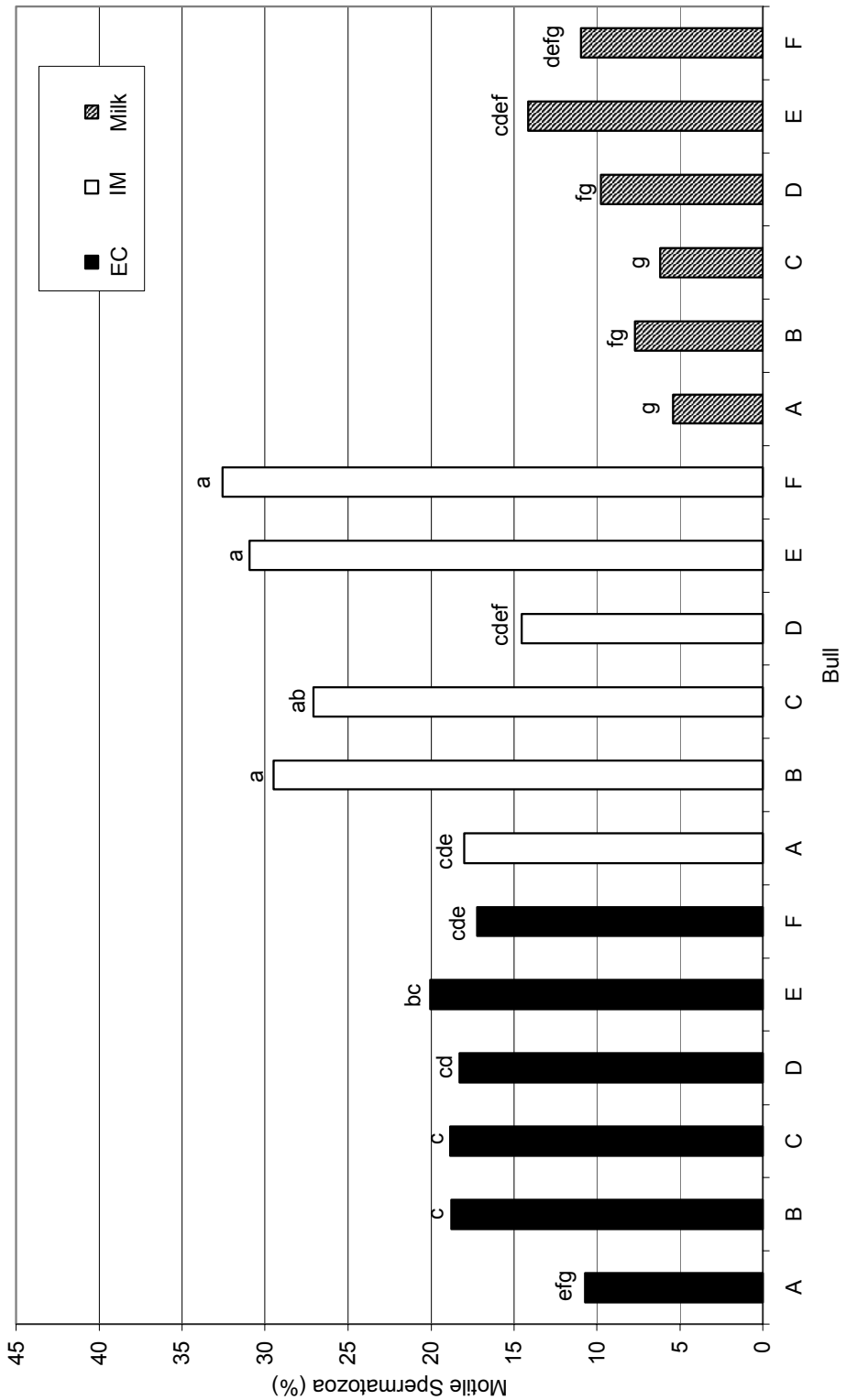

Fig. A-34. Experiment 2: Mean percentage motile spermatozoa by bull (A to F) and extender (EC = egg yolk-citrate, Milk = skim milk, or IMV) at 6 hr of incubation. Pooled SEM is2.63. ^{a,b,c,d,e,f,g} LSMeans with different superscripts differ ($P < 0.05$).

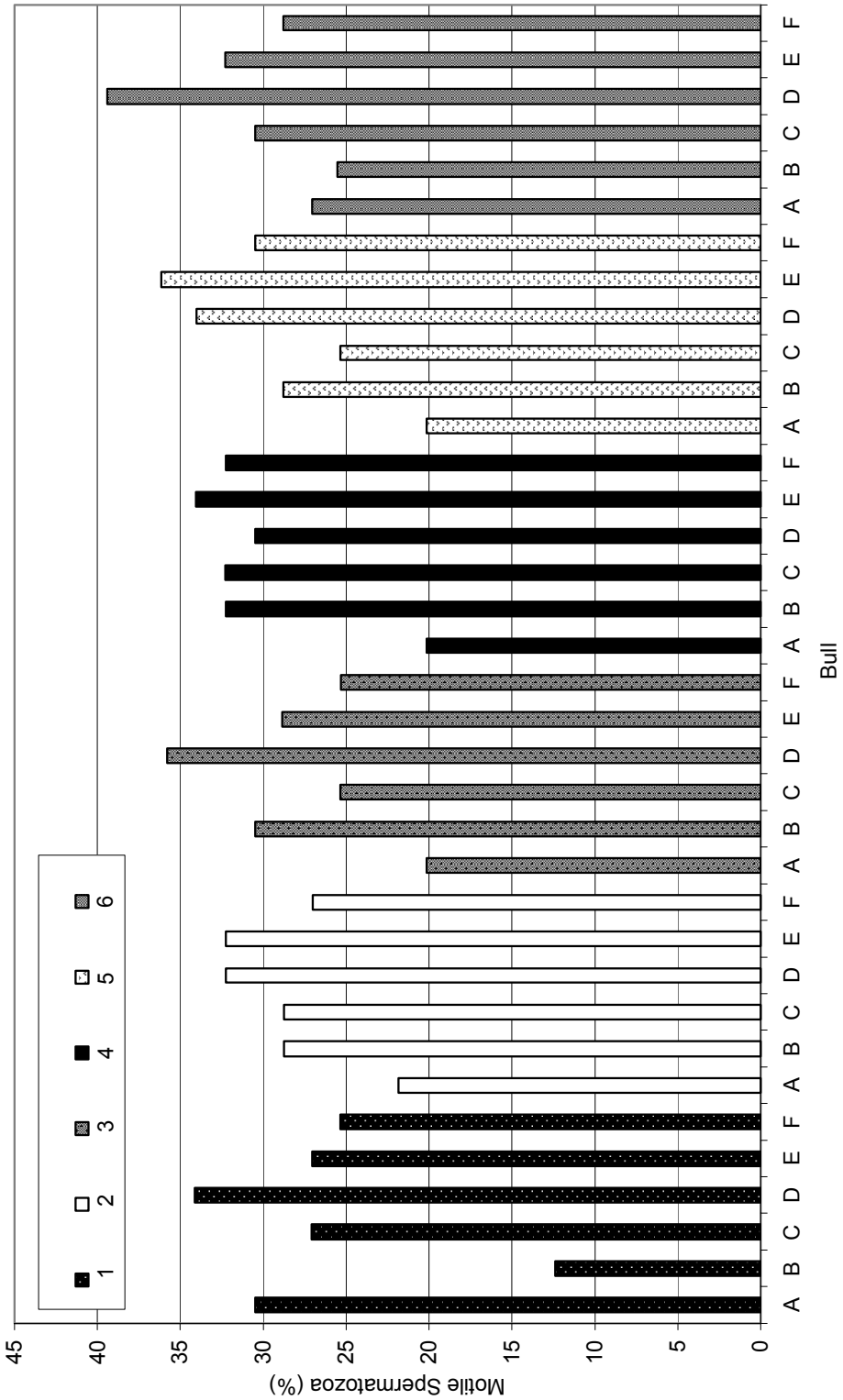

Fig. A-35. Experiment 2: Mean percentage motile spermatozoa by collection date (1 to 6), bull (A to F), and semen storage type (frozen) at 0 hr of incubation. Pooled SEM is 4.35.

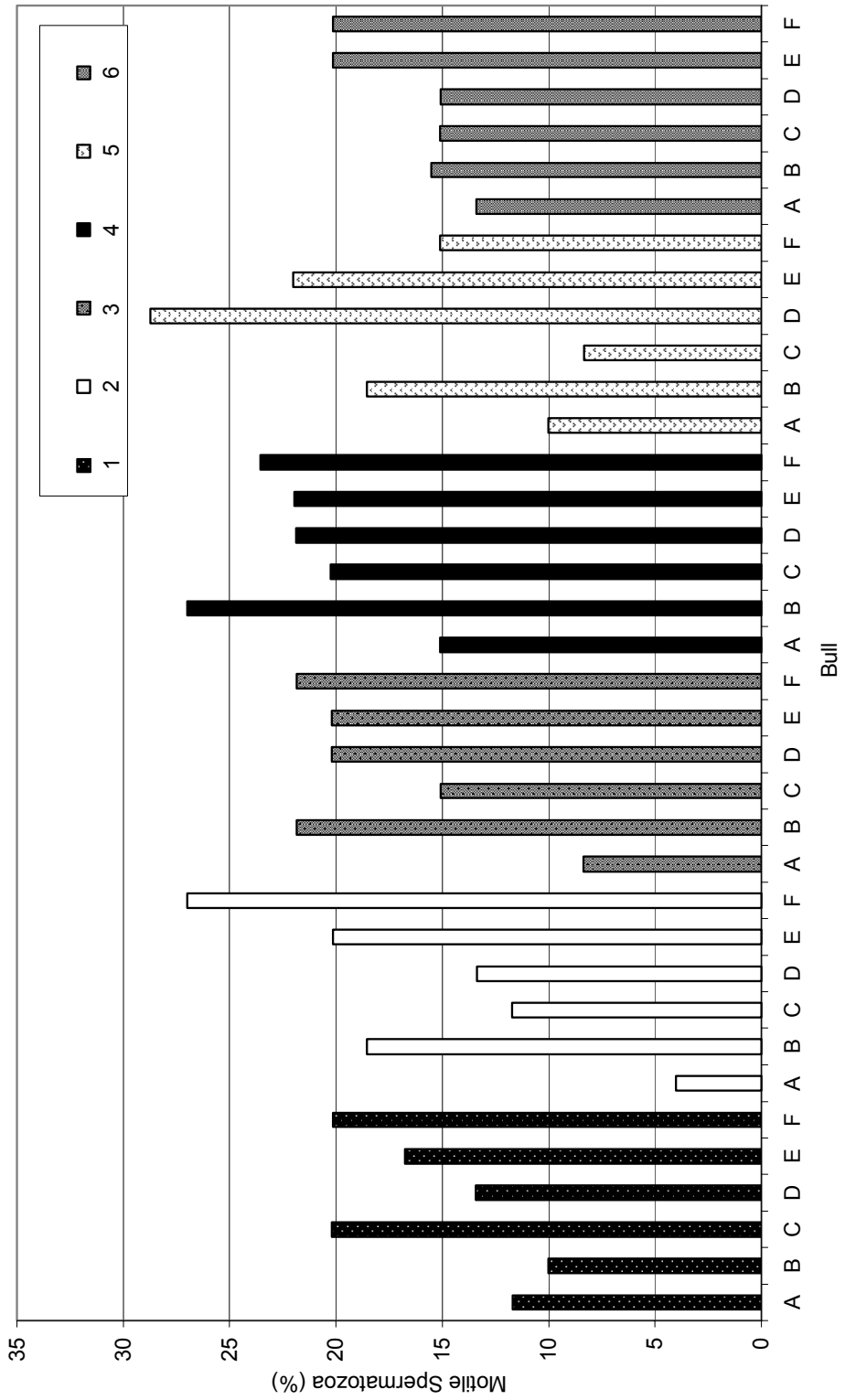

Fig. A-36. Experiment 2: Mean percentage motile spermatozoa by collection date (1 to 6), bull (A to F), and semen storage type (frozen) at 3 hr of incubation. Pooled SEM is 4.12.

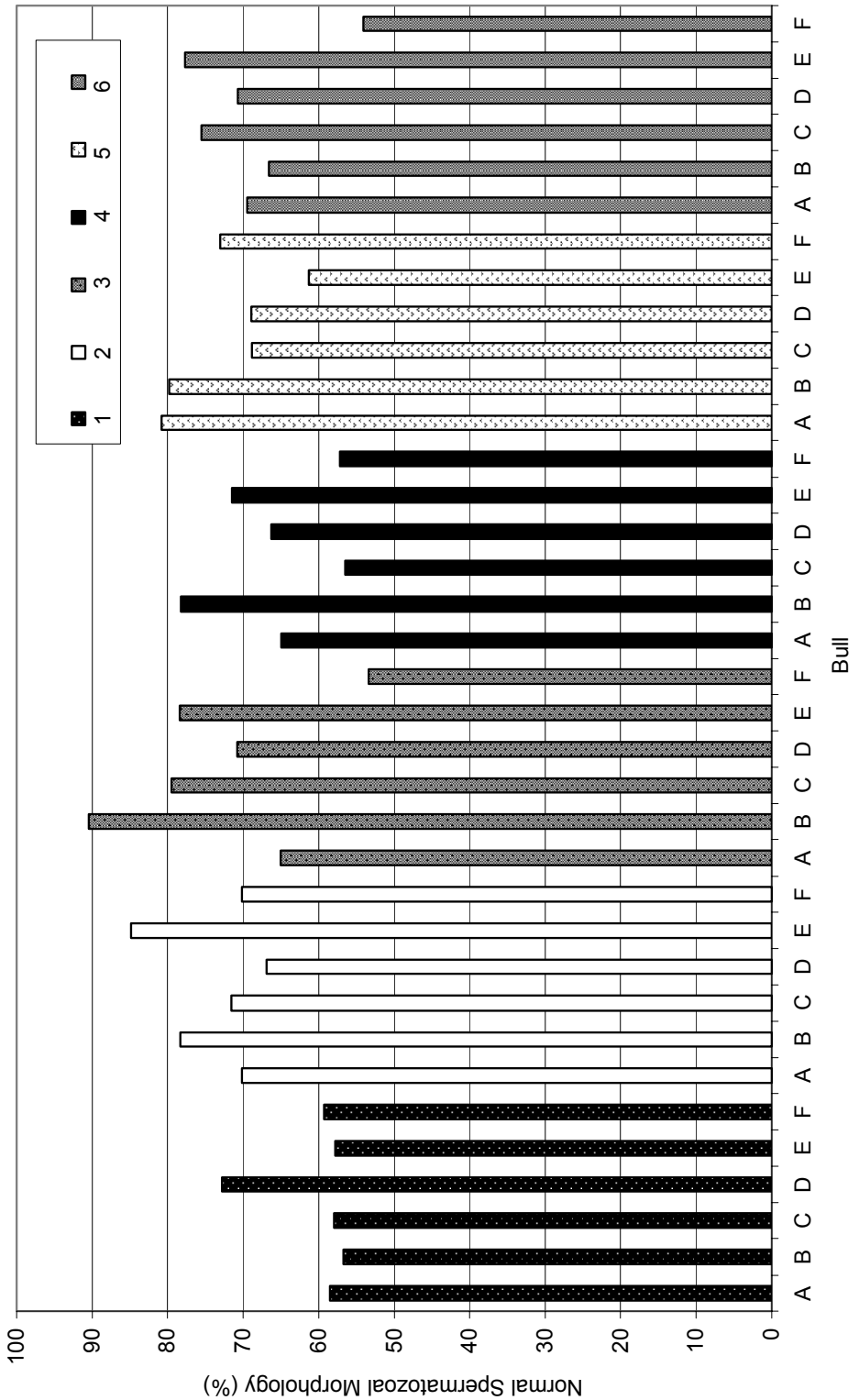

Fig. A-37. Experiment 2: Mean percentage morphologically normal spermatozoa by collection date (1 to 6), bull (A to F), and semen storage type (frozen). Pooled SEM is 5.68.

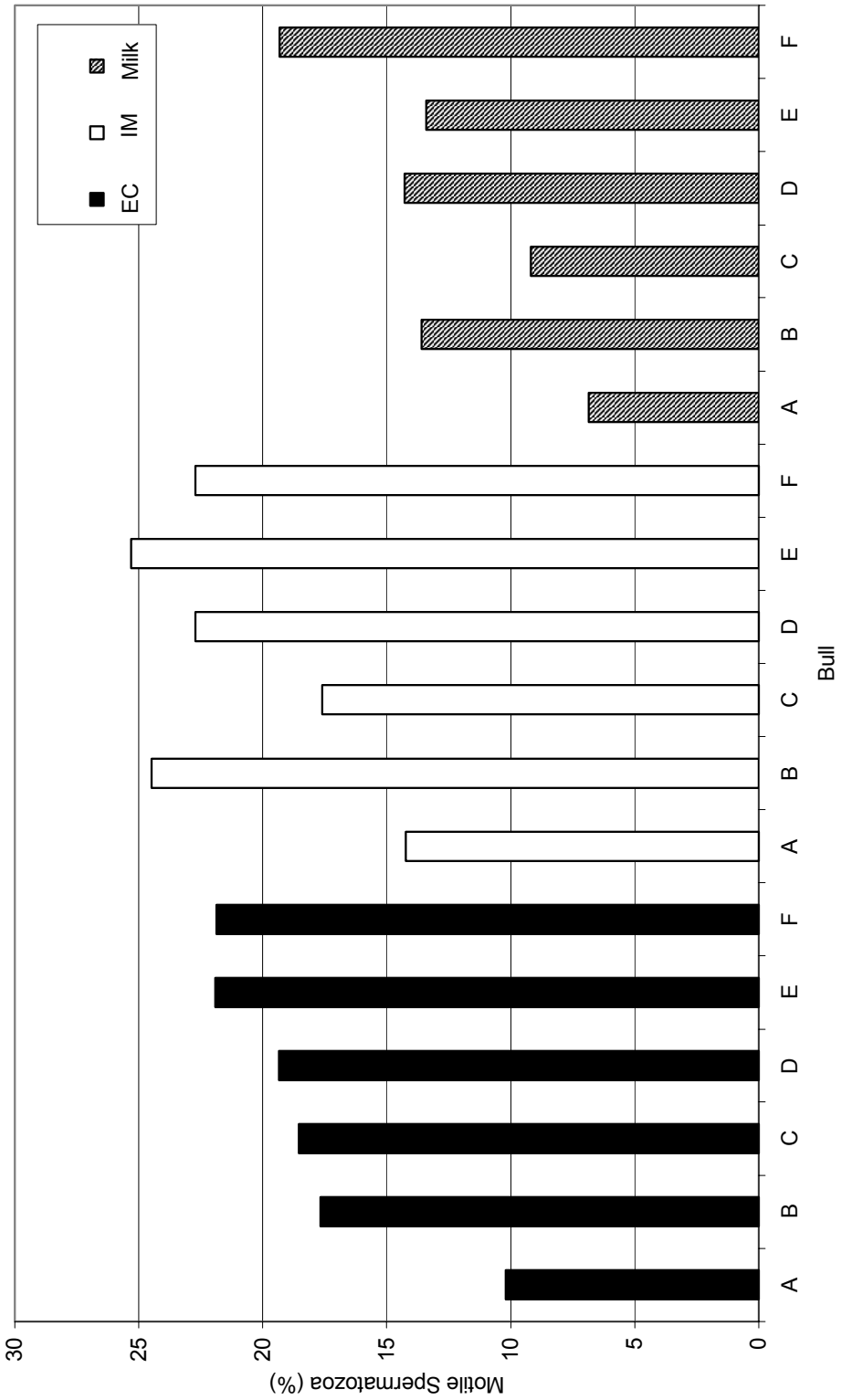

Fig. A-38. Experiment 2: Mean percentage motile spermatozoa by bull (A to F), semen storage type (frozen), and extender (EC = egg yolk-citrate, IMV, or Milk = skim milk) at 3 hr of incubation. Pooled SEM is 2.91.

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