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A Simple Test of Seminal Fluid Chemistry and Its Potential Impact on Cryopreservation

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Abstract

Cryopreservation is currently the only effective tool for long-term storage of semen in most species. However, it is well-recognized that, even in species that freeze well, some individuals resist cryopreservation. Work from this laboratory has demonstrated a relationship between maternal lipid content and the chemical constitution of the embryos they produce. The objective of the present study was to determine if a similar relationship might exist in paternal body chemistry and the animal's semen sample and if such a difference could be determined with a simple weight test. Semen samples were obtained from cattle with known differences in body composition. The samples first underwent semen analysis and were then prepared as either cell-free (CF) or neat specimens (NS). Known volumes of each sample were weighed, and the remainder of the samples was analyzed for lipids, total proteins, and total carbohydrates using a series of spectrophotometric assays and blood chemistry techniques. As expected, weight differences were seen in the CF vs NS preparations of individual semen samples ($p < 0.001$). Differences were also found in triglycerides ($p < 0.001$), glucose ($p < 0.001$), total protein ($p < 0.001$), and fructose ($p < 0.009$) of individuals with differing body composition. Statistical analysis suggested a non-linear correlation between the observed weights and total protein ($p < 0.047$) as well as triglyceride levels ($p < 0.003$). Together, these data suggest it might be possible to develop an algorithm to allow adjustment in cryoprotectants based on a simple weight procedure, allowing modification of cryoprotectants on an individual basis and potentially improving outcomes for valuable animals currently classified as "poor freezers".

Keywords

Sperm, Cryopreservation, Customization, Technique

1. Introduction

Seminal plasma is a complex combination of fluids produced in the rete testis, epididymis, and the accessory sex glands of the male reproductive tract [1] [2]. It serves as a nutrient-rich medium that promotes the development and maturation of spermatozoa in the testes and their transport during ejaculation [3]. It has also been found to increase blood flow to the oviducts and uterus and assist in stimulating female tract contractions, aiding in sperm transport [4].

It is universally accepted that cells are influenced by their noncellular environment. In the case of sperm, after ejaculation, this environment would include the various components of seminal plasma. Given that seminal plasma represents such a large portion of the ejaculate (even in cases where sperm are pelleted, there is a significant portion of seminal plasma intracellularly in the pellet), there can be little doubt that seminal plasma would be an influencing factor on the sperm cell environment during cryopreservation. In fact, studies have demonstrated seminal plasma from fertile animals can improve the cryopreservation of some sperm samples [5] [6].

Previous work from this laboratory demonstrated a relationship between oocyte/embryo cytoplasmic constituents and maternal body composition [7]. In a study with leptin receptor-deficient mice, the affected animal experienced an approximate tripling of weight due to the massive influx of lipid deposition into adipose tissue, heavily skewing overall body composition in favor of lipids. However, while maternal weight went up due to the addition of grams of lipid to an expanding body mass, the estimated weight of the early embryos, with their fixed volume, decreased, presumable as water in the cytoplasm was replaced by the lighter lipid compounds. These findings appear to confirm earlier results in a bovine model where breed differences in body composition were reflected in the oocyte and early embryo cytoplasm [8]. There can be little debate that significant changes in the oocyte/embryo cytoplasm would not only lead to changes in cellular function but would influence the uptake of cryoprotectants during the freezing process and change the freezing curve required for optimum cryopreservation rates.

Given the ratio of seminal plasma volume to spermatozoa volume in the ejaculate of all but a few mammalian species, there can be little doubt seminal plasma, a dynamic water-based fluid, would have an influence of sperm cell osmotic potentials and thus cryopreservation. Even when "removed" by centrifugation, a significant amount of plasma remains trapped intracellularly in the pellet, where it would continue to exert an osmotic influence on the sperm cells and impact the functionality of added cryoprotectants. The objective of the present study was to determine if paternal body condition exerted an influence on the chemical constituents found in seminal plasma.

2. Materials and Methods

2.1. Collection

Cattle semen samples were obtained from commercial facilities conducting

soundness checks. Animals were collected via electroejaculation into either 50-ml conical tubes (BD Falcon, Franklin Lakes, New Jersey) or 17-ml truebreed tubes (Reproductive Solutions Inc., Dallas, TX), depending on the collector's preference. Samples were obtained from 26 animals from 4 breeds: 6-Black Angus, 3-Jersey, 9-Limousine, and 8-Black Angus Cross. Information was also obtained from the initial semen analysis. Animals were 1 - 3 years old, in good body condition and being assessed for reproductive health prior to entering breeding programs.

2.2. Initial Sample Preparation

Once samples had been returned to the research lab, a repeat semen analysis was performed using the computer-assisted semen analyzer (CASA, IVOS; Hamilton Thorne, Beverly, Massachusetts). Each sample was then split into equal volumes in 15 ml conical tubes (BD Falcon, Franklin Lakes, New Jersey) and assigned a designated protocol as "centrifuged" or "non-centrifuged" to allow weighing of the neat sample containing both cells and seminal plasma versus the weight of the seminal plasma alone. To isolate the seminal plasma, the conical tubes were centrifuged (Sorvall RT6000, Block Scientific Inc., Bohemia, NY) at 3354G for 5 min at 5˚C. The supernatant was removed to a separate test tube for initial weight measurements and the pellet was labeled and frozen at −70˚C. Two aliquots measuring 0.5 ml and 100 µl of the centrifuged supernatant and neat sample were transferred to separate—1 ml Eppendorf bullet tubes (Fisher Scientific, Pittsburgh, PA) for weight measurement using a Sartorius scale (Data Weighing Systems Inc., Elk Grove, Illinois). All remaining materials were frozen at −70˚C to await biochemical analysis. However, it was later determined that cells in the non-centrifuged samples interfered with subsequent chemical analysis. Therefore, the results described below were made using the supernatant of centrifuged samples.

2.3. Biochemical Analysis

Biochemical analyses were done manually and using automated clinical human or veterinary instruments usually used for blood analysis. Individual manual analysis was made for cholesterol, triglycerides, glucose, fructose, and protein. Cholesterol determinations of each sample were made using a standard colorimetric technique (Stanbio Cholesterol LiquiColor Kit Procedure No. 1010, Boerne, TX) measured at a wavelength of 500 nm using a Bio-Rad Microplate Reader and Software (Bio-Rad Model 3550-UV, Bio-Rad, Hercules, CA). Triglyceride determinations were also made using a standard colorimetric technique (Stanbio LiquiColor Triglyceride Kit Procedure No. 2100) measured at a wavelength of 500 nm using a Bio-Rad Microplate Reader and Software. Glucose determinations were made using a standard colorimetric technique (Stanbio Glucose LiquiColor (Oxidase) Procedure No. 1070) measured at a wavelength of 500 nm using a Bio-Rad Microplate Reader and Software. Fructose concentrations were determined using a commercially available detection assay (Fructose Kit, Vitro-

life, Göteborg, Sweden/FertiPro, Istanbul, Turkey) measured at a wavelength of 490 nm using a Bio-Rad Microplate Reader and Software. Finally, protein determinations were made using a standard Bradford protein assay (*Bradford Protein Assay*, Bio-Rad)—with slight modification. Initial testing determined that the Bio-Rad reagent dye was too concentrated when following the standard protocol for Bradford protein analysis due to the high concentration of proteins in seminal fluid and had to dilute to 1:20 (determined by serial dilutions until samples could be read within the standard curve of the assay) before sample processing. With this small adjustment to the standard protocol, the resulting samples were measured at a wavelength of 595 nm using the Bio-Rad Microplate Reader and Software.

As the long-term goal of this project is to develop a system for customized modifications for individual animal cryopreservation that could be applied easily in a non-research laboratory setting (*i.e.* veterinary clinic), attempts were made to use analysis systems that might be available in the field; blood chemistry units. In addition to the independent assays listed above, attempts were made to determine concentrations of cholesterol, triglycerides, total proteins, glucose, and fructose in seminal plasma using two commercial blood chemistry systems; the Piccolo Xpress (Abaxis, Union City, CA), a system for determining concentrations of various biological molecules in human blood and the VetScan2 (Abaxis), which is an equivalent system for animal use. Three separate panels were run; the Lipid Panel Plus (Abaxis) which provided cholesterol, triglyceride, and glucose data, and the Comprehensive Metabolic Panel, which assayed for glucose, and total protein. Both of these panels were run on the human Piccolo unit. A Large Animal Profile panel was also assayed on the VetScan (Abaxis) system to provide total protein values.

While there were redundancies between cartridges, it was necessary to run all three as cross-checks, as 1) these assays had never been run on a semen product before and 2) the one instrument had been designed primarily for human blood plasma or serum, and the other instrument was designed to measure blood plasma or serum in animals; both with appropriate limits for that system. Operation of the system was simple, 150 to 200 µl of each sample was placed in the appropriate cartridge, and the cartridge was then placed onto its associated instrument (Piccolo or VetScan) to be assayed. The instruments contained electronically derived standard curves for each analyte and produced appropriate output based on blood chemistry.

2.4. Statistical Analysis

Once all data were collected, it was entered into the Statistical Package for the Social Sciences (SPSS—version 12; Chicago, SPSS Inc.) for analysis. All analyses were considered significant at $\alpha = 0.05$. The first set of analyses conducted were independent two-tailed Student's t-tests to compare means established for levels of cholesterol, triglyceride, and glucose to test for differences between the two methods/platforms for each analyte. A Levene's Test was performed prior to the

Student's t-test to test for equality of variances and ensure the selection of the appropriate P value in the Student's t-test. Comparisons of means of assays conducted across three testing platforms were performed using a one-way analysis of variance (ANOVA). Results indicated no differences in testing platforms within the analyte (discussed in detail in results), and data were combined to allow repeated measures in comparison of analytes between breeds. Once the similarity of results between testing platforms was established, an ANOVA with repeated measures was performed to test breed differences for each analyte. A comparison of weights was also performed for differences across breeds using a one-way ANOVA. Finally, a Pearson's Correlative analysis was performed to determine the relationship between differences detected in sample weight and the level of analyte present within the seminal plasma.

3. Results

As expected, the size of the ejaculate at the time of electroejaculation varied widely, ranging from 0.75 to 18.0 ml. A minimal volume of 1.2 mL was needed for the initial weight experiments. Therefore, four samples did not meet inclusion criteria and were discarded. A total of 22 ejaculates collected from 4 breeds were utilized in these experiments (n = 6 black angus, n = 3 jersey, n = 9 limousine, n = 4 black angus cross). Further, not all ejaculates had sufficient volumes for all assays, so priority was given to manual assays over the unproven automated methods. However, most ejaculates provided amply volume for all analytical comparisons.

As expected, weights per known sample volume changed in the presence or absence of spermatozoa (**Figure 1**; $p < 0.004$). There were detectable differences in the weights of seminal plasma on a per-breed basis ($p < 0.001$), suggesting differences in the chemical composition of the plasma itself.

Analysis was then conducted on the three major non-water chemical groups associated with biological fluids and tissues: lipids, proteins, and sugars. Based on the literature, lipid analysis was further broken down to cholesterol and triglycerides, while sugar determination included glucose and fructose. As stated by previous authors, chemical analysis of seminal plasma has proven difficult. A minimum of two analysis methods were used for each sample for each analyte measured.

Cholesterol analysis was done using a standard manual method as well as a human blood chemistry analyzer (**Figure 2**). Results indicated that there was no difference in the analytical technique used ($p = 0.648$) or any variation among breeds ($p = 0.228$).

The analysis also showed no interaction between breed and technique ($p = 0.325$). However, while the standard method had a much wider range of results, the automated system indicated highly similar results. As the automated system is matrix dependent and human based, the results may need more research to support its use for seminal fluid analysis.

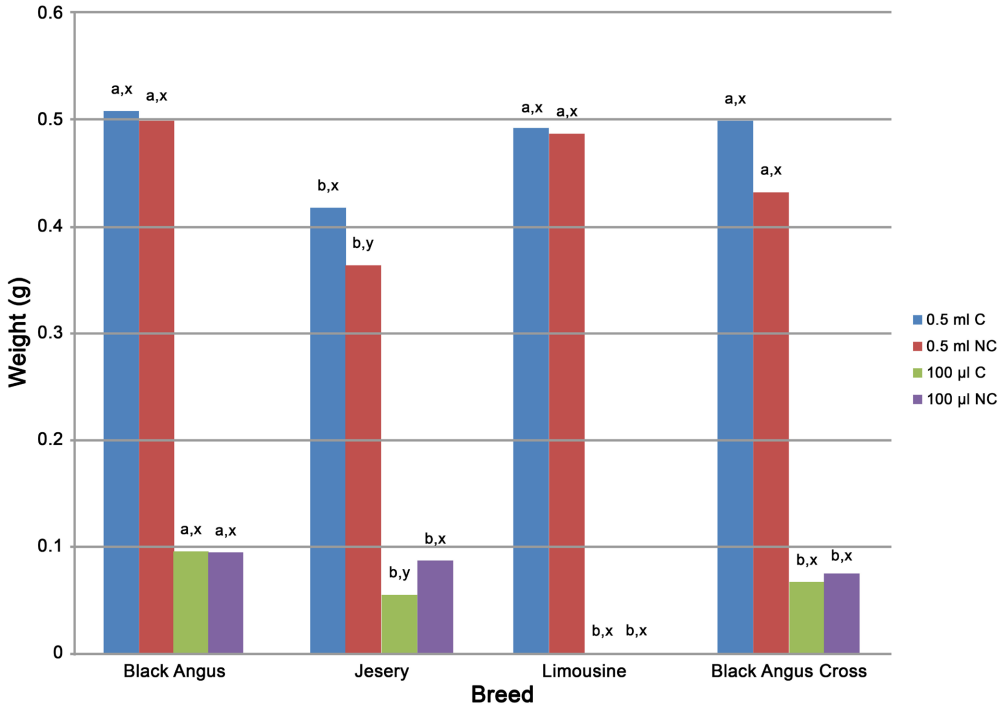

Figure 1. Mean seminal fluid weights across breeds ($p < 0.001$) and between Neat (non-centrifuged–NC) and centrifuged (C) samples ($p < 0.004$). Breed mean differences are indicated with subscripts a-b, means with the same subscript are not different. Differences between NC and C samples are indicated with subscripts x and y, means with the same subscripts are not different.

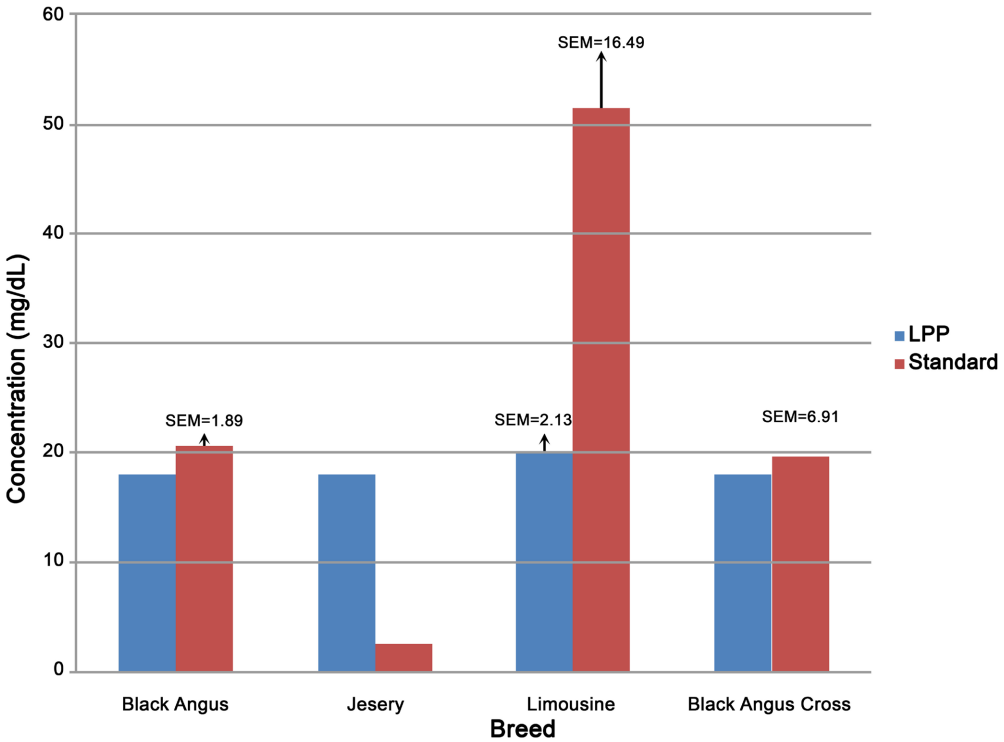

Figure 2. Cholesterol levels are shown by breed and measured using the standard assay method and lipid panel plus cartridge (LPP) ($p = 0.648$). Means of assay testing showed higher levels of cholesterol than those found in the cartridge, 31.84 mg/dL vs. 18.9 mg/dL.

Triglycerides were measured using both manual and automated techniques. Results indicated differences in the techniques used (Figure 3; $p = 0.023$). Results also indicated a difference between breeds for triglycerides in similar patterns ($p = 0.001$). However, the automated estimates are higher for each breed. Further analysis showed no interaction between the breed and technique used ($p = 0.201$).

There can be little doubt sugars play a significant role in seminal plasma chemistry. Glucose, in particular, serves as the primary energy source used for ATP production in cellular function. As gametes have a preference for fructose, both glucose, and fructose were analyzed. Glucose measurements were attempted with both manual and automated assays. However, while standards and controls in the manual assay worked as expected, the assays returned no results for experimental samples and were not reported. Figure 4 shows the results of the two automated methods. The data suggested some variation within each breed ($p < 0.001$). However, no difference in technique was detected ($p = 0.884$). No interaction between breed and technique was observed either ($p = 0.985$).

As fructose is not generally measured in blood chemistry, it was not available on either automated platform and was only detected using the manual assay. As with glucose, there appeared to be breed differences ($p < 0.009$), with only slight

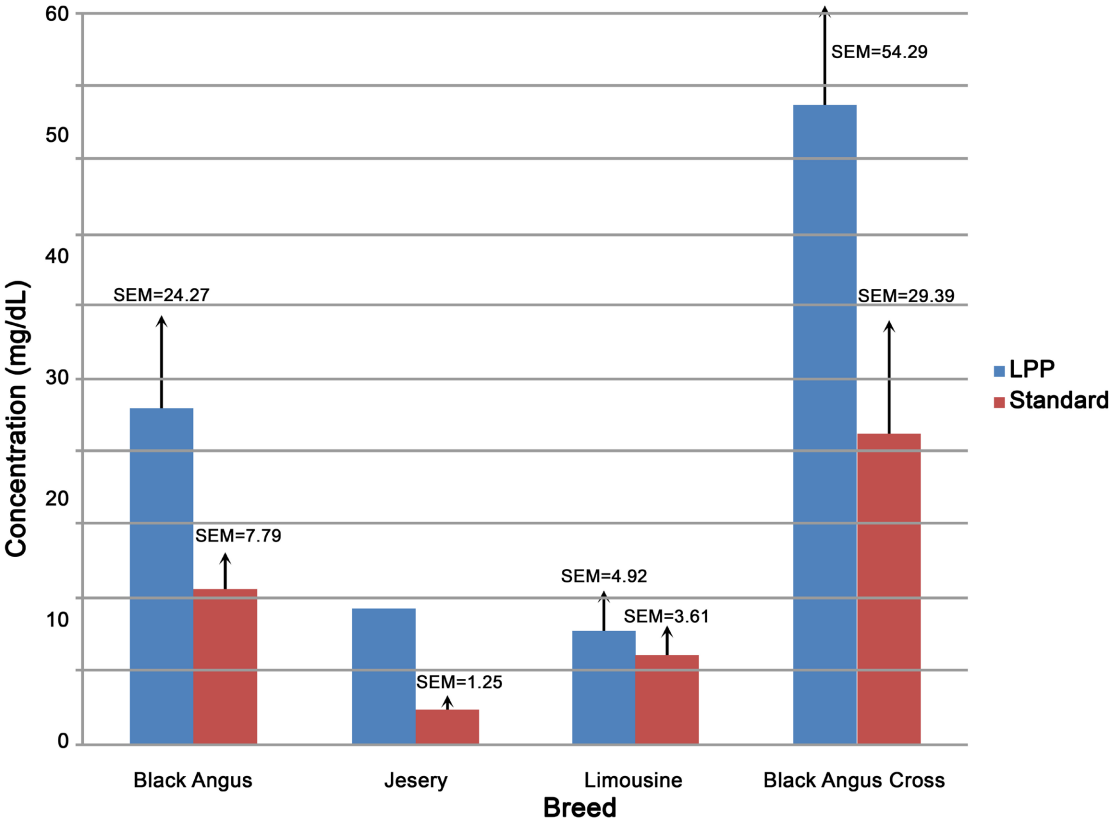

Figure 3. Triglyceride levels are shown across all breeds and measured using a standard assay method and a commercial automated lipid blood panel (LPP) ($p < 0.023$). The means of the automated method showed higher triglyceride levels than the standard assay technique, 75.39 mg/dL vs. 40.32 mg/dL.

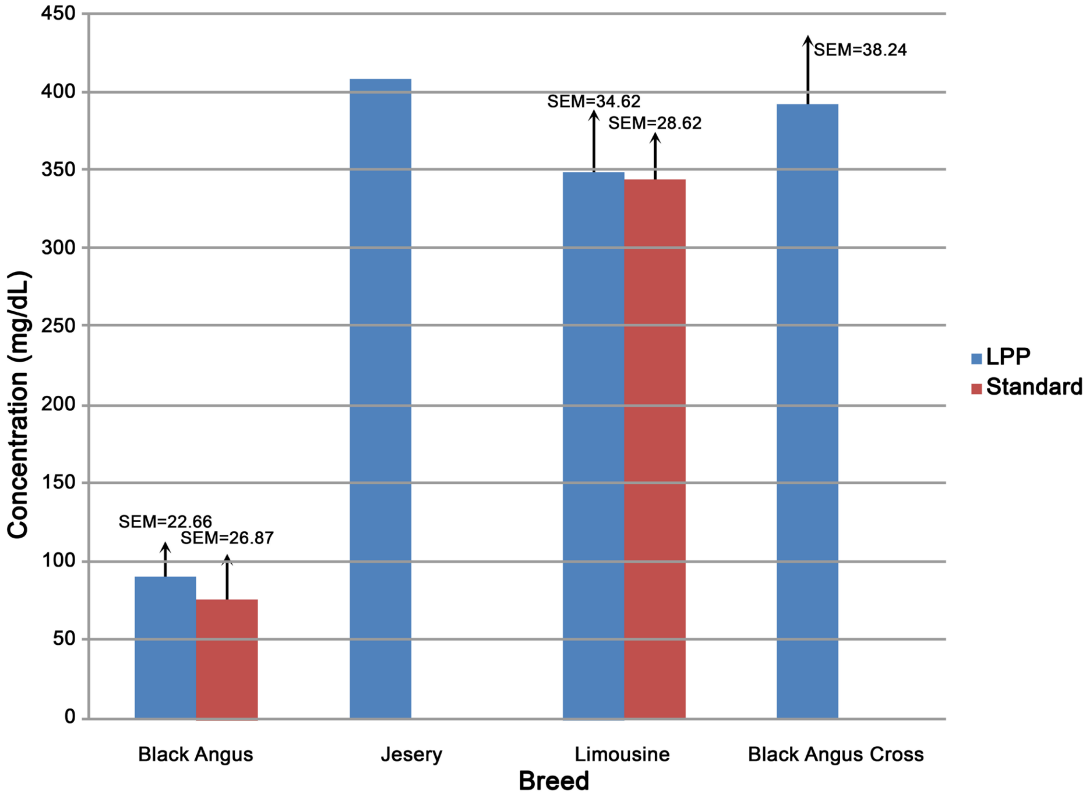

Figure 4. Glucose levels are shown by breed and measured using two cartridge methods, lipid panel plus and comprehensive metabolic panel ($p = 0.884$). Means of the two techniques proved to be close in proximity with the lipid panel plus providing slightly higher values than the comprehensive metabolic panel, 263.74 mg/dL vs. 232.67 mg/dL. Jersey bulls and Black Angus-Cross bulls were only measured in one technique due to a shortage in sample availability.

variations between the patterns observed between the two sugars (Figure 5). The similarities in these patterns suggest the automated measurements do reflect actual breed differences and no assay interference. However, the limited availability of samples for the glucose assays much make these results suspect until larger animal numbers are included in follow up studies.

The seminal fluid's protein content was assessed using the manual Bradford assay and an automated technique on each clinical machine. However, testing of the Jersey and Black Angus cross groups was limited due to sample availability. While the results indicated a difference in testing technique (Figure 6; $p = 0.018$), trends between breeds were similar in each assay. However, protein values were higher on both blood base instruments when compared to the gold standard of the manual assay. All three assays detected breed differences ($p < 0.001$), with no interactions detected between breed and assay type ($p = 0.547$).

The primary objective of this study was to determine if the weight of the seminal plasma could correlate with plasma chemistry. Both linear regression and correlative analysis were performed. Regression analysis showed no simple linear relationships between weight and chemical composition. However, correlations were found to exist among certain analytes. Analysis suggested no correlations

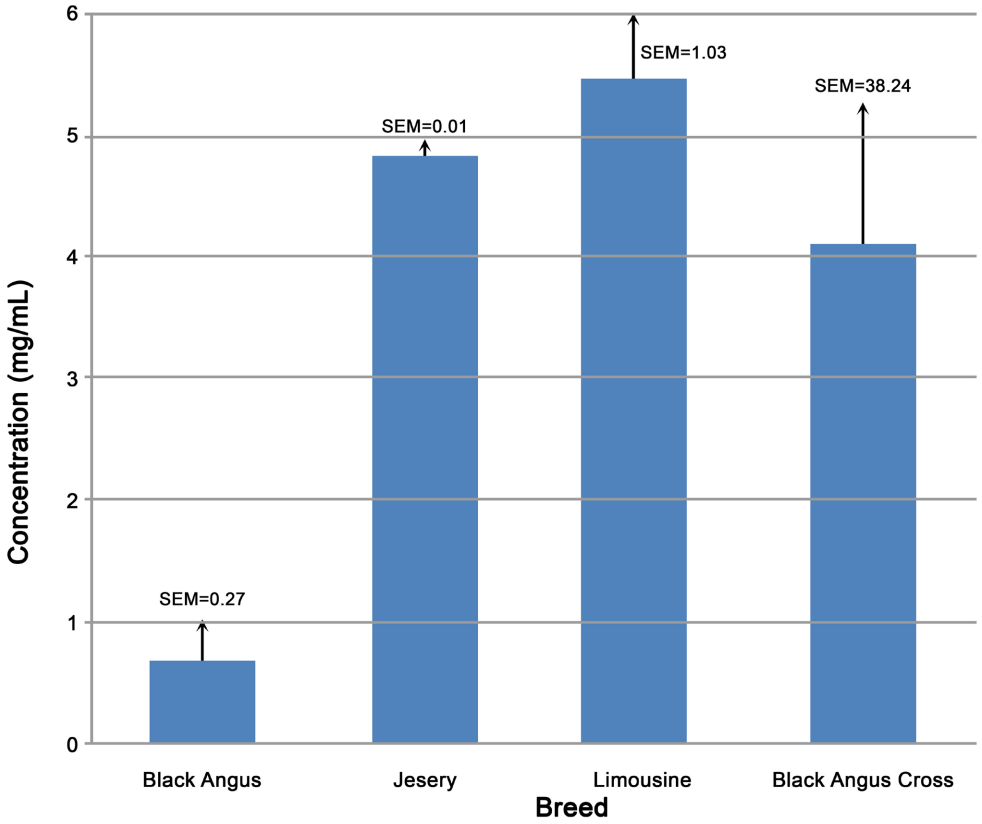

Figure 5. Fructose levels are shown across all breeds. Measurement was done using a standard fructose assay from FertiPro ($p = 0.009$). Means showed Limousine bulls to have the highest level of fructose present in seminal plasma at 5.47 mg/ml.

between cholesterol, glucose or fructose with p-values of 0.424, 0.416, and 0.405, respectively. However, triglyceride and total protein appeared to correlate with sample weight with p-values of 0.003 and 0.047, respectively. These latter data are highly suggestive and warrant further investigation. However, it must be stressed that the observations might be confounded by multiple breeds and low numbers of animals per breed.

4. Discussion

As a preliminary study, the overall goal of these experiments was to determine if there is a relationship between the chemical composition of seminal plasma and the ability of sperm to survive the cryopreservation process. Earlier work from this laboratory has suggested a relationship between maternal body condition score (BMI) and embryo quality and fitness for cryopreservation [5] [6]. Therefore the primary objective was to determine if relations might exist between the male animal's BMI, the weight of its seminal plasma, and the chemical constituents of the plasma itself; the secondary objective was to determine if plasma weight could be correlated with the chemical constituents.

Phase 1 of this experiment dealt with seminal plasma weights compared with the breeds used. As expected, cell concentrations influenced volume weight and

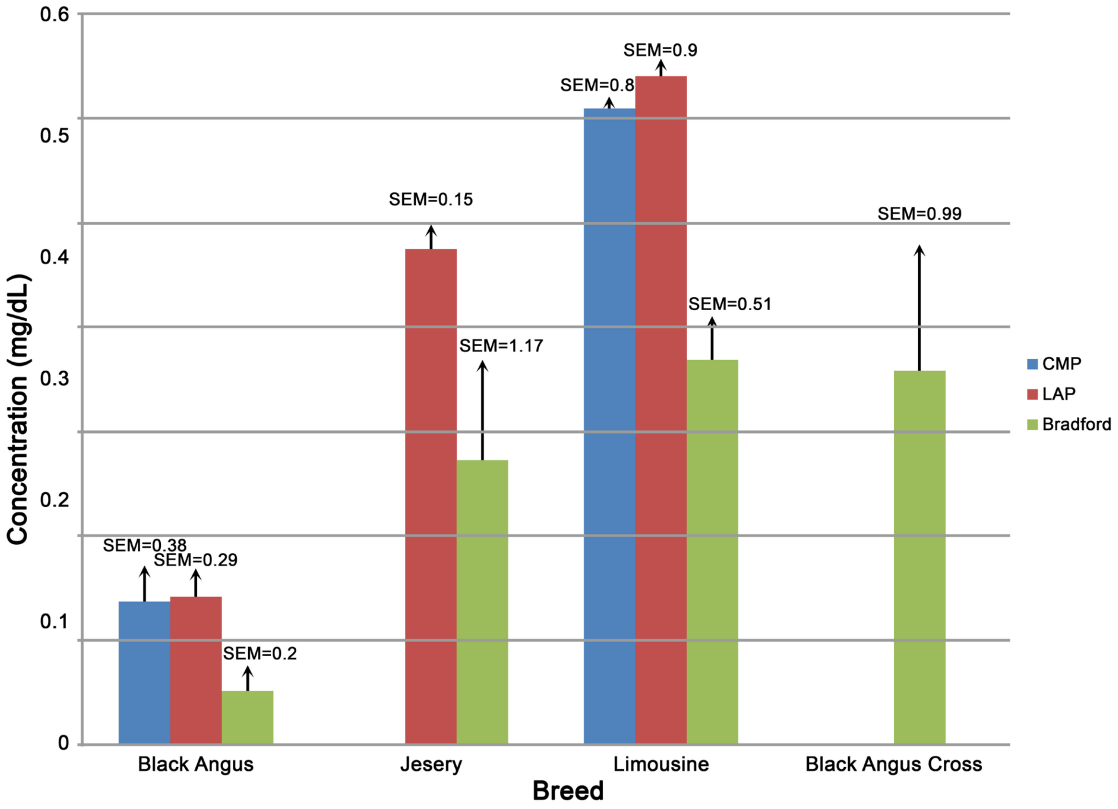

Figure 6. Total protein levels are displayed across all breed types. However, testing across assays was limited due to sample availability from the Jersey and the Black Angus cross animals. The two automated methods, the comprehensive metabolic panel cartridge (CMP) and the large animal profile cartridge (LAP) indicated higher protein content as compared to the manual Bradford method ($p < 0.018$). However, breed differences were detected and appear relatively consistent across assays ($p < 0.001$).

therefore had to be removed before determining plasma weights. While the results for just plasma demonstrated minimal variation from bull to bull and across all breeds, the *cell-free sample* demonstrated differences along breed lines, which had been pre-selected for differences in BMI. These findings suggest that plasma had differences in biochemical composition, which might influence the ability of the sample to be cryopreserved.

In Phase 2 of this experiment, an attempt was made to simultaneously evaluate the major components of the underlying biochemistry of seminal plasma. While much of the literature suggests that others have examined individual seminal components [9], to date, this appears to be the first attempt to correlate a number of factors and the survivability of semen from cryopreservation at one time. A potential reason earlier researchers had not fully elucidated seminal components is the difficulty in measuring these components in seminal plasma. We utilized redundant techniques (many of which had not been used in seminal plasma before) to ensure accurate measurements. The automated instruments were selected in collaboration with a blood chemistry expert but had not been assessed for use with seminal plasma prior to these experiments. However, it must be stressed that the preliminary outcome of these experiments was to de-

termine if sample weight could be correlated to seminal components with the standard chemistry assays. Automation of the chemistry assays would only make such assessment of any such correlations easier in a production setting. The results suggest that, while there was some variation in the absolute values obtained from each technique, they demonstrated similar patterns across the breeds measured and demonstrated that there were differences among the breeds. However, the data also suggests the older, manual techniques for chemistry determination might be best with semen as the matrix of the semen sample might interfere with the automated blood platforms.

The final phase of this study was an attempt to correlate the observed differences in plasma weight to its chemical composition. In the earlier work by Weathers [7] [8], it was found that total lipids as a function of the individual's BMI appeared to correlate with embryo weight. In the present study, cholesterol, a major component of plasma lipids [10], did not appear to correlate with plasma weight but triglycerides did. Given the importance of triglycerides to membrane integrity [11], it is easy to postulate how differences in plasma triglycerides could affect cryopreservation.

It is well documented in the literature that semen from certain species, breeds and even individuals (including humans) do not cryopreserve well. The ability to determine seminal plasma chemistry may allow simple modification of the level of cryoprotectant used and allow customization of cryopreservation techniques on a per-animal basis, allowing improved results from individuals who do not freeze well under normal conditions. Current data suggest a possible relationship between paternal body condition and triglycerides in the seminal fluid. A much larger study would appear warranted to establish such a relationship firmly further, while the current data demonstrated similar patterns between breeds for the automated verses manual assay techniques. It is acknowledged that the automated systems may not provide reliable results due to the matrix differences between blood and seminal plasma (as demonstrated by the need for dilution of the protein assays). However, f a relationship can be firmly established, then a simple weight test of the cell-free seminal plasma described might allow adjustment of cryoprotectants to customize and maximize the cryopreservation potential of samples currently thought of as "non-freezers".

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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