1-20-2022

Proteomic Analysis of Epididymal and Ejaculated Sperm and Respective Fluids

Saulo Menegatti Zoca

Emmalee J. Northrop-Albrecht

Julie Walker

Robert A. Cushman

George Perry

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Objective

The objectives of these studies were to identify differences in proteins found both in the environment (fluid) and loosely attached to spermatozoa from both the epididymis and ejaculate, and to evaluate the effect of pH on sperm longevity.

Study Description

Ejaculated and epididymal semen was collected from mature Angus bulls (n = 9), and then centrifuged to separate sperm and fluid. Fluids were collected and sperm pellets were resuspended in a high ionic solution and vortexed to remove loosely attached proteins. Sperm samples were centrifuged, and the supernatant was collected. Samples collected for protein analysis were snap frozen in liquid nitrogen and stored at -80 ºC. Protein analysis was performed by liquid chromatography with tandem mass spectrometry (LCMS/MS). Yearling Angus cross bulls (n = 40) were used for sperm cultures. Ejaculated (n = 20) and epididymal (n = 20) sperm were collected, diluted and cultured in a commercial media at pH 5.8, 6.8 and 7.3, at 4 ºC and evaluated for motility and viability every 24 h until motility was below 20%. There was an effect of pH, time and pH by time interaction for motility and viability for both ejaculated and epididymal sperm ($P \leq 0.05$). At 216 h of incubation epididymal sperm at pH 7.3 and ejaculated sperm at pH 6.8 dropped below 20% motility. Overall, in all samples, a total of 458 unique proteins were identified. It was identified that ejaculated fluid and ejaculated sperm had 178 and 298 proteins, respectively. Also, it was identified that epididymal fluid and epididymal sperm had 311 and 344 proteins, respectively. There were Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways significantly enriched (FDR < 0.05) for ejaculated fluid (n = 8), epididymal fluid (n = 24), ejaculated sperm (n = 10), and epididymal sperm (n = 18). The most important KEGG pathway identified was the metabolic pathway. Within the metabolic pathway the glycolysis/gluconeogenesis, pentose phosphate, and glutathione metabolism pathways were significantly enriched among proteins only present in epididymal samples. Other proteins identified that may be related to epididymal sperm's increased longevity were peroxidases and glutathione peroxidases for their antioxidant properties.

Take Home Points

In the epididymis, energy metabolism appears to be more glycolytic compared to ejaculated. Also, there was a larger number of antioxidants present in the epididymis which may help maintain sperm in a quiescent state and increase sperm longevity. Epididymal sperm was able to maintain viability longer than ejaculated sperm when cultured under the same conditions. Understanding mechanisms associated with epididymal sperm's increased longevity compared to ejaculated sperm has the potential to assist in improvement of sperm storage.

Introduction

During final maturation, spermatozoa lose their ability to biosynthesize, repair, grow, and divide, and become very simple in their metabolic function (Hammerstedt, 1993). This results in spermatozoa becoming completely

dependent on their external environment to survive and function. While in the epididymis, spermatozoa are stored for a long period of time in a relatively quiescent (dormant) state. It has been hypothesized that this is due to both quiescence and prevention of premature activation of the spermatozoa prior to ejaculation (Sullivan et al., 2005). Upon ejaculation or dilution of the fluid of the caudal epididymis, motility is increased (Acott and Carr, 1984; Carr and Acott, 1984). A consequence of this increased motility is a reduction in viability to only several hours in most species (Austin, 1975).

Glycoproteins coat the spermatozoa plasma membrane (Magargee et al., 1988; Mahmoud and Parrish, 1996; Geussova et al., 1997) and several proteins that have been identified in epididymal fluid are enzymes that can modify proteins or lipids at the spermatozoa surface. A subset of these proteins are implicated in spermatozoa protection against oxidative stress (Girouard et al., 2011), and some of the proteins that are transferred to the spermatozoa are also proposed to modulate motility (Frenette et al., 2003; Frenette et al., 2004; Frenette et al., 2005). For example, macrophage migration inhibitory factor (MIF) protein, present in the epididymis, associates with the spermatozoa flagella impacting acquisition of motility (Eickhoff et al., 2004), and MIF concentration were negatively correlated to motility (Sullivan et al., 2005). Epididymal fluid pH range from 5.8 to 6.8 in bulls, and pH has been associated with sperm motility. Lower pH environments have been reported to inhibit sperm motility while greater pH increased sperm motility (Wales et al., 1966; Acott and Carr, 1984; Carr and Acott, 1984; Carr et al., 1985). Uterine pH decreased (~7.1 to ~6.8) at the initiation of standing estrus (Elrod and Butler, 1993) and was also decreased in animals that exhibited standing estrus (~6.8) prior to fixed-time AI compared to animals not exhibiting standing estrus (~7.1); also, uterine pH from animals that exhibited standing estrus rose prior to ovulation (~7.1; Perry and Perry, 2008a, b). Estrus expression prior to fixed-time insemination increased the number of spermatozoa that reached the site of fertilization (Larimore et al., 2015) and had a linear relationship with pregnancy success (Grant et al., 2011). It is hypothesized that the decrease in pH at onset of estrus would increase sperm longevity and the rise in pH prior to ovulation would increase sperm motility (Perry and Perry, 2008a, b). Thus, the objectives of these experiments were to identify differences in proteins found both in the environment (epididymal fluid and seminal plasma) and loosely attached to spermatozoa from both the epididymis and ejaculate to evaluate the effect of pH on sperm longevity.

Experimental Procedures

Experimental design

Study I

Ejaculated semen was collected via electro-ejaculation from nine sexually mature (4-yr old) Angus bulls with a history of successful breeding. After semen collection, bulls were rested for six weeks to renormalize epididymal reserves and then slaughtered. Testes and epididymides were collected and transported back to the laboratory. Epididymides were dissected and epididymal fluid and spermatozoa were collected from the caudal section of the epididymis. Ejaculated and epididymal sperm were diluted (~3 × 109 sperm/mL) and evaluated for motility, viability, and mitochondrial membrane potential at the time of semen collection. Ejaculated sperm was evaluated at pH 7.3 (most semen extender pH and uterine pH before and after estrus) and epididymal sperm was evaluated at physiological pH (5.8) and at pH 7.3. Epididymal semen from a subset of bulls (n = 3) were cultured for 310 h at 4 °C in three different pH; pH 5.8, pH 7.3, and pH 6.8 which has been reported to be the uterine pH at estrus (Perry and Perry, 2008a, b). The remainder of samples were processed for protein analysis.

Study II

Semen from 20 yearling (12- to 15-mo old) Angus crossed bulls were collected by electro-ejaculation and 20 different yearling (12- to 15-mo old) Angus crossed bulls were slaughtered and testes and epididymides were collected at a commercial slaughter facility. Epididymides were dissected and epididymal fluid and spermatozoa were collected from the cauda section of the epididymis. Ejaculated and epididymal semen were diluted (~42 × 10^6 sperm/mL and ~60 × 10^6 sperm/mL, respectively) and incubated at three different pH (5.8, 6.8, and 7.3). Epididymal sperm was transported back to the laboratory in culture, thus, first evaluation at 24 h

incubation. Samples were evaluated every 24 h, until total motility were below 20%, then no further evaluation was made for total motility, progressive motility, or viability.

Sperm analyses and culture

For both studies, aliquots of each sample (ejaculated and epididymal) were evaluated at collection, 0 h for ejaculated and 24 h after slaughter for epididymal. Samples were diluted (42 × 106 to 3 × 109 sperm per mL; details in experimental design) and cultured in a commercially available media (OPTIXcell, IMV technologies, France) and adjusted to different pH (5.8, 6.8, or 7.3), at 4 °C. Samples were evaluated for motility and viability by a computer-assisted sperm analysis machine (CASA; Hamilton Thorne IVOS II, Beverly, MA), and mitochondrial membrane potential procedure (study I only), by MitoTracker red (Thermo Fisher, Eugene, OR) staining following manufacturer's label. Mitochondrial membrane potential was evaluated using a Nikon Fluorescence microscope, and the NIS-Elements software package was used to outline 100 individual spermatozoa and fluorescence intensity was determined. Samples were evaluated every 24 h, ejaculated samples from 0 h and epididymal samples from 24 h after collection (due to transport to the lab a true 0 h was not possible) until 310 h incubation for motility and viability (study I) or until total motility were below 20% (study II).

Protein isolation

Samples were centrifuged (700 × g for 10 min) to separate spermatozoa and fluids (epididymal fluid or seminal plasma) for protein analysis. Fluids were collected and snap frozen in liquid nitrogen and stored at -80 °C until analyzed. Spermatozoa pellets were then washed with a high ionic solution (Rifkin and Olson, 1985) and vortexed for 1 min to remove proteins loosely attached to the spermatozoa. Samples were then centrifuged (700 × g for 10 min) to separate spermatozoa from stripped proteins. Stripped proteins were collected, snap frozen in liquid nitrogen and stored at -80 °C until analyzed. This resulted in four types of samples: 1) epididymal fluid, 2) ejaculated fluid, 3) epididymal sperm stripped proteins (epididymal sperm), and 4) ejaculated sperm stripped proteins (ejaculated sperm).

Liquid chromatography with tandem mass spectrometry analysis

Protein samples were shipped to the University of Minnesota Mass Spectrometry facility for identification by LCMS/MS. All LCMS/MS samples were analyzed using Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version 2.1.0.81). Sequest was set up to search the bovine (taxid 9913) protein sequence database from Uniprot.org with canonical and isoforms included and merged with the common lab contaminant protein database (thegpm.org/crap/index, 109 proteins). Scaffold (version Scaffold_5.0.0, Proteome Software Inc., Portland, OR) was used to validate LCMS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 99.0% probability by the Scaffold Local false discovery rate (FDR) algorithm. Protein identifications were accepted if they could be established at greater than 7.0% probability to achieve an FDR less than 1.0% and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on tandem mass spectrometry (MS/MS) analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Further analysis was conducted using peptides identified as exclusive and unique to each protein. Total spectrum counts for proteins were used for abundance comparisons (proteins found in one sample but not the other or found in both samples) and statistical analysis. Comparisons were made for total spectrum counts between epididymal and ejaculated fluid proteins and between epididymal and ejaculated spermatozoa surface proteins. Significant P-values were adjusted for multiple testing using the Benjamini-Hochberg calculation to correct the FDR. For each comparison, proteins that were identified in the samples were entered into DAVID v 6.8 (Huang et al., 2008, 2009) using their official gene names to determine the top Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways associated with those proteins. For the significant KEGG pathways within each sample, that had a physiological meaning for the study objective, the proteins contributing to that KEGG that were exclusive for one of the samples were entered into the STRING database (Szklarczyk et al., 2015; Hu et al., 2018) to determine the network interactions of those proteins.

Statistical analyses

Study I

Differences of spermatozoa parameters between ejaculated and epididymal (pH 5.8 and 7.3) at collection were evaluated using the GLIMMIX procedures of SAS (v 9.4). The model used was treatment (ejaculated, epididymis 5.8, epididymis 7.3) as a fixed effect and bull as a random effect. For fluorescence intensity, area measured was included as a covariate. Cultured spermatozoa parameters were evaluated using the GLIMMIX procedure of SAS (v 9.4) for repeated measures. The model included the fixed effect of treatment (pH 5.8, 6.8, and 7.3), time of incubation and the interaction. Time points 286 and 310 h incubation were removed from progressive motility analysis because all values equaled zero. Significance was declared when $P \leq 0.05$ and tendency when $P > 0.05$ but $P \leq 0.10$.

Study II

Ejaculated and epididymal sperm parameters were evaluated separately. Total motility, progressive motility and viability were evaluated using the methods described above for repeated measures. Significance was declared when $P \leq 0.05$ and tendency when $P > 0.05$ but $P \leq 0.10$.

Results and Discussion

Females need to be in estrus or under the influence of estrogen for efficient transport of spermatozoa from the site of deposition to the site of fertilization (Hawk, 1983). Estrogen may influence fertilization rates through both spermatozoa transport and fertilization efficiency by altering the uterine environment (pH). It has been reported that uterine pH decreased at the initiation of standing estrus (Elrod and Butler, 1993) and was also decreased in animals that exhibited standing estrus prior to fixed-time AI compared to animals not exhibiting standing estrus (Perry and Perry, 2008a, b). Estrus expression prior to fixed-time insemination increased the number of spermatozoa that reached the site of fertilization (Larimore et al., 2015) and had a linear relationship with pregnancy success (Grant et al., 2011). It is hypothesized that the decrease in pH at onset of estrus would increase sperm longevity and the rise in pH prior to ovulation would increase sperm motility (Perry and Perry, 2008a, b). Thus, sperm was diluted and incubated at three different pH: physiological pH of the epididymis (5.8), physiological pH upon ejaculation which is similar to the uterine pH at time of ovulation and most semen extender (7.3) and uterine pH at onset of estrus (6.8). In study I, treatment influenced mitochondrial membrane potential ($P < 0.01$; Fig. 1), percentage of total motility, progressive motility, and viability ($P \leq 0.01$; Fig. 2). Also, there was an effect of treatment on the mitochondrial membrane potential ($P < 0.01$; Fig. 1). Epididymal sperm had increased mitochondrial membrane potential compared to ejaculated ($P < 0.01$), also, epididymal sperm at 5.8 tended to have a greater mitochondrial membrane potential compared to epididymal sperm at 7.3 ($P = 0.07$). Upon dilution of caudal epididymis fluid motility was increased (Acott and Carr, 1984; Carr and Acott, 1984), but when epididymal sperm was incubated in caudal epididymal fluid, motility was inhibited (Carr and Acott, 1984). When epididymal sperm was diluted and pH altered to 7.3 there was an increase in sperm motility. The washing and dilution of caudal epidydimal fluid were sufficient to cause an increase in sperm motility which explains the lack of statistical difference between epididymis sperm at pH 5.8 and ejaculated sperm at pH 7.3 in study I and is similar to what has been reported by others (Acott and Carr, 1984; Carr and Acott, 1984; Carr et al., 1985). Interestingly, when the pH of epididymal sperm was adjusted to 7.3 total motility and progressive motility were increased to above ejaculated sperm. This is consistent with the increased mitochondrial membrane potential of epididymal sperm and agrees with the increase in sperm motility reported by others (Ericsson et al., 1993).

In study I, when epididymal sperm were cultured at pH 5.8, 6.8 and 7.3 there was an effect of pH by time interaction on total motility and progressive motility ($P \leq 0.05$; Fig. 3 and 4); however, the pH by time interaction was not significant for viability ($P = 0.16$; Fig. 5). There was an effect of pH on total motility ($P < 0.01$) and viability ($P < 0.01$; Fig. 5), but there was no effect of pH on the percentage of progressive motility ($P = 0.59$). There was also an effect of time on total motility, progressive motility, and viability ($P < 0.01$). In Study II, there was an effect of pH, time and pH by time interaction for total motility ($P \leq 0.04$; Fig. 6), progressive motility ($P \leq 0.03$; Fig. 7) and viability ($P \leq 0.02$; Fig. 8) for both ejaculated and epididymal sperm. In study I, epididymal

sperm were able to maintain motility for a longer period of time when it was incubated at pH 6.8 compared to pH 5.8 or 7.3. This is consistent with what was hypothesized by Perry and Perry (2008a, b) that a decrease in uterine pH at the onset of estrus would increase sperm longevity. In study II, ejaculated sperm at pH 6.8 and epididymal sperm at pH 7.3 maintained total motility above 20% longer than other samples, at 216 h of incubation motility decreased below 20% for both ejaculated 6.8 and epididymal 7.3 (17.1% and 18.9%, respectively). The percentage of sperm displaying progressive motility at 216 h was 1.6% and 1.1%, and viability 51.3% and 95.4%, for ejaculated 6.8 and epididymal 7.3, respectively. Perry and Perry (2008a, b) hypothesis held true for ejaculated sperm (pH 6.8 had the greatest longevity), in study II; however, epididymal sperm at pH 7.3 had greater longevity (total motility) compared to sperm at pH 6.8. Animals in study I and study II were different, the main differences between the two groups of bulls (age) may have caused the observed differences. In study I, animals were mature bulls (4-yr old) with proven fertility and study II animals were 12- to 15-mo old that had just reached puberty and passed a breeding soundness exam.

When comparing sperm viability (study II) between ejaculated and epididymal sperm, even though sperm motility decreased during incubation, epididymal sperm had at least 15 percentage points more viable sperm compared to ejaculated sperm at any time point regardless of media pH (Fig. 8). In the cauda epididymis, sperm are stored for a long period of time. After differentiation and maturation, sperm has a relatively simple metabolism and is highly dependent on its environment (Hammerstedt, 1993). Sullivan et al. (2005) hypothesized that the increased longevity of epididymal sperm is due to both quiescence and prevention of premature activation of the spermatozoa. In agreement with Sullivan et al. (2005), the increased viability of epididymal spermatozoa compared to the ejaculated, was not only due to the relatively quiescent state it was in, but also due to proteins associated with these spermatozoa, because even after dilution and initiation of motility epididymal sperm had increased viability compared to ejaculated sperm in the present study.

Proteins were identified in ejaculated and epididymal samples in order to investigate which proteins may be involved in increased viability of epididymal sperm. An overall total of 458 unique proteins were detected between all samples, 178 were detected in ejaculated fluid, 298 were detected in ejaculated sperm, 311 were detected in epididymal fluid, and 334 were detected in epididymal sperm. There were 103 proteins detected in the fluids that were present in both ejaculated and epididymal samples, ten proteins had increased abundance in ejaculated fluid ($P \leq 0.05$) and 29 had increased abundance in epididymal fluid ($P \leq 0.05$). There were 221 proteins detected in the sperm samples that were present in both ejaculated and epididymal samples, 12 proteins had increased abundance in the ejaculated sperm ($P \leq 0.05$) and 109 proteins had increased abundance ($P \leq 0.05$) in the epididymal sperm. There were eight significant KEGG pathways (FDR < 0.05) for ejaculated fluid proteins and 24 KEGG pathways for epididymal fluid proteins. There were ten significant pathways for ejaculated and 18 for epididymal proteins that were stripped from the sperm. When comparing proteins stripped from the sperm and in the fluids between ejaculated and epididymis samples, the metabolic pathway had the greatest number of proteins and was identified as the most important KEGG pathway for this data set. This was expected as sperm are maintained in a quiescent mode in the epididymis which allows sperm to be stored for several days. The KEGG metabolic pathway can be subdivided into other pathways, as proteins may have function in multiple pathways [e.g., Fructose-bisphosphate aldolase A (ALDOA) is present in the metabolic pathway, glycolysis/gluconeogenesis pathway, pentose phosphate pathway and, fructose and mannose pathway]. The total number of proteins identified in ejaculated (n = 305) and epididymal sperm (n = 384) suggests that epididymal sperm metabolism and environment are more regulated by proteins than ejaculated sperm, especially, since 153 proteins were present only in epididymal samples compared to 74 proteins present in ejaculated samples only.

Metabolic pathway associated proteins in the fluid samples included: 15 proteins that were present in both ejaculated and epididymis samples, nine proteins that were only present in ejaculated fluid and 55 proteins that were only present in epididymis fluid. The proteins related to the metabolic pathway only present in ejaculated fluid were not highly related as seen by few connections between proteins [protein-protein interaction (PPI) enrichment P = 0.22]. The proteins present only in epididymis fluid and related to metabolic pathway differed from ejaculated fluid and were highly interactive with a complex network (PPI enrichment P < 0.01). Eleven proteins in this network were related to glycolysis/gluconeogenesis pathway, five proteins were related to the oxidative phosphorylation pathway, eight were related to the pentose phosphate pathway, and four proteins

were related to fructose and mannose metabolism. There were 36 proteins present in both ejaculated and epididymis sperm samples related to the metabolic pathway. Nevertheless, 11 proteins were only present on ejaculated sperm and 32 were only present on epididymal sperm. Proteins related to the metabolic pathway only present in ejaculated sperm were not highly related with few connections between proteins, similarly to proteins only present in ejaculated fluid (PPI enrichment P = 0.09). The proteins only present in the epididymis sperm samples and related to the metabolic pathway, different from ejaculated sperm and similarly to those from epididymis fluid, were highly interactive with a complex network (PPI enrichment P < 0.01). There were seven proteins related to the pentose phosphate pathway, five proteins related to glycolysis/gluconeogenesis pathway and two proteins related to the fructose and mannose metabolism pathway that were present only in the epididymis sperm samples compared to the ejaculated sperm samples.

Bovine sperm can utilize both anaerobic (without oxygen) and aerobic (with oxygen) methods of energy production to maintain similar levels of motility (Krzyzosiak et al., 1999). Proteins found only in the epididymal samples that were involved in the glycolysis/gluconeogenesis pathway (11 and five in fluid and sperm, respectively) and that were associated with oxidative phosphorylation (five proteins identified in fluid) were identified. Two different proteins were present in ejaculated sperm that related to oxidative phosphorylation. The glycolysis/gluconeogenesis pathway had seven and 11 proteins that were present in both ejaculated and epididymis samples, fluid and sperm, respectively. Human patients with asthenozoospermia (low sperm motility) had increased levels of ALDOA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), alcohol dehydrogenase (AKR1A1), L-lactate dehydrogenase B chain (LDHB) in seminal plasma compared to control patients (Wang et al., 2009). In this data set, the proteins elevated in seminal plasma of patients with asthenozoospermia, ALDOA, GAPDH, and AKR1A1 were only detected in epididymal fluid. Also, AKR1A1, LDHB and GAPDH were only present on epididymal sperm (ALDOA was present in ejaculated sperm but in lower abundance compared to epididymal sperm). Our results suggest that energy production, specifically through glycolysis, in the epididymis is more regulated compared to after ejaculation. Thus, these proteins only detected in epididymal samples may need to be removed or diluted to undetectable concentrations to facilitate and promote energy production and, consequently, sperm motility.

It is possible that the increased number of proteins related to glycolysis in epididymal samples is a mechanism to reduce the production of reactive oxygen species (ROS) which is a by-product of oxidative phosphorylation and is not produced during glycolysis. Reactive oxygen species are oxygen containing molecules that can be found as free radicals or non-radical oxidants, these molecules remove electrons from specific reactants. The presence of ROS is necessary for normal sperm function; however, the deleterious capacity of ROS is determined by its concentration. The increase in sperm intracellular cyclic adenosine monophosphate (cAMP) is caused by ROS which leads to a cascade of biochemical events that lead to sperm capacitation (Aitken et al., 2015; Aitken, 2017); however, when in elevated concentrations, ROS can cause oxidative stress which leads to lipid peroxidation. Lipid peroxidation has been reported to increase DNA fragmentation, decrease plasma membrane integrity (viability), and reduce motility in bovine sperm (Kasimanickam et al., 2007). Antioxidant proteins can remove ROS from the media and prevent harmful elevated concentrations of ROS. Aldehyde dehydrogenase (ALDH2) was identified in all samples except for ejaculated fluid, this protein was identified in the metabolic pathway, and it has been reported to provide antioxidant properties in stallion sperm (Gibb et al., 2016; Hall et al., 2017). The glutathione metabolism pathway was present in epididymal fluid but not in ejaculated fluid and was present in both ejaculated and epididymal sperm. The glutathione S-transferases proteins have been reported to be antioxidants in stallion (Gibb et al., 2016; Hall et al., 2017) and have been suggested to be involved in bovine sperm protection (Girouard et al., 2011). It was identified that the protein glutathione S-transferase P (GSTP1) was only present in epididymal fluid and sperm; however, glutathione S-transferase Mu (GSTM1) was present in epididymal fluid and both epididymal and ejaculated sperm. Another pathway that has been reported to have antioxidant properties is the pentose phosphate pathway (Williams and Ford, 2004). Peroxidases (PRDX1, PRDX2, PRDX4, and PRDX6) are important antioxidants that have been reported to protect sperm from oxidative stress, and they were identified in epididymal samples but not ejaculated samples. Additionally, glutathione peroxidases were identified in both ejaculated and epididymal samples (GPX5) or only in ejaculated samples (GPX6; O'Flaherty, 2019).

Implications

In the epididymis, sperm energy metabolism appears to be more glycolytic compared to sperm after ejaculation, based on the greater number of proteins related to this pathway only present in epididymal samples. Sperm also has a greater number of antioxidants available in the epididymis that is likely to be maintaining ROS at low concentrations to inhibit premature sperm activation. This is supported by a greater mitochondrial membrane potential of epididymal sperm compared to ejaculated sperm and the fact that epididymal sperm was able to maintain viability longer than ejaculated sperm when cultured under the same conditions. In addition, when both ejaculated and epididymal sperm were cultured at uterine pH (7.3), epididymal sperm had greater motility. More research is necessary to better understand the specific roles of the proteins only identified in the epididymis with the increase in sperm longevity, regulation of sperm activation, and their possible role in bull fertility and ability to dominate a breeding pasture (Abell et al., 2017; Bennett et al., 2021). An increased understanding of mechanisms associated with epididymal sperm increased longevity compared to ejaculated sperm has the potential to improve sperm storage.

Acknowledgements

This project was supported by the National Science Foundation BREAD EAGER [Grant no. 1446886]. Names are necessary to report factually on available data; however, neither South Dakota State University nor the USDA guarantees nor warrants the standard of the product, and the use of names by South Dakota State University and the USDA implies no approval of the product to the exclusion of others that may also be suitable. South Dakota State University and USDA are an equal opportunity providers and employers.

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Figures

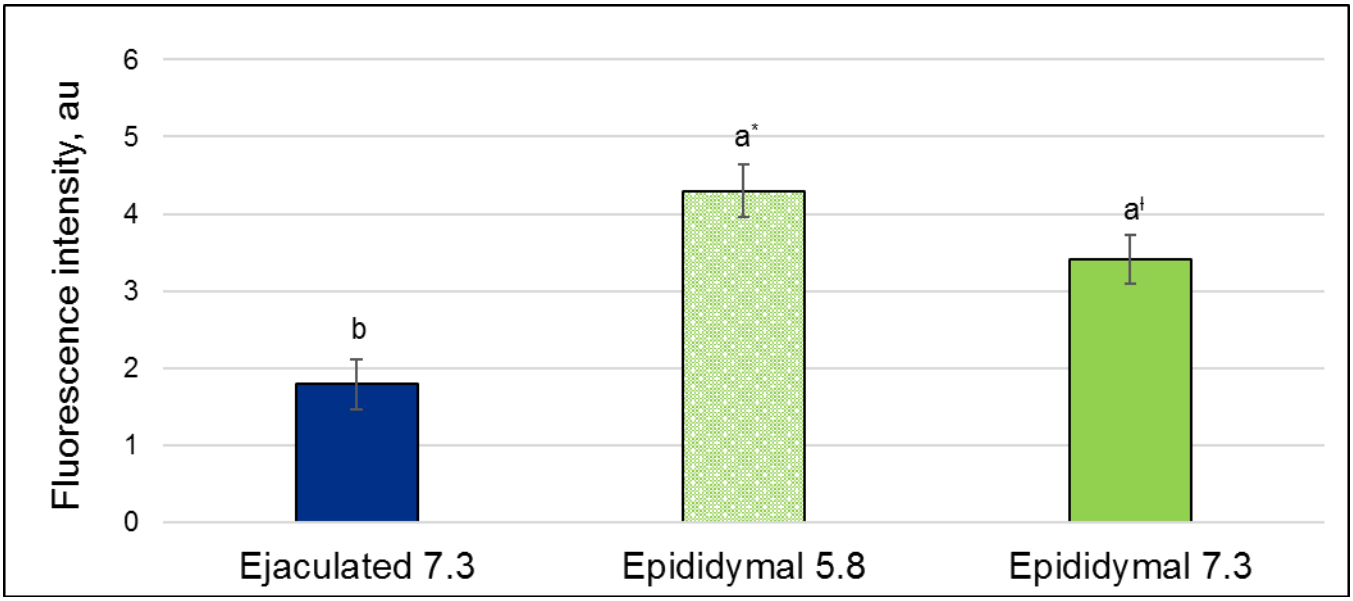

Figure 1. Mitochondrial membrane energy potential for ejaculated sperm at pH 7.3, and epididymal sperm at pH 5.8 and 7.3 (study I). There was a significant effect of treatment $P < 0.01$. The greater the value the greater the mitochondrial membrane energy potential.
[a,b]Bars with different letter superscripts differ $P < 0.01$.
[*,¦]Bars with different symbols superscript differ $P = 0.07$.

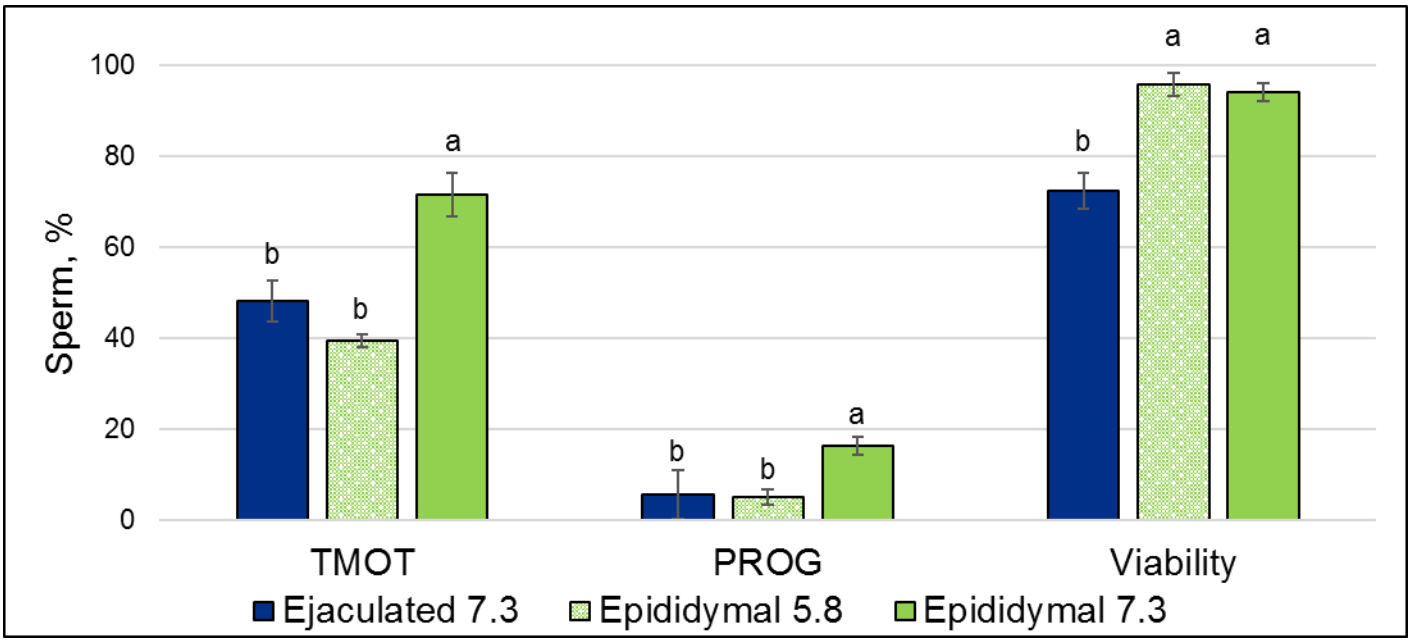

Figure 2. Percentage of total motility (TMOT), progressive motility (PROG), and viability for ejaculated sperm at pH 7.3, and epididymal sperm at pH 5.8 and 7.3 (study I). There was a significant effect of treatment for TMOT, PROG and Viability $P < 0.01$.
[a,b] Bars with different letter superscripts differ $P \leq 0.05$.

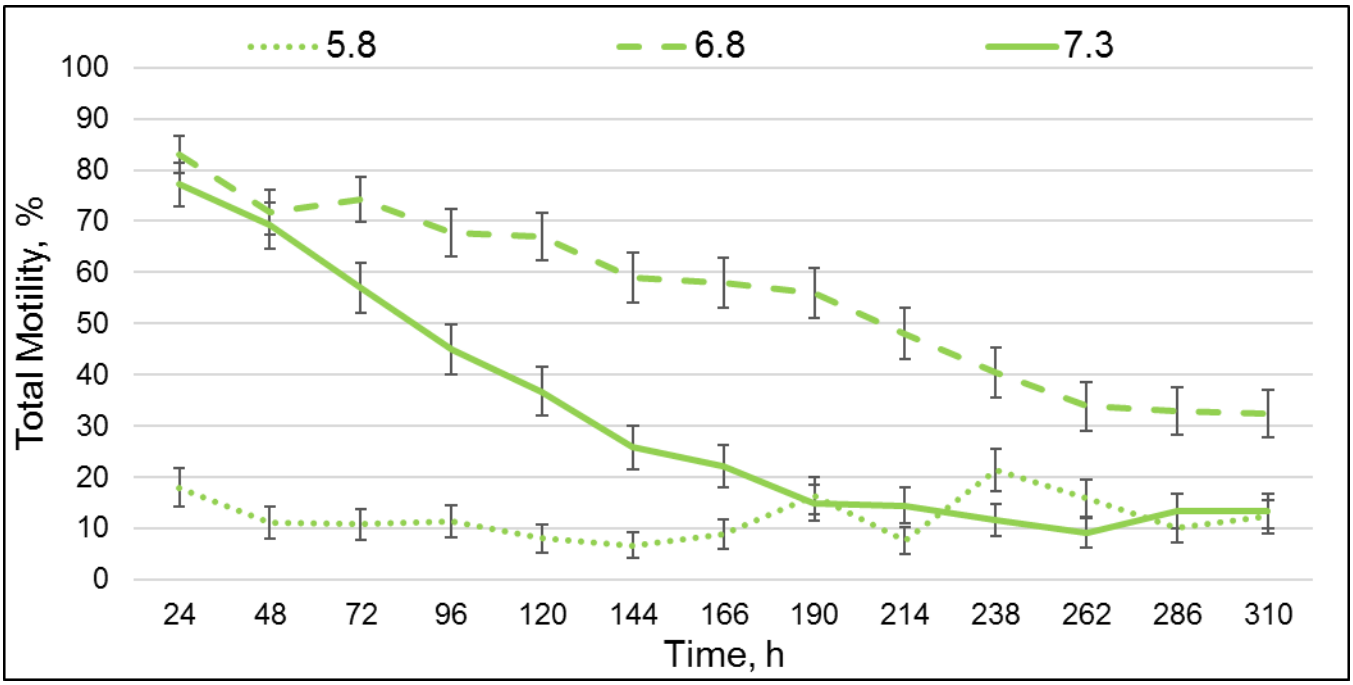

Figure 3. Percentage of total motility for epididymal sperm culture at pH 5.8, 6.8, and 7.3 (Study I). There was a significant pH, time, and pH by time interaction ($P < 0.01$).

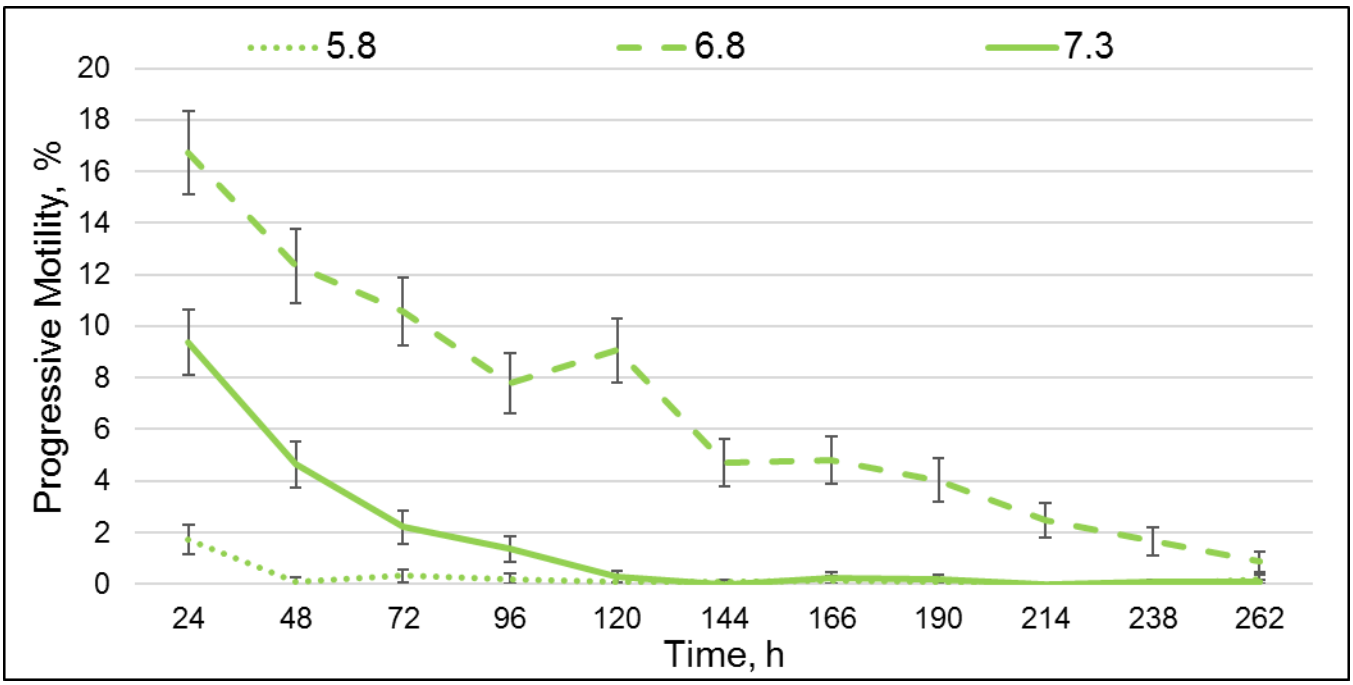

Figure 4. Percentage of progressive motility for epididymal sperm cultured at pH 5.8, 6.8, and 7.3 (Study I). There was a significant time and pH by time interaction ($P \leq 0.05$); pH was not significant ($P = 0.59$).

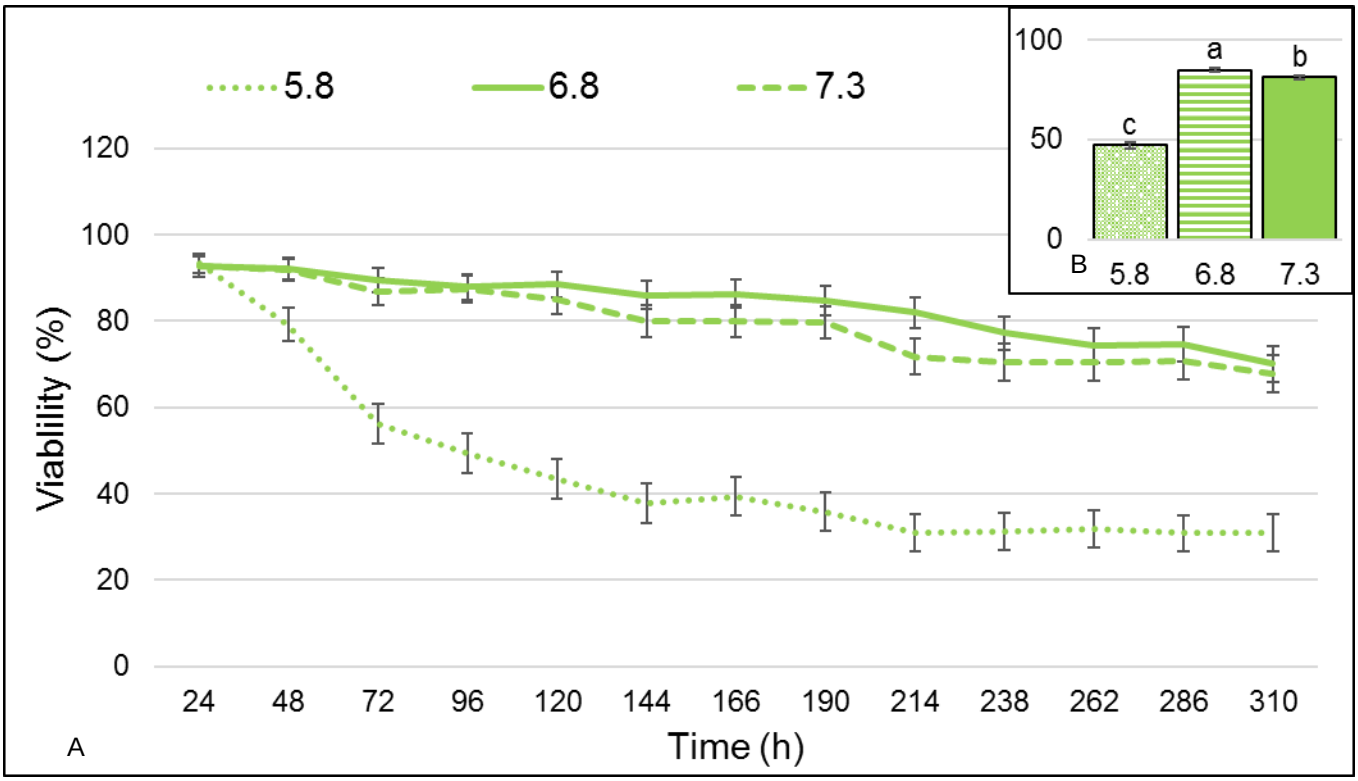

Figure 5. Percentage of viable epididymal sperm cultured at pH 5.8, 6.8, and 7.3 (Study I). There was not a significant pH by time interaction (A; $P = 0.16$). There was a significant pH (B; Y-axis represents viability % and X-axis represents pH treatment) and time effect ($P < 0.01$).
[a-c] Bars within figure not sharing a common superscript differ $P \leq 0.05$.

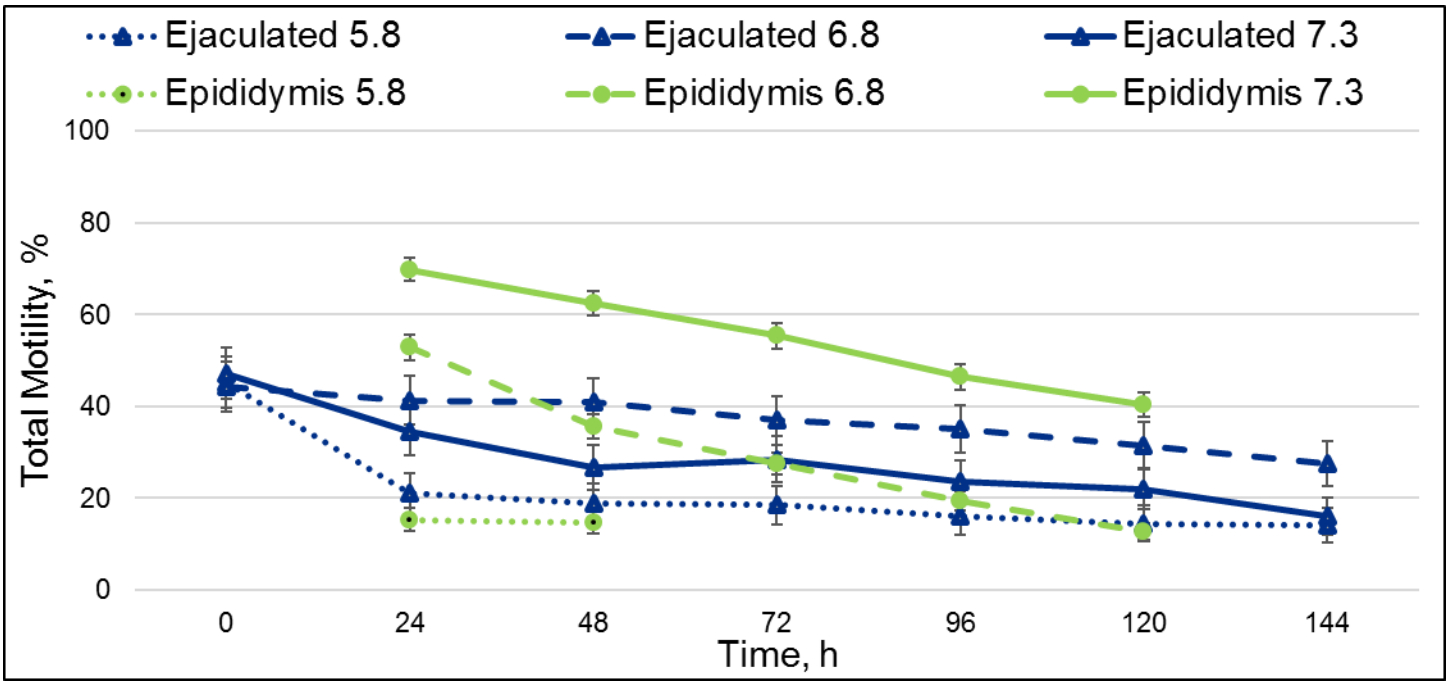

Figure 6. Percentage of total motility for epididymal and ejaculated sperm cultured at pH 5.8, 6.8, and 7.3 (Study II). Samples were considered non-viable when total motility decreased below 20%. There was a significant pH, time and pH by time interaction for both epididymal and ejaculated sperm ($P \leq 0.04$).

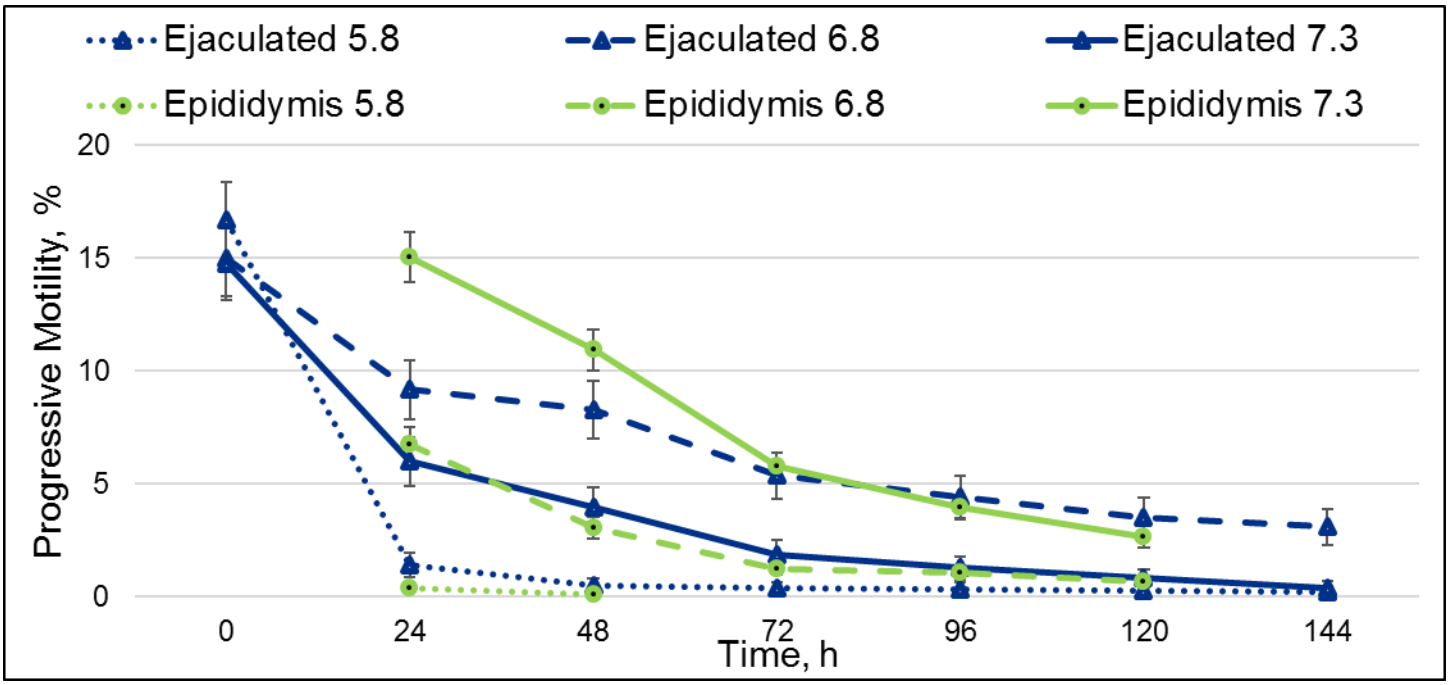

Figure 7. Percentage of progressive motility for epididymal and ejaculated sperm cultured at pH 5.8, 6.8, and 7.3 (Study II). Samples were considered non-viable when total motility decreased below 20%. There was a significant pH, time and pH by time interaction for both epididymal and ejaculated sperm ($P \leq 0.03$).

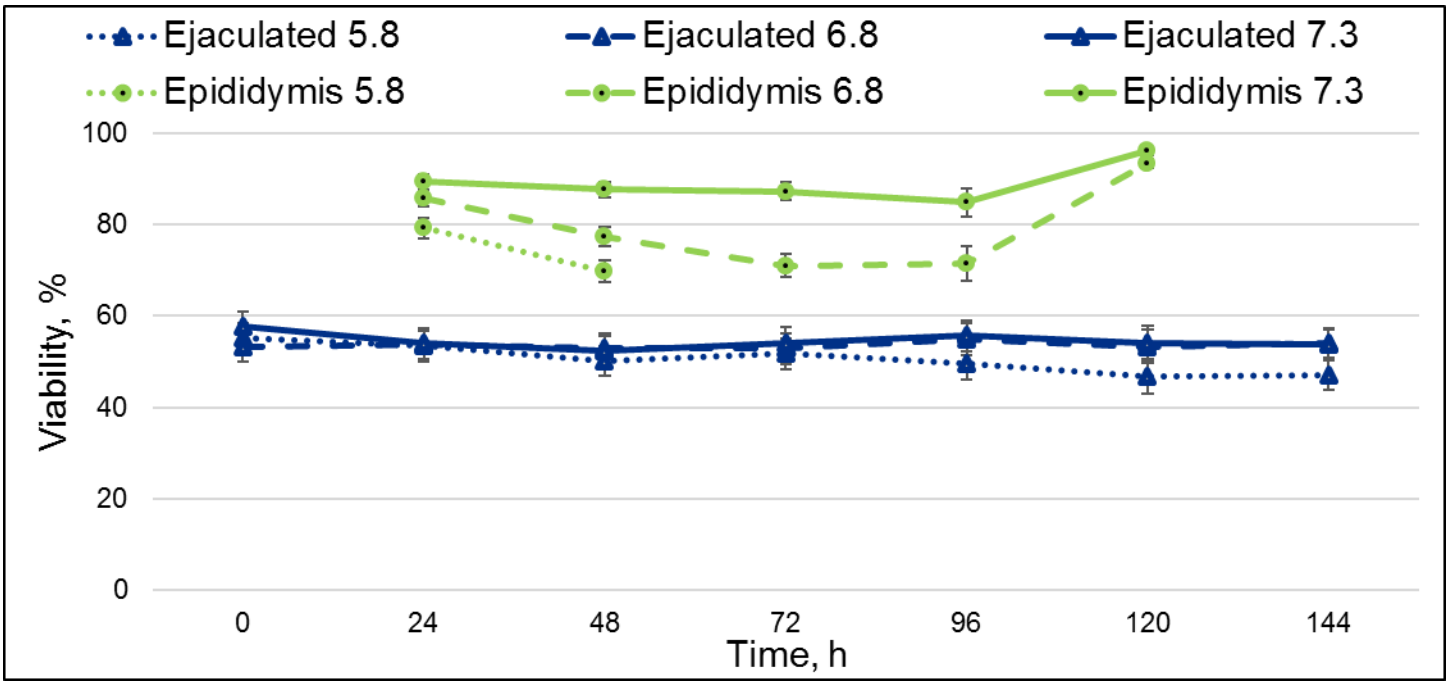

Figure 8. Percentage of viable for epididymal and ejaculated sperm cultured at pH 5.8, 6.8, and 7.3 (Study II). Samples were considered non-viable when total motility decreased below 20%. There was a significant pH, time and pH by time interaction for both epididymal and ejaculated sperm ($P \leq 0.02$).