

# **Bison seminal plasma factor affects sperm plasma membrane integrity**

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## ABSTRACT

The bison industry has been growing exponentially over the last few decades; with a 35 % increase in the number of bison within Canada from 2001 - 2006 and bison meat selling at a record high. This growth is important for the Canadian and American economy, especially in light of recent setbacks in the beef market. Presently, bison ranchers have not made use of reproductive technologies that are commonly practiced in the dairy and beef industries. In order to optimise financial gain it would be advantageous to make use of these technologies within the bison industry. One issue that has interfered with ranchers being able to make use of reproductive technologies is; bison are wild animals being kept in captivity. This means that bison become highly stressed while being handled in close contact with humans. When stressed, the bison become unpredictable and dangerous, possibly hurting themselves and the handlers. Moreover, stressed bison are difficult to electroejaculate and the collected semen is low quality.

The first objective of this study was to find a way to lower the stress in bison during handling in order to improve methods for semen collection. It was hypothesised that a long acting tranquilizer (Piportil<sup>®</sup>) would lower the stress levels in the bulls, and improved the quality of collected semen. The results of the study that make up this thesis demonstrated that the animals treated with 200 mg/bull of Piportil<sup>®</sup> were less stressed when moved through the hydraulic-controlled chute system ( $P < 0.05$ ). Also, treated bulls had lowered endocrine stress indicators during handling and semen collection while testosterone production was increased for treated bulls compared to their control (corticosterone:  $0.101 \pm 0.01$  vs.  $0.145 \pm 0.02$  ng/mL; testosterone:  $9.107 \pm 1.68$  vs.  $5.327 \pm 0.74$  ng/mL) respectively ( $P < 0.05$ ). Moreover, the quality of semen collected from bulls treated with 200 mg of Piportil<sup>®</sup> was significantly better when compared to untreated bulls ( $P < 0.05$ ). No detrimental side effects were observed when using Piportil<sup>®</sup> on the bison bulls, allowing us to suggest that this drug provided a way of lowering level and increasing semen quality.

It has been suggested, that there maybe a factor in bison seminal plasma that affects the sperm plasma membrane causing increased damage during cryopreservation; resulting in low post thaw motility. However, this factor appears to be captured in the low density lipoprotein (LDL) fraction of an animal based extender. The objective of this part of the study was to identify the bison seminal plasma proteins that were captured by the LDL in Triladyl<sup>®</sup>.

Bison seminal plasma proteins that were associated with either LDL from Triladyl<sup>®</sup> or the phospholipids from Andromed<sup>®</sup> were analysed using 2 dimensional electrophoreses (2DE) (47 and 21 protein spots respectively). From these spots, 17 protein spots of interest were identified by Matrix-Associated Laser Desorption Ionization Time of Flight (MALDI-TOF). Once the protein identities were known, the main functions of these proteins were identified. It was found that 6 of the proteins had functions directly involved with the spermatozoa. From these 6 proteins, only one type had a function that could possibly be associated with sperm being unable to survive the cryopreservation procedures. These proteins are called binder of sperm proteins (BSPs) and they are involved with cholesterol and phospholipid efflux from the sperm plasma membrane, initiating capacitation. However, a procedure must still be developed to prove whether BSP is the only interfering factor.

In conclusion, the use of LAN will help to reduce stress in bison bulls during handling and electroejaculation and may increase the quality of collected semen. This semen can then be used in a variety of reproductive technologies within the bison industry. The use of these reproductive technologies will be further enhanced once a method to successfully cryopreserve bison semen is developed. The first step in this process will be to identify the seminal plasma factor that is interfering with the sperm ability to survive cryopreservation and then to develop a method to neutralize its actions. The realization of these goals will be beneficial to bison producers in Canada and the United States to increase economic gain in the future.

## **DEDICATION**

This thesis is dedicated to my mother Simone Gratton for all her support and hard work helping me with editing and grammar throughout the process, also to my father Ron Gratton for supporting me and never giving up on me during this journey.

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## LIST OF ABBREVIATIONS

2DE	Two dimensional electrophoresis
μA	Microampere
μg	Micrograms
μl	Microlitres
AI	Artificial insemination
BPB	Bromophenol blue
BSP	Binder sperm protein
CAGR	Canadian Animal Genetic Resources program
CASA	Computer assisted sperm analysis
CNRC	Canadian National Research Counsel
COSEWIC	Committee on the Status of Endangered Wildlife in Canada
g	Grams
GAG	Glycosaminoglycan
GC	Glucocorticoids
HDL	High density lipoprotein
IEF	Isoelectric focusing
im	intramuscularly
I <sub>p</sub>	Isoelectric point
IVF	<i>In vitro</i> fertilization
IVP	<i>In vitro</i> production
LAN	Long acting neuroleptic

LDL	Low density lipoprotein
MALDI-TOF	Matrix-Associated Laser Desorption Ionization Time of Flight
mg	Milligram
mL	Millilitre
mM	Molar mass
n	Number
ng	Nanogram
PBS	Phosphate-buffered saline
PAF	Platelet activating factor
PAF-AH	Platelet activating factor –Acetylhydrolase
PM	Progressive motility
Rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SP	Seminal plasma
TGS	Triglycine sulfate
TM	Total motility
USDA	United States Department of Agriculture
USD	United States Dollar
V	Volts
VAP	Velocity average path
VCL	Velocity curve linear
VSL	Velocity straight linear

## CHAPTER 1

### 1. GENERAL INTRODUCTION

The North American Bison (*Bison bison*) is the largest native land mammal in North America. Traditionally two subspecies have been classified, the Plains bison (*Bison bison bison*) and the Wood bison (*Bison bison athabascae*) [1]. However, recent genetic testing has shown a degree of hybridisation between all Wood bison with Plains bison leading to a change to merge both subspecies [2]. In the early 1900's, these animals were hunted to near extinction, but with the combined conservation efforts of governments and ranchers both subspecies have made a remarkable comeback [3]. Plains bison are no longer considered threatened in the United States or Canada, but Wood bison were still considered threatened by Committee on the Status of Endangered Wildlife in Canada (COSEWIC) at the last assessment done in the year 2000 [4, 5].

The near loss of the North American bison has raised awareness of the importance of bison in both industry and the environment. Canada signed the Global Plan of Action for Animal Genetic Resources at the International Conference on Animal Genetic Resources for Food and Agriculture in 2007 [6]. This resulted in the creation of the Canadian Animal Genetic Resource programme (CAGR) whose mandate is to preserve the genetic diversity of all livestock, through both management of live animals and the creation of a bank of genetic material [7]. Bison are included in CAGR because of their economic importance. Bison meat is seen as a new low fat alternative to beef for consumers and the price of bison meat is at a record high [8]. There are few producers outside of Canada and the US, and North America is one of the few sources of bison products for export around the world [9].

Bison have evolved for thousands of years in the North American climate which makes them an easily managed animal that needs little human interference [9]. At this point, most bison are ranched with a more holistic approach allowing them to be grass fed on large ranches with little human contact until they are ready for slaughter [8]. This is not the most economical way to produce bison and growth within the industry is forcing producers to begin to adopt ranching practices already in use in the bovine industry. This movement has led to the realization that there is very little actually known about bison physiology and reproductive biology. Recently, there has been an increase in interest and research to fill these voids. The research presented in this thesis will be focusing mainly on reproductive aspects of bison; particularly bulls.

Bison are non-domesticated animals being raised in a ranch situation; as a result bison can be unpredictable animals to work with because they are easily stressed. In a modern ranch setting the animals must come into contact with humans in many situations including; being vaccinated, checked for breeding soundness and being prepared for slaughter. When bison come into close contact with humans, they become stressed and can easily cause injuries to themselves and their handlers. Long Acting Neuroleptics (LAN's) have been shown to calm wild animals for a prolonged period of time without causing any permanent harm [10]. For this study, a LAN was examined to see if it is an appropriate method to keep bison bulls calmer while being handled by humans over an extended period of time.

Within the bovine industry, bulls are subject to breeding soundness evaluations at approximately 1 year of age [11]. This type of evaluation is vital to ensure economical ranching practices by removing bulls that may be poor breeders for a variety of reasons. This practice has only recently been introduced to bison ranching and typically follows established bovine protocols [11]. An increase in semen collection for breeding purposes in bison has led to greater interest in cryopreserving semen to preserve genetics from bulls for later use. Cryopreservation of semen has been used since the 1950's and is extensively used in the bovine dairy industry. Success of semen cryopreservation can be very species dependent and this holds true for bison.

When typical bovine methods are used to cryopreserve electroejaculated bison semen, the post thaw results are far below typical bovine standards. Bison show a more than 50 % decline in total motility in post thaw samples and semen has been observed to have different survivability depending on which cryopreservation extender is used (Triladyl<sup>®</sup> 35 % vs. Andromed<sup>®</sup> 9 % total motility) [12]. Interestingly, when bison epididymal sperm was removed and cryopreserved the post thaw motilities were over 50 % for both extenders [13]. However, the use of epididymal semen is not a viable alternative in the bison ranching industry as it is dependent on collecting from deceased or castrated bulls. In order for the bison industry to be able to use cryopreserved bison semen, an improved protocol must be established. The fact that sperm collected from the epididymis have a much higher post thaw survival compared to ejaculated sperm has led us to suggest that there might be an interfering factor found within seminal plasma that is reducing post thaw survival of bison sperm after cryopreservation . This factor appears to be captured by the LDL fraction of Triladyl<sup>®</sup> extender, but not by the phospholipids in Andromed<sup>®</sup> extender [12]. The goal of this study was to determine whether unique bison seminal plasma proteins are being captured in the LDL fraction of Triladyl<sup>®</sup>.

A better understanding of the reproductive biology of bison bulls should lead to the creation of advanced cryopreservation procedures. This would allow for greater movement of bison bull genetics between herds and increase the economic viability of bison ranching. Finally, advances in these technologies will allow for important genetics to be saved in cryogenic banks for future use. [14]



## CHAPTER 2

### I. LITERATURE REVIEW

#### 2.1. Natural History of Bison

Just three hundred years ago North American bison ranged from the Arctic Circle to Mexico and from Oregon to New Jersey [1, 15]. Their numbers were estimated to be around 30 million head [1]. Bison are the largest land mammals in North America; they are sexually dimorphic animals with an average weight of approximately 2000 lb in males and an average of 1100 lb in females [16]. Bison acted as a keystone species for the Great Plains as their grazing habits disproportionately influenced the type of vegetation [17].

Bison can be divided into two subspecies plains bison (*Bison bison bison*) and wood bison (*Bison bison athabasca*). Traditionally plains bison were found in the USA and Southern Canada, while wood bison lived in northern Alberta and the North West Territories of Canada [16]. The two subspecies are very similar in most physiological aspects but some morphological differences have been noted. Wood bison tend to be larger animals with humps that peak before their shoulders, where as the plains bison's hump is centered over their shoulder or slightly behind [18]. Wood bison tend to have a slightly darker hair and the hair on their head tends to be longer and straighter than the plains bison. Recent advances in genetic testing has revealed that all Wood bison contain some Plains bison genetics, this knowledge has led to controversy about whether keeping the subspecies designation [2]. At this point in time bison are still referred to as their separate subspecies but the future trend maybe to combine them into just one species (*Bison bison*) [2]. Both subspecies of bison are herd animals, but most of the year the males and females live in different herds [1]. Females tend to form larger herds than males, but herd size and

composition changes depending on grazing resources and time of year [17]. Males often form small bachelor groups of 2-3 individuals, but this association stops during the rut when the males drift through herds of females seeking to mate [16, 19].

Early European colonisers used bison for meat and their hides for clothing and leather [15]; however, bison were also, excessively hunted for sport [15]. By the mid 1870's unregulated hunting of bison led to a virtual extinction of the species [3, 20]. Within Canada by 1890 all plains bison had been destroyed and only a very small herd of wood bison consisting of around 250 individuals remained in the north [20]. The wood bison population was further devastated when, from 1925-1928, the Canadian Government introduced 6673 plains bison into the recovering wood bison herd at Wood Bison National Park (Alberta and North West Territories, Canada) allowing for crossbreeding [18]. This almost resulted in the loss of the wood bison subspecies [18]. In the 1900's a few ranchers residing in the United States noticed the rapid decline of the Plains bison population and captured some of the few remaining animals and kept them at five different private ranches [3, 20]. Along with the ranches, a small herd of about 100 plains bison were protected within the sanctuary of Yellowstone National Park (Wyoming, Montana, Idaho, USA).

The remarkable comeback of the bison was spurred by private individuals, government involvement and conservation groups like the American Bison Society [15]. Private ranchers thought that Plains bison could be bred with their cattle to create offspring that would be better adapted to North America's cold climate [4]. The resulting offspring called cattalo, or beefalo, had low fertility rates and were not economically viable [4]. Soon these same ranchers realized the value in raising bison and began to market the meat and other products from their captive animals [21]. Individual citizens and ranchers within America created the American Bison Society in 1905 and were able to lobby for the creation of several public conservation herds within the USA [3]. The establishment of the National Bison Range was one of their early successes [21]. These state parks worked so well in conserving the existing bison populations that

in 1966 Custer State Park (South Dakota) held its first auction to slim down the herd [22]. This auction brought in ranchers from all over North America to buy these animals and it was considered to be start of the modern commercial bison industry [22]. Within Canada the government, through the national parks system also began to show an interest in the conservation of Plains and Wood bison [3]. In 1907, a herd of Plains bison was purchased from a ranch in Montana and shipped to Elk Island National Park and eventually to a park in east-central Alberta [3]. This was the beginning of the return of Plains bison within Canada. Wood Buffalo National Park was created in 1922 to save the remaining wood bison in Canada [23].

Presently, bison in both private and conserved herds are descended from those few remaining animals; creating a genetic bottle neck leading to the loss of genetic diversity [24]. Compounding this uncertain genetic situation, it is also believed that about 1% of the bison population have some cattle ancestry due to early crossbreeding attempts [20]. This loss of genetic diversity may affect the population's ability to adapt to future changes in their environment [24].

### **2.1.1. Bison Industry**

Raising bison has become a lucrative business for many ranchers. Part of the appeal of bison meat to consumers is that it is lower in fat and higher in zinc than traditional beef [25]. Also, the animals are adapted to live in the North American climate and as a result some ranchers find them to be a hardy, low maintenance livestock. A Canadian Census of Agriculture done in 2006 found that there were 195,728 bison in the country, a 34.9 % increase from the 2001 census [4]. The American herd has also seen exponential growth over the last decades with over 70,000 bison slaughtered in 2008 [26]. Most Bison producers in Canada are concentrated within the prairies including Alberta, Saskatchewan and Manitoba. The Agricultural censuses found that Saskatchewan supported the second largest herd with 29.3 % of the national herd from 2001-

2006 [4]. At this time the number of head in Saskatchewan increased from 34,781 to 57,395 [4]. Canadian producers ship large numbers of bison over the border to the United States, with 3,736 head recorded in 2008 [26]. In the month of August 2011, 761 live bison were exported into the USA from Canada [8].

With increased knowledge of bison reproduction and development of an improved protocol for cryopreservation of germplasms can be established. Once protocols are established the industry can ship bison germplasm between countries similar to the present day cattle industry. Use of frozen germplasm would enable the movement of unique genetics, allowing the genetic diversity of bison to be saved and would also reduce the transmission of disease between herds.

### **2.1.2. Handling Stresses on Ranched Bison**

It has only been in the past 100 years that bison have been kept in ranch situations. This short period of time has not allowed for much domestication to take place. Therefore, bison act more like wild animals than domestic cattle and must be treated differently and with more caution. A difference when handling bison compared to cattle is that they do not like to walk single file like cattle in a chute system. Bison are much calmer when able to move side by side, forcing single file movement can result in animals crowding each other and jumping onto one another causing injuries [27]. Being a prey animal, bison have a keen sense of smell and hearing. Interestingly, bison behaviour can be modified through training and habituation, but this is a long process that is not always practical on a modern bison ranch [12, 28]. Ranchers must become alert to the slightest change in bison behaviour to prevent stress in the animals as they work with them. Subtle signs of stress in bison include licking, increased blinking, huddling together, raising their tails, circular movement (milling), backing up and balking [28]. As stress continues behaviours such as laboured breathing, frothing in the mouth, vocalizing, bulging eyes, running,

pushing, goring, sitting, laying down as well as jumping or climbing out of their enclosure may be observed [28]. If stress and fear become too great for the animals to handle they are known to go into a state of tonic immobility [28]. Tonic immobility, is a form of shock characterised by a state of motor inhibition; this state is reversible [29]. This knowledge is of utmost importance to ranchers because stressed animals can be unpredictable and dangerous to themselves and anyone working near them.

It has been shown that handling stress can also influence reproductive success in some species [30]. When wild animals are held in captive situations, unnatural stresses combined with natural stresses may affect many aspects of the animals wellbeing over a longer term [31]. With heifers selling for up to \$1500 and meat prices at a record high since the USDA began tracking [8], it is economically important for ranchers to keep their animals as stress free as possible.

### **2.2.2 Physical effects of stress on Animals**

Stress can affect animals in many physical and physiological ways. When faced with a stressful situation the body will automatically shift energy away from non essential functions like the digestive tract and reproductive organs to essential areas including the heart and lungs [32]. There are two main pathways that the stress response can act on; the neural and the endocrine pathways [33]. The neural pathway involves the release of catecholamines (dopamine, epinephrine and norepinephrine) from the adrenal medulla which in turn elevates heart rate, blood pressure and respiration rate [33]. The endocrine pathway acts through the hypothalamo-pituitary-adrenocortical axis causing secretion of glucocorticoids (GC's) from the adrenal cortex [33]. Cortisol, corticosterone and aldosterone are included in the GC's family of steroid hormones [32, 33]. Within the body, GC's have a wide area of effects including the immune system, muscle production and reproduction [32, 33]. Both pathways play an important role in allowing the animal to have heightened senses and to enter what is often referred to as a "fight or

flight” state, but long term exposure to the products of the pathways can cause detrimental effects on the animal. Prolonged exposure to GC’s (chronic stress) can lower the immune response in animals leaving them open to infections [16]. The stress response involves initiation of hypothalamo-pituitary-adrenocortical axis which in turn has been shown to reduce the levels of killer T cells and the function of macrophage cells in the body, leaving the body more susceptible to pathogens [34].

Bison are social animals that live in a dominant hierarchy society; this in itself can impose stress on individuals. Interactions between males to establish a higher rank causes increased cortisol levels as monitored in fecal samples [33]. Cortisol levels are highest during the rut period when males are fighting to be able to mate with females [35]. Analysis of fecal testosterone levels have indicated that males tending females preparing to mate have higher cortisol levels than other males. Fecal analysis has shown that cortisol levels increase with dominance rank for bison [33]. Testosterone also increases with bison rank, thus dominate males produce the most testosterone and in turn have the highest levels of GC’s [35]. When bison are kept in captivity they are more likely to encounter stressful situations. Examples of these stressful situations include, humans coming into close contact while handling bison and bison being moved into new herds where social interaction increase because of the need to re-establish dominance order in the group.

## **2.2. Tranquilizers**

Humans have been using substances to alter their moods and feelings for as long as history can date. In the past, natural forms of chemicals were used including alcohol, opium smoking or chewing certain plant leaves and even extracting poisons from animals to eat [36]. Some of the first man-made chemical tranquilizers were developed in the early 1900 with the invention of “Veronal”, a commercially available barbiturate for humans [37]. It was not until the late 1950’s that tranquilizers became a common phenomenon in everyday society [36]. Tranquilizers are categorized as either minor or major. Minor tranquilizers include some of the

most widely prescribed tranquilizers like valium; these drugs do not have antipsychotic properties [37]. Major tranquilizers have antipsychotic properties and are typically used to manage psychosis including schizophrenia and bipolar disorder in humans [37]. Chlorpromazine the first major tranquilizer was discovered in 1952 when it was used as a surgical anaesthetic. It was soon being used in extreme mental patients instead of a lobotomy (surgical cutting the nerve fibres connecting the frontal lobes to the thalamus of the brain) [38]. Since the introduction of chlorpromazine, several more major tranquilizers have been developed which act in slightly different ways on the nervous system but all have similar sedative effects including perphenazin, haloperidols and pipothiazine [39].

### **2.2.1. The Use of Tranquilizers on Wild Animals**

Wild animals interpret humans as a threat to their survival, and will not allow humans to come in close contact. But in order to gain in-depth knowledge about these wild animals close contact is necessary. However, this contact can increase stress on animals causing them to act in unpredictable and dangerous ways towards humans. Moreover, captured wild animals kept for long periods of time have high incidences of stress-related death [40]. The only way for humans to successfully work closely with wild animals is to completely immobilize them with a hypnotic-sedative tranquilizer like a barbiturate or long acting neuroleptics (LANs) which chemically lower stress levels in animals making them less fearful of humans. The focus of this thesis is on the use of LAN's in wild life; hypnotic-sedative tranquilizers will not be covered.

### **2.2.2. Long Acting Neuroleptics (LANs)**

LANs are long acting sedative drugs that work by having the active ingredient dissolved in oil (like sesame, vegetable or other medicinal oils) that does not break down immediately once

it enters the body. The oil helps to delay the hydrolysis of the active ingredients thus slowing absorption into the blood [41]. Neuroleptics were first designed to help with the treatment of psychosis in the early 1950's [42]. The first long acting neuroleptic Fluphenazine was released in 1963 it was designed for use in non-compliant patients [42]. Since the first LAN, many more have come onto the market including penfluridol, zuclopethixol, clopenthixol and pipotiazine [39]. LAN's can be administered orally but work best as an intramuscular injection within humans. These drugs typically begin to act quickly for humans and the duration of the effects depends on the drug; some drugs have been shown to last up to four weeks before re-injection is needed [39]. Antagonist drugs are not needed to counteract the sedative effects of LANs, because the active ingredients are metabolised by the body. This property is advantageous when working with captive wild animals to minimize the number of injections and human contact needed.

### **2.2.3. The Use of LAN's on Wild Animals**

Over the last 20 years LANs have proven to be an excellent alternative form of tranquilization compared to hypnotic-sedative drugs for handling wild animals [43]. Because LANs help to reduce stress, they allow wild animals to be kept in enclosures for longer periods of time [44]. This facilitates behavioural observation of animals in close contact with humans [45-47]. Under the influence of LAN's animals have lower heart rates, increased smooth muscle relaxation, lower cortisol levels and a decreased flight zone [10, 40, 48]. Another benefit of LANs is that they are metabolized by the body and therefore antagonist drugs are not needed and fewer injections are required in the animals [39].

A serious side effect of LANs involves repeated involuntary movement of body parts; this condition is called tardive dyskinesia [39, 43]. This condition can interfere with normal functions like eating, drinking and walking [39]. Appetite suppression was observed in captured wild



impalas injected with the LAN Piportil [40]. No thermoregulation issues were seen when LANs were used in domesticated goats [10, 40].

A commonly used LAN for wildlife is Piportil L4<sup>®</sup> (Pipothiazine Palmitate). Like all LANs, it was designed as an antipsychotic drug with long-acting sedative effects for use in non-agitated chronic schizophrenic patients [41]. Its duration of action can last from 3-6 weeks in humans but repeated injections every 4 weeks are common [41]. This drug has been used for translocation of animals, especially in Africa where large mammals are often being moved between game reserves to help stabilize populations [10, 40]. The long duration of action (2-4 weeks) of Piportil<sup>®</sup> makes it appropriate for use in animals [40, 43]. Piportil<sup>®</sup> also has been successfully used in female bison with no visible side effects (Dr. McCorkel, personal communication). The drug lasted approximately 2 weeks in the female bison before re-injection was performed (Dr. McCorkel, personal communication).

### **2.3. Bison Reproduction**

A pre-requisite to a successful bison breeding programme is an understanding of the reproductive characteristics of bison. This is important for the growing bison industry where ranchers are constantly striving for more economical ways to produce bison.

#### **2.3.1 Females**

The main focus of this thesis is on bison bulls, but it is important to mention females when talking about reproduction. Bison breed only once a year during the breeding season which

begins July and finishes late September [49]. Bison heifers typically breed for the first time during the second breeding season having their first calf at 3 years of age [49]. Gestation lasts 262-293 days; calves are born weighing between 30-50 lb [50]. Single calves are most common but twins may be born; in the wild one of the twins will often be left by the mother because she does not have enough milk for both [51]. Most females will only be mated once during the rut; however they have a naturally high rate of conception and up to an 80 % birth rate [1, 52, 53]. Bison cows generally are long lived often producing calves until the age of 25 years [49]. Recently the use of ultrasound technology has revealed that bison cows have smaller ovaries, follicles and corpora lutea than similarly aged bovine females [49].

### **2.3.2. Males**

There has been little research of male bison physiology up to this point in time. We know that during the breeding season males will grow extremely thick hair on their foreheads, most likely for protection when fighting for dominance against other males [1]. Bulls also grow more beard hair and on the back of the forelegs to display their virility to females and other males [1]. When a female is in estrus bulls will exhibit tending behaviour, e.g., The bull will stay near to the female, often rest the chin on the females back as well as sniffing and licking the female until she is ready to copulate [1]. It is known that male bison have similar reproductive organs to male cattle but some morphological differences do exist. Bison males tend to have smaller testes than cattle. At one year of age a male bison testis weighs an average of  $53.3 \pm 8.6$  grams, while a one year old bovine bull's testes averages  $89.9 \pm 26.2$  grams [54]. Bison also have thicker scrotal skin and more hair covering their testis than male cattle; this may be an environmental adaptation for male bison living in cooler climates [54]. Bison tend to grow slower than domestic cattle. While domestic cattle reach sexual maturity at 1 year of age male bison reach this benchmark at 2 years of age [54]. In bison it has been shown that sperm is present in ejaculates between 13-15 months of age but most sperm show abnormal morphology including proximal cytoplasmic droplets [5, 55]. Quality of the semen increases significantly with increased age; the most improvement was

seen between 19 and 23 months [55]. In comparison bovine sperm is first found in the ejaculate between 8-12 months of age [55]. Bison bulls reach puberty between 14 and 18 months when puberty is defined as ejaculate containing a minimum of 50 million sperm and at least 10 % have progressive motility [55]. There is more variation in age of puberty in bison than in bovine [55]. This is likely the result of artificial selection pressure for reproductive efficiency in bovine bulls that has not been forced on bison bulls [55]. Within wild herds, bison males have been observed successfully breeding when they enter their third breeding season, but because of dominance hierarchies males between 7-9 years of age do most breeding [1, 50]. Compared to their wild counterparts ranched bison have less male competition for breeding. Often bulls as young as two years of age are released on their own with a herd of females needing to be bred. Male bison have also shown a circannual reproductive rhythm having highest sperm concentrations ( $700\text{-}800 \times 10^6/\text{mL}$ ) and normal sperm morphology (approximately 70 %) in the months leading up to and during the rut [56]. However, these semen values, as well as sperm motility do not vary much between seasons in bison bulls [56]. Therefore, bison bulls are capable of breeding at any time of year and seasonal reproduction may be due more to variation in female cyclicity.

## **2.4. Production of Semen**

This thesis will focus on the biology of bison bull semen, so I will briefly review the production of semen and the various proteins involved in semen production and quality.

### **2.4.1. Spermatogenesis**

Spermatogenesis is the process in which germ cells (diploid) develop into spermatozoa (haploid). This process takes place mostly within the testes in long convoluted tubes called seminiferous tubules. These tubules act as the scaffolding where the spermatozoa mature from

germ cells to almost functioning cells [57]. There are three main cell types involved with the development of sperm in the testis they are sertoli, myoid and Leydig cells. Sertoli cells are found within the seminiferous tubules; they surround the developing sperm cells [58]. Sertoli cells contribute to many functions for the developing sperm cells: they providing nutrients, are a scaffolding to grow in, help with phagocytosis of defective or abnormal germ cells and taking part in steroid metabolism [58]. Myoid cells provide the structure for the sertoli cells to rest on and use contractile action to propel fluid and sperm through the tubules [57, 58]. Leydig cells are found outside the seminiferous tubules, within the interstitial spaces [58]. The Leydig cells are the main source of androgens, including testosterone, which are needed to sustain spermatogenesis [58].

Spermatogenesis in bovine bulls is divided into 3 main stages: proliferative, meiotic and spermiogenic stages. In bovine bulls it takes approximately 59 days for a sperm cell to develop through all the stages [59]. The proliferative stage is when germ cells undergo rapid successive division which take the spermatogonial stem cell through mitosis up to primary spermatocytes. As spermatogonia the cells go through renewal back to stem cells or go through mitosis to become primary spermatocytes [58, 60, 61]. During the meiotic stage each spermatocyte recombines its genetic material and splits from diploid chromosomes to haploid resulting in round spermatids [60, 61]. The meiotic phase takes 23 days in a bovine bull [60]. In the spermiogenic stage, the nucleus condenses and changes from a spherical shape to the compact flattened shape of the mature spermatozoa [60, 61]. At the spermiogenic stage the flagellum elongates into a tail and the acrosome is formed [60, 61]. Spermiation, is the release of the spermatozoa from the Sertoli cells and this signifies the end of spermiogenesis. This process takes 21 days in bovine bulls; when it is finished spermatozoa look mature but are not able to fertilize an oocyte yet [60]. For bison bulls, the seminiferous tubules were seen to be smaller in diameter during the non-breeding season compared to breeding season [56]. More elongated and round spermatid numbers are also found during the breeding season, but no work has been done to look at the exact stages and time for spermatogenic cycles in bison [56]. The stages and time of spermatogenesis in bison has to be investigated to increase knowledge of bison physiology.

## 2.4.2. The Epididymis

Spermatozoa travel from the testis through the efferent ducts into the caput epididymis. The epididymis acts as both a maturation and storage site for sperm before ejaculation [62]. There are three parts to the epididymis: caput, corpus and cauda. For bovine bulls it takes approximately 11 days for the sperm to traverse the epididymis [61]. The importance of the epididymis as a whole can be seen in *c-ros tyrosine kinase receptor* knock out mice [63]. This mutation causes defects in the regionalization and differentiation of the epididymis [63]. The animals are healthy but are sterile even though actual spermatogenesis is not affected [63]. Sperm that have reached the caput epididymis are immature and do not have the ability to swim in a forward motion or bind to the zona pellucida and fertilize an oocyte [62]. A major function of the caput is absorption of excess luminal fluid from the testis and concentration of the spermatozoa [64]. Sperm continue to travel along the epididymal tube into the corpus of the epididymis; this is the region where remodelling of the sperm plasma membrane begins to take place [65]. Epididymal plasma proteins bind to the sperm surface and prepare it to move and fertilize an egg [62, 65]. In the cauda epididymis sperm are highly concentrated [62]. Within the cauda,  $\text{Na}^+$  and  $\text{Ca}^{2+}$  have been reabsorbed and  $\text{K}^+$  has been secreted into the epididymal fluid left around the sperm [65]. The pH of epididymal fluid tends to be acidic, (pH 5.8 for bovine bulls) due to a lack of bicarbonate compared to an average pH of between 6.0 and 6.5 of ejaculated bovine semen [65]. Bicarbonate is essential for initiation of sperm motility, thus sperm are immobile within the cauda epididymis [65]. Sperm in the cauda of the epididymis have a distally positioned cytoplasmic droplet, which is shed when sperm are mixed with vesicular gland fluid [65]. During epididymal storage the metabolic rate of sperm is suppressed by 3-5-fold compared to ejaculated sperm [62]. This could be a mechanism to protect sperm from reactive oxidative species during storage [62]. Sperm move continuously by peristalsis from the cauda epididymis to the ampullae via the vas deferens. The ampullae are the main sperm storage site from which sperm are ejaculated. However, there is continuous movement of sperm out of the ampullae into the urethra, thus a population of young viable sperm are always available for ejaculation regardless of the level of sexual activity [58].

### **2.4.3. Accessory Sex Glands**

At ejaculation, sperm move from the ampullae into the urethra where they mix with secretions from the accessory sex glands. The types and sizes of these glands is species dependent but most animals have ampullary glands, seminal vesicles, prostate gland and bulbourethral glands. Each gland releases different fluids that all work together to produce the seminal plasma that is ejaculated with the sperm. The main function of the ampullary gland is to act as a sperm reservoir and only contribute a small volume to the seminal plasma in bovines [66]. Ampullary glands are well developed in equine, their main contribution is a protein called ergothioneine [66]. Ergothioneine helps sperm utilize fructose and inhibits the binding of cupric ions which would inhibit sperm mobility [67]. The seminal vesicles in bovine and bison contribute a significant volume to seminal plasma. Within the secretion are proteins that are involved in sperm capacitation and the acrosomal reaction [68]. Vesicular glands produce fructose which is used as a metabolic source of energy for the mitochondria found in the midpiece of sperm [69]. The prostate surrounds the pelvic urethra and secretes a watery solution containing potassium, citric acid, phosphorylcholine and prostaglandins which contribute to seminal plasma [66]. For canines that lack vesicular glands, the prostate is the major accessory gland [66]. A glycoprotein secretion released from the bulbourethral gland helps to remove excess urine from the urethra prior to ejaculation and provides lubrication for coitus [66, 70]. The bulbourethral glands are well developed in boars and they produce the gel portion of ejaculates [71]. Accessory sex gland secretions are influenced by testosterone and estrogen [69]. Contractions of smooth muscle lining the glands is controlled by oxytocin which is secreted by the posterior pituitary [69].

### **2.4.4. Seminal Plasma**

Seminal plasma is a complex mixture of proteins, sugars, lipids and inorganic molecules such as calcium and zinc [72]. The amount of seminal fluid that each accessory sex gland

contributes depends on the species. For bison the greatest contribution is from the vesicular glands [73]. The fluid released from these glands mixes with spermatozoa at the moment of ejaculation. Proteins in seminal plasma play a variety of roles including: enhancing sperm transport through the female genital tract, helping to regulate the uterine immune response, keeping the environment surrounding the sperm at an optimum pH of approximately 7.5, helping in establishment of an oviductal sperm reservoir, regulating sperm capacitation and acrosome reaction and finally playing a role in gamete interaction and fusion [69, 73]. All of these actions accumulate towards the eventual goal of fertilization.

The protein profile of seminal plasma varies between species. For mature Holstein bulls 2-dimensional SDS-PAGE analysis showed that their seminal plasma had an average of  $52 \pm 5$  protein spots [74]. Bovine seminal plasma contains a large proportion of binder sperm protein (BSP), along with proteins involved in sperm membrane protection including albumin, clusterin and 5'-nucleotidase and proteins involved in sperm motility like spermadhesin Z13 and acidic seminal fluid protein (aSFP) [72-74].

For this review I will focus on 5 important proteins found commonly in seminal plasma; albumin, clusterin, binder of sperm proteins, platelet activating factor and spermadhesin. In addition to proteins there are countless types of inorganic molecules, lipids and sugars that all play essential roles in reproduction [69]. For a more in-depth look at the complex make up of seminal plasma from different species please refer to reviews by Rodríguez-Martínez et al., 2011 [73] and Aldo Poiani, 2006 [69].

#### **2.4.4.1. Albumin**

The Albumin family of proteins are small non glycosylated proteins often found in the blood plasma of mammals, but different forms of this protein have been found in both cauda epididymal and accessory sex gland fluids in bovine bulls [74]. Albumin plays a role in sperm motility, capacitation, acrosome reaction and protecting the sperm membrane against lipid peroxidation [75]. Interestingly, albumin does not actually bind to the sperm membrane but is

able to inhibit peroxidation of lipids on the sperm plasma membrane [75]. Albumin is able to act as a sink for cholesterol removing it from the sperm plasma membrane initiating the capacitation processes [76, 77]. There are currently many unanswered questions about the exact role of albumin on all these aspects of the sperm. The average albumin concentration in bovine semen is 0.32 mg/ml [78]; albumin has not been studied in bison semen to my knowledge at this time.

#### **2.4.4.2. Clusterin**

Clusterin proteins are disulfide-linked heterodimeric molecules [79]. These proteins were originally discovered in the rete testis where they were observed to be involved in cell aggregation [79]. Further study found that clusterin is secreted by cauda epididymal fluid and seminal vesicles of bovine [74, 80]. Clusterin helps to prevent oxidative damage and helps to aggregate and bind abnormal spermatozoa so they can be disposed of by the body [74]. Clusterin acts as a chaperone to help protect sperm from the effects of protein precipitation that can happen during transit through the female tract due to a change in pH [74, 81]. Moreover clusterin inhibits complement-induced sperm lysis and some metalloproteinases, meaning it stops the female's antibody defences produced by neutrophils from destroying the sperm cells [74, 81]. The clusterin found in the male reproductive tract has a pH of between 8.0-8.5 and isoelectric point (Ip) ranging from 4.8-5.2, depending on degree of glycosylation [81, 82].

#### **2.4.4.3. Binder of Sperm Proteins (BSP)**

There are three main Binder of Sperm proteins (BSPs); BSPA<sub>1/A2</sub> (PDC-109) (molecular weight of approximately 15-16 kDa and Ip of 3.6 - 5.2), BSP 3 (molecular weight of approximately 15-16 kDa and Ip of 3.6 - 5.2) and BSP5 (molecular weight of 28 - 30 kDa and Ip of 3.9 - 4.6) [83]. The BSP proteins bind to the acrosomal and equatorial regions of the sperm head along with the midpiece section of sperm [75]. In bovine bulls, approximately 65 % of the total seminal plasma proteins consist of BSPs, and approximately 30 % and 20 % of the total protein from ram and goat seminal plasma were BSPs [72]. In equine, seminal plasma proteins



SP-1 and SP-2 are orthologs of BSP proteins and account for up to 70 % of the total proteins [84]. At this point no one has looked at the complete seminal plasma profile of bison, but it is known that BSP proteins make up approximately 25 % of the seminal vesicle secretions [85]. When BSP proteins contact sperm at the time of ejaculation, they initiate the first cholesterol and phospholipids efflux from epididymal sperm [86]. This lipid efflux modifies the composition of the plasma membrane and helps to initiate capacitation [87]. A number of BSPs continue to be attached to the sperm membrane during traverse of the female tract [87]. The physical shape of BSP proteins include two fibronectin type II N-terminal domains, which increase heparin binding sites on the outside of the sperm [87]. The heparin sites allow the sperm to interact with the epithelial cells of the female tract, where they assist in the creation of a sperm reservoir within the isthmus of the oviduct [83]. This reservoir prolongs the life of the sperm and allows the sperm to be slowly released to meet the oocyte when it is prepared for fertilization [83]. As the oocyte reaches maturity, there is an influx of high density lipoproteins (HDL) and glycosaminoglycans [88] within the female tract; which interact with the heparin binding sites found on the BSPs causing a further efflux of cholesterol and phospholipids allowing the sperm to capacitate [83, 87]. This action results in additional destabilization of the lipid membrane, releasing the BSPs from the oviductal epithelium and helping with initiation of the hyperactivity of sperm [83, 87].

BSPs are necessary for fertilization as they help sperm cells to undergo capacitation to be able to fertilize the ovum. However, the actions of BSPs can negatively affect sperm during cryopreservation [87, 89]. The removal of the phospholipids and cholesterol from the sperm membrane destabilizes the plasma membrane of the sperm, allowing an increase of damage during cryopreservation [89]. Egg yolk or milk based extenders are able to bind BSP proteins so the proteins are unable to interact with the sperm membrane. The LDLs (low-density lipoproteins) found in egg yolk bind to the BSP proteins [89]. Within milk based extenders, it is the milk casein micelles that are able to acquire the BSP proteins thus stopping BSPs from interacting with the plasma membrane and causing the lipid efflux [90].

#### **2.4.4.4. Platelet Activation Factor (PAF) and Platelet Activation Factor Acetylhydrolase (PAF-HA)**

Platelet activation factor (PAF) (1-0-alkyl-2-acetyl-sn-glycero3-phosphorylcholine) are signalling phospholipids with a wide array of functions throughout the body including platelet aggregation, roles in inflammation, changes in vascular permeability and lipid mediators associating with HDL and LDL in the blood [91, 92]. PAF have also been associated with sperm capacitation though the movement of lipids in the sperm membrane [93]. The primary role of PAFs involve inflammation and immune responses [91]. PAF proteins have been found in the seminal plasma of most animals tested including mice, rats, boar, bulls and humans [94]. A primary role of PAF proteins is to help initiate hyperactivation of sperm [94, 95]. The PAF proteins are able to influence hyperactivation by initiating a cascade resulting in calcium mobilization within the cell, [94, 95].

PAF-AH (PAF-Acetylhydrolase) is a PAF deactivation enzyme produced by the epididymis, seminal vesicles and ampulla in bovine [92, 96]. In bovine only one form of PAF-AH has been discovered; it has a molecular weight of approximately 60 kDa [96]. The role of PAF-AH is to regulate the activity and production of PAF [94, 96]. Decapacitation factors act to keep the sperm from starting capacitation early and thus maintain the fertilizing ability of the sperm [93, 96]. PAF-AH is thought to work by hydrolyzing acetate from position 2 of the individual PAF protein, its removal is essential for PAF synthesis and motility and proper fertilization by the sperm [92, 94].

#### **2.4.4.5. Spermadhesins**

Spermadhesins are a family of 12-16 kDa polypeptides that have been found in a variety of species including equine, porcine, bovine and humans [88, 94, 97]. Spermadhesin proteins have the ability to bind a variety of ligands including: carbohydrates, phospholipids and protease inhibitors [98]. This ability to associate with so many different ligands helps spermadhesin to be involved in many aspects of fertilization including capacitation, lipid modification and acrosome reaction [98]. Members of this protein family, including AQN-1 and AWN, have been shown to coat the sperm membrane and help in stabilization of the membrane associated with the acrosomal vesicle [97]. Also, AQN-1 was shown to be part of the sperm mobility factor complex [88]. This factor is thought to affect sperm mobility by interfering with the dynein arm function, thus inhibiting the mobility of the sperm flagella [88]. A member of the spermadhesin family AQN-3, has been shown to be associated with acrosin-inhibitor acceptors, helping to prevent premature acrosome reaction [94, 99]. Spermadhesin (AQN-3 and AWN) is also important for sperm-oocyte interactions by acting as primary receptors for the zona pullucida of the oocyte [73, 74, 99, 100]. Although there are many members of the spermadhesin family, they all perform essential functions necessary for the sperm survival and the ability to fertilize.

## **2.5. Reproductive Technologies in Bison**

The cattle industry has been at the forefront of reproductive technologies; AI (artificial insemination) was being used as early as the 1920's to allow for superior bulls to breed more cows [101]. At present 70 % of the cattle used in the American dairy industry are bred through the use of AI [102]. In Northern Europe, up to 90 % of dairy cattle are bred artificially [103]. Bovine bulls are checked for strict breeding soundness standards before being used. They must be in good physical condition with a large scrotal circumference (greater than 34 cm) [104]. Fresh semen should have progressive motility of 65 % or higher and normal sperm greater than 70 % [105] After cryopreservation, bovine semen has sperm motility as high as 70 % [106]. The high post thaw motility achieved recently is due in part to advances in cryopreservation techniques and increased genetic selection for bulls whose semen survives cryopreservation. In

the last decade new technologies have allowed for semen to be sexed before fertilization, this is extremely important to the dairy industry where females calves are preferred over male calves [107].

With the growth of the bison ranching industry, more research has been done to improve reproductive technologies in including AI, semen collection by ejaculation and cryopreservation. These technologies are often taken from the bovine industry. Artificial insemination with ejaculated bison semen has been successfully performed and the first calf was born in 1994 using this procedure [49]. Recently, our group used frozen thawed wood bison semen to inseminate 22 bison cows, which resulted in 5 confirmed pregnancies. Three of these pregnancies were allowed to develop to full term and three female bison calves were born (Dr. Adams, personal communication). Moreover, embryo transfer using chilled semen for AI of Wood bison females resulted in the birth of 2 bison calves (Dr. Adams, personal communication). Additional knowledge on female reproduction and cryopreservation of semen is needed to improve pregnancy rates in bison before AI can be an economical option for producers. Ovarian synchronization and superstimulation is possible within the bison breeding season using progesterone and estradiol injections (Dr. Adams, personal communication). Estrus cycles have been induced outside of the breeding season with the use of Synco Mate-B a type of progesterone [49]. Collected sperm and oocytes are being used in labs for IVP (*in vitro* production) of embryos, but the produced embryos are of poor quality when compared to IVP of bovine embryos [108].

Much of the knowledge gained from the cattle industry could be applied to the bison industry. Bison semen can be collected using an electroejaculator [12]. Breeding soundness evaluations also follow similar guidelines in bison as cattle i.e., sperm motility must be > 60 % with normal sperm morphology > 70 % [11]. Bison bull scrotal circumference should be > 29 cm at approximately 2 years of age when breeding soundness evaluations usually take place [11].

### **2.5.1. Limitations of Reproductive Technologies in Bison**

Despite all the information that has been learned about bison, significant problems are still being faced by bison producers where reproductive technologies are involved. A central issue is the difficulties encountered in the cryopreservation of bison semen. Frozen thawed semen collected by electroejaculation shows a loss of over 50 % total motility when compared to fresh semen, and progressive motility often is less than 20 % [12]. Interestingly, when sperm is retrieved from the epididymis and cryopreserved there is only a loss of approximately 20 % total motility post thaw [13]. The higher post thaw total motility of cryopreserved epididymal sperm compared to ejaculated sperm indicates the presence of a factor within the seminal plasma affecting sperm ability to survive the cryopreservation procedure. This factor must be secreted from the accessory sex glands as epididymal sperm do not have contact with it. The use of cryopreserved epididymal sperm is not viable for use in the bison industry, because the bull must be dead or castrated in order to collect epididymal sperm.

Cryopreserved semen is important for many aspects in the study of the reproductive physiology of bison. Frozen thawed semen is frequently used in AI of females; it allows the genetics of superior bulls to be used in numerous females thought out the world. Cryopreserved semen can also be used for the study of *in vitro* fertilization (IVF). Technologies like IVF allow large numbers of high quality embryos to be produced that can eventually be transferred into cows. Another area in which cryopreserved semen is used is to gene bank important animal genetics. The Canadian government has created the Canadian Animal Genetic Resources (CAGR) programme for this purpose. Without advances in the ability to cryopreserve bison semen, these technologies will not be able to advance to accommodate the needs of the bison industry.

### **2.5.2. Cryopreservation of Sperm**

Cryopreservation is defined as the preservation of cells or tissues at extremely low temperatures [38]. Cryopreservation of semen was first developed in the early 1950's, by Christopher Polge [109, 110]. He accidentally used glycerol from a miss-labelled fructose bottle when attempting to cryopreserve semen and the resulting post thaw semen was motile and viable [109, 110]. In 1951, five bovine calves were born from cows inseminated with cryopreserved semen [109].

Cryopreservation can be accomplished by both slowly or rapidly cooling cells; the exact rates of cooling being species dependent [111]. For successful cryopreservation, intracellular water must be removed to avoid intracellular ice formation [111]. Too much water left within a cell will cause damage to the interior of the cell which eventually can lead to cell death [111]. This is not as important for sperm because they have very little intercellular water left at the time of ejaculation. Cryoprotectants are used to help with the removal of internal water through osmosis [111]. There are two types of cryoprotectants: penetrating (eg. dimethyl sulfoxide and glycerol) and nonpenetrating (eg. glucose and lactose) [111, 112]. Both types of cryoprotectants work by create an osmotic gradient that displaces the internal water from the cell thus dehydrating the cell prior to freezing [111].

Penetrating cryoprotectants consist of small, non-ionic molecules that are soluble in water at low temperatures [111]. The penetrating cryoprotectants are able to move into the cell, replacing the intracellular water as it moves out [111]. They also act as a buffer for some of the intracellular salts so they do not become too concentrated as temperatures are lowered [111]. Penetrating cryoprotectants are able to form hydrogen bonds with any remaining intercellular water, stopping the water from being able to form ice [111, 112]. Nonpenetrating cryoprotectants are long-chain polymers that are soluble in water but are too large to penetrate into the cells [111]. These molecules interact with the plasma membrane of the cell and create a protective layer which help the cells to avoid damage caused by extracellular ice [111]. Penetrating

cryoprotectants provide the most protection from injuries occurring during slow-cooling of the cells while, nonpenetrating cryoprotectants provide protection against rapid-cooling injuries; this demonstrates that both types of cryoprotectants are important for the safe freezing of cells [111].

As a rapid cooling method, vitrification (flash freezing of cells) has become popular [110]. The process results in a glassy solid phase, avoiding intracellular ice crystal formation, so less damage results to the cells [110]. This technology is popular for cryopreserving oocytes and embryos but is not used for sperm because of the cryotoxic effects and low survival rates [113].

### **2.5.3. Commercial Extenders**

As cryopreservation has become such a vital part of modern reproductive techniques, the commercial companies involved in cryopreservation industry have begun creating extenders for semen freezing in a variety of species. Typical extenders contain the same basic ingredients including; buffers, carbohydrates (glucose, lactose, raffinose, saccharose and trehalose), salts (sodium citrate, citric acid), cryoprotectants (egg yolk or milk) and antibiotics (penicillin, streptomycin) [114]. Extenders are often designed to be species specific to optimise survival of sperm by the cryopreservation procedures, because semen from different species has different pH and osmolarities [109]. There are many different varieties of commercial extenders being used including: Triladyl<sup>®</sup> (Triladyl), Andromed<sup>®</sup> (Andromed), Androhep<sup>®</sup>, SpermAid<sup>®</sup> and MR-A medium<sup>®</sup> to name just a few. Citrate-sugar-based extenders were widely used for freezing semen prior to the 1960's [109]. Lactose-based extenders have been used for freezing ram semen and are often combined with egg yolk [109]. Tris-extenders are the most common extender used in bulls, rams and bucks. This common extender is combined with egg yolk in many commercial extenders including Triladyl which has been used for several species including bison. Egg yolk and milk have been traditionally used in extenders as a means of protecting sperm against initial cooling to 4°C. Protection for sperm during the cryopreservation procedures is provided by the LDL from egg yolk and the casein from milk [89, 90]. The LDL and milk casein are able to bind

specific proteins that are involved in sperm capacitation [90, 115]. Once the proteins are bound to the LDL or milk casein the cooling can no longer cause the destabilization of the plasma membrane, allowing the sperm to survive cryopreservation [90, 115].

Commercial extenders have many advantages over laboratory prepared extenders as commercial extenders are produced to ensure that all the procedures are done in a consistent manner for each batch and companies have quality control procedures to check function of the final product. This can eliminate some experimental errors (e.g. inappropriate pH or osmolality and miss-calculation of ingredients) that can happen when extenders are produced in a laboratory. The two commercial extenders that will be focused on in this thesis are Triladyl<sup>®</sup> and Andromed<sup>®</sup>; the products of Minitube (Ingersoll, Ontario, Canada). Triladyl<sup>®</sup> has been used for cryopreservation in a variety of species with good results including: ovine, bovine, bison, African buffalo, equine and canine [12, 109]. Egg yolk is added to Triladyl<sup>®</sup> before it is used; the yolk provides the LDL that is important for protecting sperm during initial cooling [115].

The use of animal products like egg yolk and milk proteins has recently caused controversy. It is felt that consistency cannot be accomplished as the exact composition of the egg or milk can change based on the individual source animal (chicken or cow) that produces the product used. On an international level, there is fear that the animal product in extenders may increase disease transmission [116, 117]. Additionally, there are worries that the cryopreserved cells may become contaminated with drugs and hormones from the animal that produce the yolk or milk [116, 117]. Egg yolk has been shown to have significant amounts of progesterone and pregnenolone [117, 118] and progesterone is known to promote the acrosome reaction in sperm. Thus progesterone might predispose sperm to damage prior to the cryopreservation procedures [117]. Finally, there is a chance that foreign animal proteins in extenders might cause an immune response in the female tract that could cause extra damage to sperm [116, 117].

These concerns have led to research in the use of plant based phospholipids as an alternative to egg yolk or milk proteins [119]. The first commercially available extender not containing any animal products was developed in the early 1990's, but results were not up to the AI standard of the time [117]. Andromed<sup>®</sup> came onto the market for AI use in the year 2000 and



since then different varieties of plant based extenders have been developed for use in a variety of animals including AndroMed<sup>®</sup>-E for equine semen and AndroMed<sup>®</sup> for bovine, porcine, ram, African buffalo, bison and buck semen [12, 117, 119-122].

Andromed<sup>®</sup> contains phospholipids are derived from soybeans [121]. The phospholipids are intended to provide protection for cells in the same way as LDL from egg yolk does. Andromed<sup>®</sup> does not contain large protein particles which can interfere with sperm evaluation using CASA or sperm protein studies involving flow cytometry [13]. For bison, there was no difference noted for fertilizing ability between bison epididymal semen frozen in Triladyl<sup>®</sup> or Andromed<sup>®</sup> [13]; however, no post thaw progressive motility was observed when Andromed<sup>®</sup> was used to cryopreserve electroejaculated bison semen [12].

## **2.6 Comparison of Abilities of Andromed<sup>®</sup> and Triladyl<sup>®</sup> to Protect Bison Sperm**

As previously stated, when electroejaculated bison semen was cryopreserved it lost more than 50 % total motility after thawing [12]. However, there was only a 20 % loss of motility when epididymal sperm was cryopreserved [13]. This indicates that there may be a factor found in seminal plasma causing a higher loss in bison sperm motility during cryopreservation. Post thaw total and progressive motilities were approximately 35 % and 15% respectively, when the egg-yolk-based extender Triladyl<sup>®</sup> was used to cryopreserve electroejaculated bison semen [12]. In contrast, the post thaw total motility was approximately 15 % and there was no progressive motility observed when Andromed<sup>®</sup> the plant based extender was used for cryopreservation [12]. No substantial loss of progressive motility was observed when epididymal sperm was frozen with Andromed<sup>®</sup> compared to when ejaculated bison sperm was cryopreserved with Andromed<sup>®</sup> [13]. This suggests that a seminal plasma factor affecting survival of bison sperm might be captured by Triladyl<sup>®</sup> but not Andromed<sup>®</sup>. Triladyl<sup>®</sup> contains egg yolk and LDLs from yolk bind to certain seminal plasma proteins [72]. The LDL fraction of Triladyl<sup>®</sup> might capture the interfering factor in seminal plasma preventing its effect on the sperm during cryopreservation

## II. General Objectives and Hypothesis

The goal of this study was to improve semen collection in order to enhance procedures for freezing bison semen for storage in gene banks. The first general hypothesis is that reducing stress in bison bulls will improve the quality of semen collected by electroejaculation. The second general hypothesis is that the low-density lipoprotein (LDL) fraction of egg yolk based extenders can bind the interfering factor in bison seminal plasma that affects its ability to cryopreserve.

### *Specific Objectives and Hypothesis*

Objective 1 (Chapter 3): To lower stress in bison and improve the methods of semen collection

Hypothesis 1 (Chapter 3): The use of a LAN (Pipartil<sup>®</sup>) in bison bulls will lower stress levels during handling and facilitate semen collection by electroejaculation.

Objective 2 (Chapter 4): To isolate proteins captured in the LDL fraction of extenders.

Hypothesis 2 (Chapter 4): The egg yolk derived LDL fraction of a cryopreservation extender Trilady<sup>®</sup> is better able to bind unique proteins from bison seminal plasma than the soy derived phospholipid of Andromed<sup>®</sup>

### Appendix

Objective 3: To identify the specific bison seminal plasma protein that interferes with bison semen cryopreservation.

Hypothesis 3: Specific BSP proteins affect sperm survival during cryopreservation.

### Chapter 3

#### **Effects of Piportil<sup>®</sup>, a long-acting sedative drug, on decreasing the stress of handling bison bulls for semen collection and on semen motility characteristics**

**Authors** G. Gratton\*, B. Toosi\*, R. McCorkell, M. Woodbury and C. Lessard

Author	Role
G. Gratton*	Data collection and evaluation of bison semen Equal participation in writing of paper
Dr. B. Toosi*	Interpretation of behavioural data and hormonal analysis Equal participation in writing of paper
Dr. R. McCorkell	Experimental design and hormonal analysis
Dr. M Woodbury	Experimental design
Dr. C. Lessard	Experimental design and write up on hormonal analysis

### 3.1. Abstract

Handling North American bison induces a high level of stress on the animal and can lead to injuries for both handlers and animals. Moreover, this induced stress can result in variations in the quality of semen collected by electroejaculation. The objective of this study was to investigate if a long acting neuroleptic (LAN) can lower stress in bison, allowing for safer handling of the animal and improvements in the quantity and quality of collected semen. In each trial, bulls were randomly divided in 2 groups: LAN-treated (N=3) and non-treated control (N=3). For the treatment groups 100 and 200 mg per bull doses of Piportil<sup>®</sup> were used. All bison were collected by electroejaculation. To evaluate stress heart and respiration rates were recorded before and after each collection. In addition, the time to capture bulls within the squeeze chute for semen collection was recorded. Blood was collected to quantify testosterone, corticosterone and cortisol. The volume of semen samples was recorded, concentration, total motility, progressive motility, curvilinear velocity (VCL), average path velocity (VAP) and straight line velocity (VSL) were analysed by CASA. Results indicated that 100 mg-treated group took less time to be captured and they struggled less once captured compared to the control group ( $P < 0.05$ ). The semen and hormone parameters investigated were not significantly different between treated and control bison for bulls treated with 100 mg ( $P > 0.05$ ). When bulls were treated with 200 mg, the time to capture was less than from control bulls ( $P < 0.05$ ). Corticosterone levels were lower and testosterone levels were higher than in control bulls (corticosterone:  $0.101 \pm 0.01$  vs.  $0.145 \pm 0.02$  ng/mL; testosterone:  $9.107 \pm 1.68$  vs.  $5.327 \pm 0.74$  ng/mL) respectively, ( $P < 0.05$ ). Sperm collected from the 200 mg-treated bulls were significantly more motile and had higher velocity parameters compared to control bulls ( $P < 0.05$ ). These results indicate that a 200 mg dose of Piportil<sup>®</sup> lowered the stress response in bison bulls allowing for safer handling of the animals and increased the quality of semen.

### 3.2. Introduction

The bison ranching industry has become important to the Canadian economy as indicated by a 34.9 % increase in the number of bison between 2001 and 2006 [4]. The Canadian Bison industry has historically resisted the use of reproductive technologies which would aid industry expansion and growth. Also, the application of reproductive technologies has been hindered by an inadequate knowledge of the biology and behaviours of this species, concerns of infectious diseases, and stress induced by handling of the animals [1, 4, 49, 55]. Bison are non-domestic wild animals being kept in close contact with humans. When wild animals are held in captivity stresses can become chronic and may affect the animals wellbeing over the long term [31]. In bison, signs of extreme stress include stamping feet, stampeding, heavy breathing, frothing in the mouth, pushing, attacking, sitting and jumping or attempting to climb out of pens [28]. These unpredictable actions can lead to bison harming themselves and the handlers [28]. Stress may also influence reproductive success within herd and may interfere with semen collection by electroejaculation [30]. Training bison to accept handling procedures prior to semen collection has proven to reduce some of the stress effects, but this process can be lengthy and is not always feasible on a large scale ranch situation [12, 27, 28].

Bison are seasonal breeders; their breeding season lasts from June to October [16]. Better sperm motility and morphology are observed during this period, but good semen may be produced all through the year [56]. Evaluation of bison semen during their breeding season shows potential for normal sperm morphology of > 70 % and total motility of > 65 % [56]. Puberty is reached by approximately  $16.5 \pm 2.5$  months of age. At this time, semen production reaches concentrations of  $50 \times 10^6$  million/mL, progressive motility 10% and normal sperm morphology 65 % [55]. Semen quality continues to increase as the bison age, but is of lower quality compared to bovine semen collected by electroejaculation, which should be > 70 % in both motility and normal morphology [56, 123]. Electroejaculation is currently the only method that can be safely used to collect bison semen when the animals are properly restrained [12, 55, 56].

Tranquilizers have been utilized to facilitate handling of wild animals [10, 44]. Forms of tranquilizers, specifically long acting neuroleptics (LANs), are employed for translocation of animals, especially in Africa where large mammals are often moved between game reserves [10, 40]. A commonly used LAN in wildlife is Piportil L4<sup>®</sup> (Pipothiazine Palmitate); designed as an antipsychotic drug with long-acting sedative effects for use in non-agitated chronic schizophrenic patients [41]. The duration of action for this drug can last from 3-6 weeks in humans [41]. However, repeated injections every 4 weeks are commonly administered [41]. Piportil's<sup>®</sup> long duration of action reduces the number of occasions the animal is handled. The advantage of using Piportil is that it has been successfully used in a study involving female bison; the animals did not demonstrate any adverse effects (Dr. McCorkel, personal communication).

In the present study, we evaluated the effect of Piportil<sup>®</sup> to alleviate stress caused by the handling of the bison that might lead to improved semen quality collected by electroejaculation.

### **3.3. Methods and Materials**

#### ***3.3.1. Animals***

All Animal experimentation was performed according to the guidelines of the Canadian Council on Animal Care and was approved by the University of Saskatchewan animal care committee. Sexually mature (> 2 years of age) bison bulls (n = 3, Wood bison and n = 3, Plains bison) with a mean body weight of  $517.0 \pm 17.0$  kg ( $X \pm SD$ ) were used in this study, between May and November 2010. The first treatment was given from May until July 2010 while the second treatment was done from August to November 2010. Bison bulls were kept at the Native Hoofstock Centre near Saskatoon, SK, Canada; 52° N latitude. Animals had access to pasture and hay with fresh water and iodized salt licks available ad libitum. All bison handling procedures were followed as previously described [12].

### ***3.3.2. Experiment design and treatment***

The current study included eight trials. For each trial, bulls were randomly selected to receive Piportil L4<sup>®</sup> (n = 3; Sanofi Aventis, Laval, QB, Canada) or to serve as untreated controls (n = 3). In the first four trials, 100 mg of Piportil<sup>®</sup> was given intramuscularly (im) while in the last four trials 200 mg of Piportil<sup>®</sup> was administered im. For each trial, treatment was done on Fridays and semen was collected on the Tuesdays and Thursdays over the following 2 weeks (four semen samples/trial/bison). All bulls were given a week rest before the next trial.

### ***3.3.3. Semen collection and evaluation***

Semen was collected using an electroejaculator (Pulsator IV, Lane Manufacturing, Denver, CO, USA). After collection, the total volume of the ejaculate was recorded. The semen sample was then kept in an incubator at 37°C until transferred to the laboratory for semen analysis (< 2 hrs). Each sample was analysed for concentration, total motility, progressive motility, curve linear velocity (VCL), average path velocity (VAP), and, straight line velocity (VSL) using computer assisted sperm analyses (CASA; Sperm Vision 3.0, Minitube Canada, Ingersoll, ON, Canada).

Semen smears stained with eosin-nigrosin were prepared after semen evaluation was completed for each bull [124]. This was done by placing a small drop of semen on a microscope slide near to a drop of the eosin-nigrosin stain. The semen and the stain were then mixed and a smear was created on the slide. The smear was air dried before analysis. Sperm from each bull was analysed for morphology and live/dead sperm using a light microscope under oil immersion (100x).

### ***3.3.4. Blood Sampling***

Blood samples (10 ml) were collected prior to treatment and semen collection, by jugular venipuncture, using vacuum tubes. Blood samples were allowed to clot for 1 to 2 h at room temperature, followed by centrifuged at 1500g for 15 min. Serum was harvested and stored at -20°C until assayed.

### ***3.3.5. Hormone assays***

All serum samples were analyzed for circulating concentrations of testosterone, cortisol and corticosterone by validated liquid chromatography-mass spectrometry. Bison serum samples were thawed in a warm water bath. Protein precipitation was performed using zinc sulfate containing internal standards. Fifty microlitres of zinc sulfate solution was added to 50 µL of serum, vortexed, stored at -20°C for 15 min, centrifuged at 14,000 rpm for 10 min, and 50 µL of supernatant was removed for analysis.

The serum samples were analyzed using an AB SCIEX QTRAP<sup>®</sup> 5500 mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source and an Agilent 1200 liquid chromatograph system. Forty microlitres of sample was loaded onto a Phenomenex Kinetex 2.6 µ C18, 100 x 3.00 mm column maintained at 40°C. Mobile Phase A consisted of water and Mobile Phase B consisted of methanol. The elution gradient was 10 % B for 0.50 min, 10-40 % B from 0.50-1.50 min, 40-70 % B from 1.50-5.50 min, 70-80 % B from 5.50-8.50 min, 80-95 % B from 8.50-8.70 min, 95-95 % B from 8.70-11.50 min, 95-10 % B from 11.50 to 11.70 min, and held at 10 % B from 11.70-15.00 min at a flow rate of 0.550 mL/min.

A scheduled MRM scan was performed in positive mode with a 30 sec detection window. Nitrogen was used as the source, nebulizer, and collision gases. Mass resolution in Q1 and Q3 was set to unit resolution. Two transitions were monitored for each analyte: a quantifier



transition (-1) and a qualifier transition (-2). Data processing and quantitation were performed using Analyst 1.5.1 software (AB SCIEX).

### ***3.2.6. Behavioural data collection***

The hydraulic handling system (Berlinic Manufacturing, Quill Lake, SK, Canada) used in this study is composed of a chute (waiting area) ending in a squeeze chute. At the beginning of each semen collection session, all bison bulls (n = 6) were herded into the chute waiting area. The entrance order of all bulls into the chute system was recorded. Once all animals moved into the chute waiting area, the chute entrance gate was closed and animals were allowed to move freely and rest within the waiting area for five minutes. The standing order of animals was recorded after five minutes with the bull closest to the squeeze chute ranked first and the bull farthest from squeeze chute ranked the sixth. After this first rest period, the first bull was moved into the squeeze. The squeeze chute entrance gate was closed immediately after a bull was located completely inside. The neck gate was then opened to allow the bull to move farther in order to capture and restrain the head and neck. The time interval between the closing the entrance gate to closing the neck gate was recorded. The bull was allowed to rest for three minutes and any struggling was recorded during this second rest period. The degree of animal struggling in the squeeze chute after restraining the head and neck was scored as zero (no struggling), one (struggling 1 to 3 times), two (struggling 4 to 6 times), three (struggling 7 to 8 times), four (struggling more than 8 times) and five (sitting or laying down in system). After the second rest period, heart and respiration rates were recorded before collecting the blood sample. A semen sample was collected next and immediately after semen collection heart and respiration rates were recorded again.

### ***3.3.7. Statistical methods***

Changes in animal behaviour, and serum concentrations of testosterone, cortisol and corticosterone were analyzed by two-way analysis of variance (SigmaStat, Statistical Software for Windows Version 3.5, 2005, SPSS Inc., Chicago, IL) for the main effects of treatment (Piportil-treated and untreated control) and the dose (100 mg vs. 200 mg). Since the control values of bison treated with 100 or 200 mg of Piportil were significantly different between trials; the main effect of the treatment dose was excluded from analysis. Comparisons were made between treatment and control groups within each dose (100 or 200 mg) category by Student's t-test (SigmaStat, Statistical Software for Windows Version 3.5, 2005, SPSS Inc., Chicago, IL). Two way ANOVA (SigmaStat) was used to analyze the changes in heart rate and respiration rate with main effects of time relative to semen collection (before and after collection) and treatment (Piportil-treated and untreated control), Multiple comparisons were made by the method of Fisher's least significant difference [125]. All values are means  $\pm$  SEM and statistical significance was set as  $P < 0.05$ . Semen parameters were evaluated using SAS 9.1 (SAS Institute, Cary, NC, USA). The inability to compare the treatment groups together resulted in a multiple variable ANOVA with repeated measures being used.

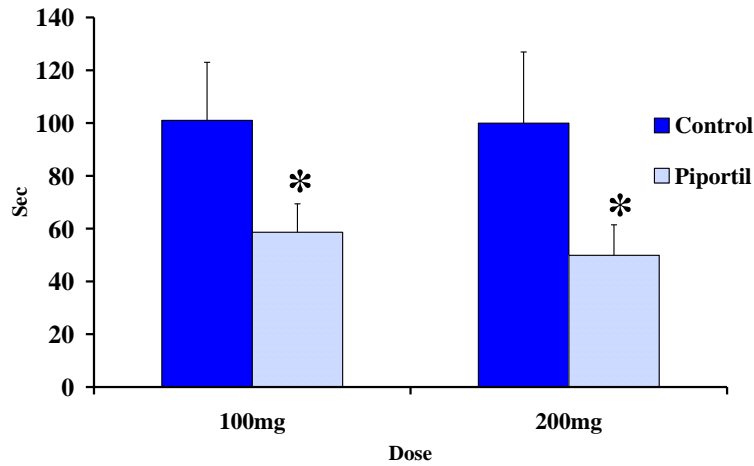
### **3.4. Results**

For this study, the control groups done at the same time as the treatments were analysed and found to be significantly different from one another in all treatment parameters ( $P < 0.05$ ); resulting in the inability to test the treatment groups against one another. This could be a result of the two treatments happening before and during the breeding season.

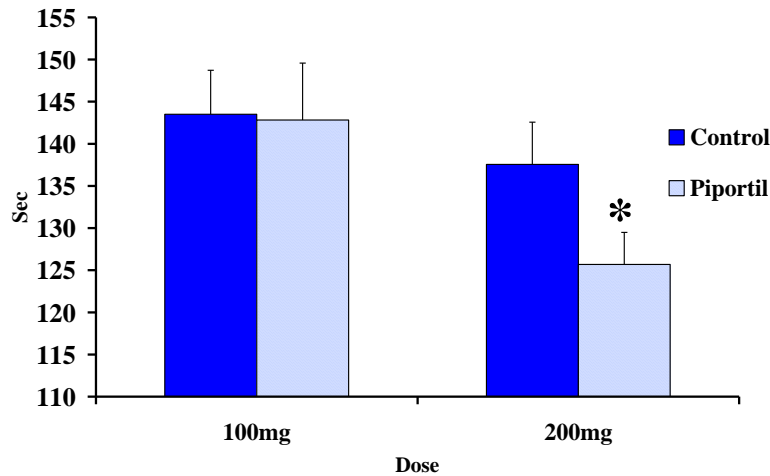
#### **3.4.1. Behaviour responses to handling procedures**

The capturing time of a bison, from back gate closed to neck captured in the squeeze chute, was found to be significantly less for both 100 and 200 mg treatment groups compared to their respective controls (100 mg;  $58 \pm 10$  vs.  $101 \pm 21$  sec; 200 mg;  $49 \pm 11$  vs.  $99 \pm 26$  sec) ( $P < 0.05$ ; Figure 1A respectively). No significant difference was detected for total time of semen collection for control and 100 mg treatment group ( $P < 0.05$ ). However, semen collection time was significantly less for 200 mg treated group compared to control bison ( $125 \pm 3$  vs.  $137 \pm 5$  sec) ( $P < 0.05$ ; Figure 1B). The mean struggle score within the squeeze chute (range of 0, no struggling to 4, struggling more than eight times) was lower when bison bulls were treated with 100 mg of Piportil<sup>®</sup> compared to their control group ( $1.5 \pm 0.1$  vs.  $2.3 \pm 0.3$ ) ( $P < 0.05$ ; Figure 1C). Finally, no significant differences were observed for respiration and heart rates recorded before and after each collection period in all groups (control and treated) (data not shown).

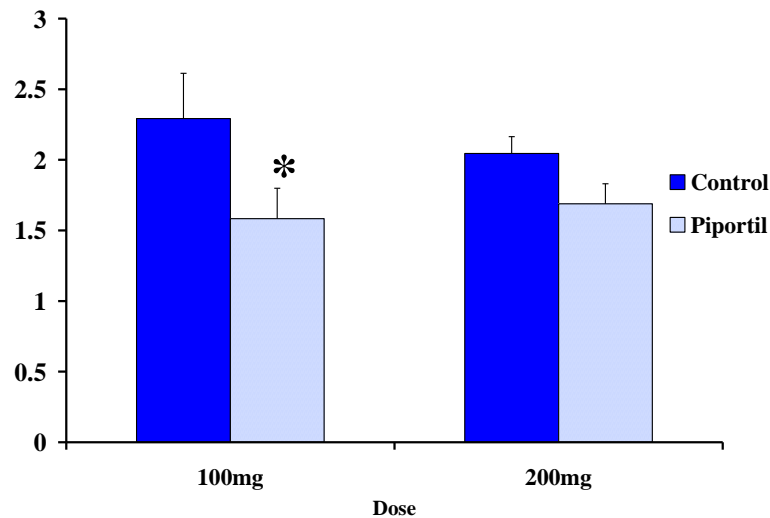
A



B



C

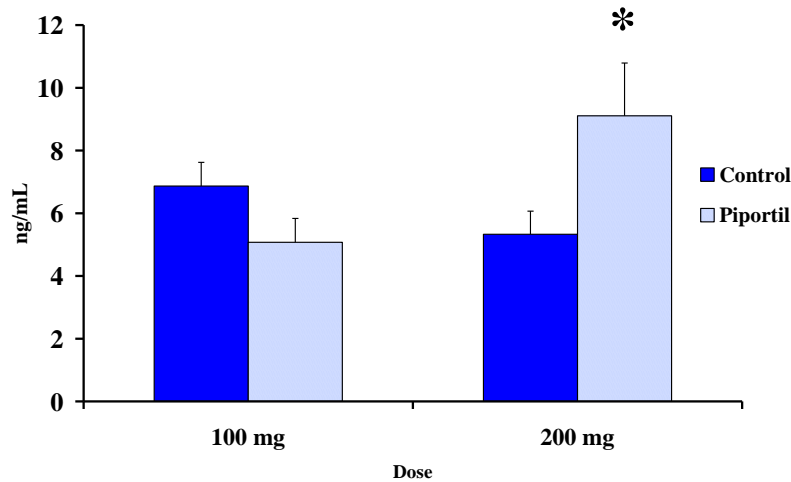


**Figure 3.1:** Behavioural responses of bison bulls treated with or without Piportil® (100 and 200 mg dose) in relation to: A) mean time to restrain in squeeze chute, B) mean duration of semen collection and C) mean struggling score once restrained (Significant differences in relation to control  $P < 0.05$  are represented with an asterisk \*)

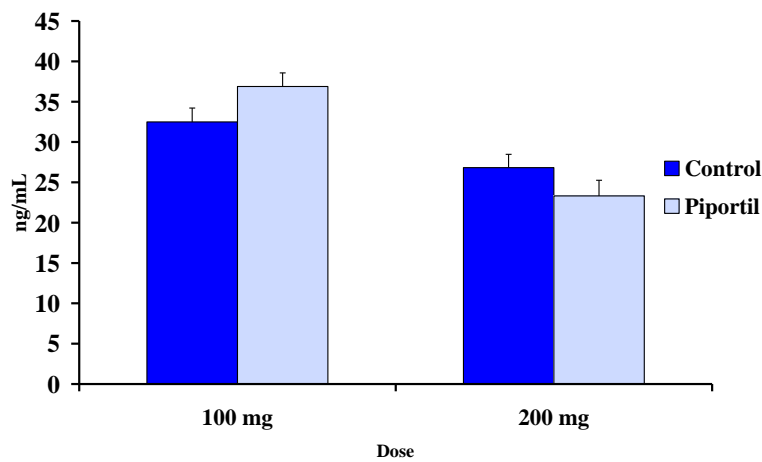
### **3.4.2. Endocrine stress indicators to the handling procedures**

The lower dose of Piportil<sup>®</sup> administered to bison bulls showed no significant differences for any of the stress hormones tested when compared to control (Figure 2ABC;  $P > 0.05$ ). When the dose of Piportil<sup>®</sup> was increased to 200 mg, the levels of testosterone were increased compared to controls (Figure 2A;  $P < 0.05$ ), but in contrast, the corticosterone levels decreased compared to controls (Figure 2C;  $P < 0.05$ ). Interestingly, the levels of cortisol were not significantly different between treatment and control groups (Figure 2B;  $P > 0.05$ ).

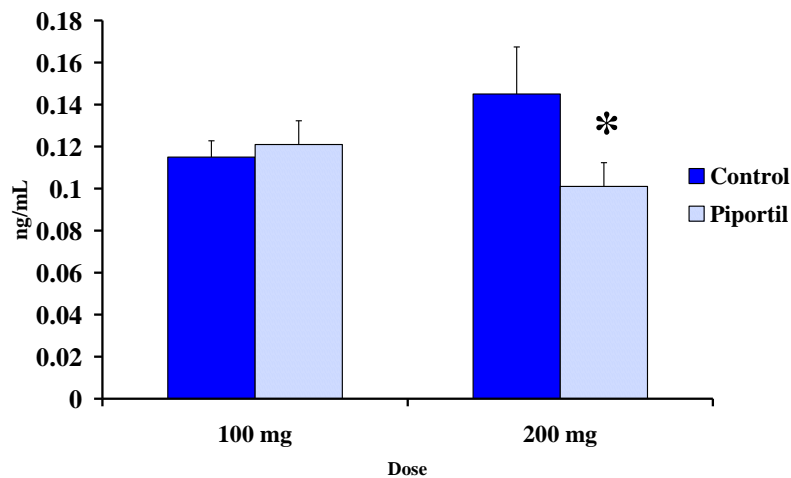
A



B



C

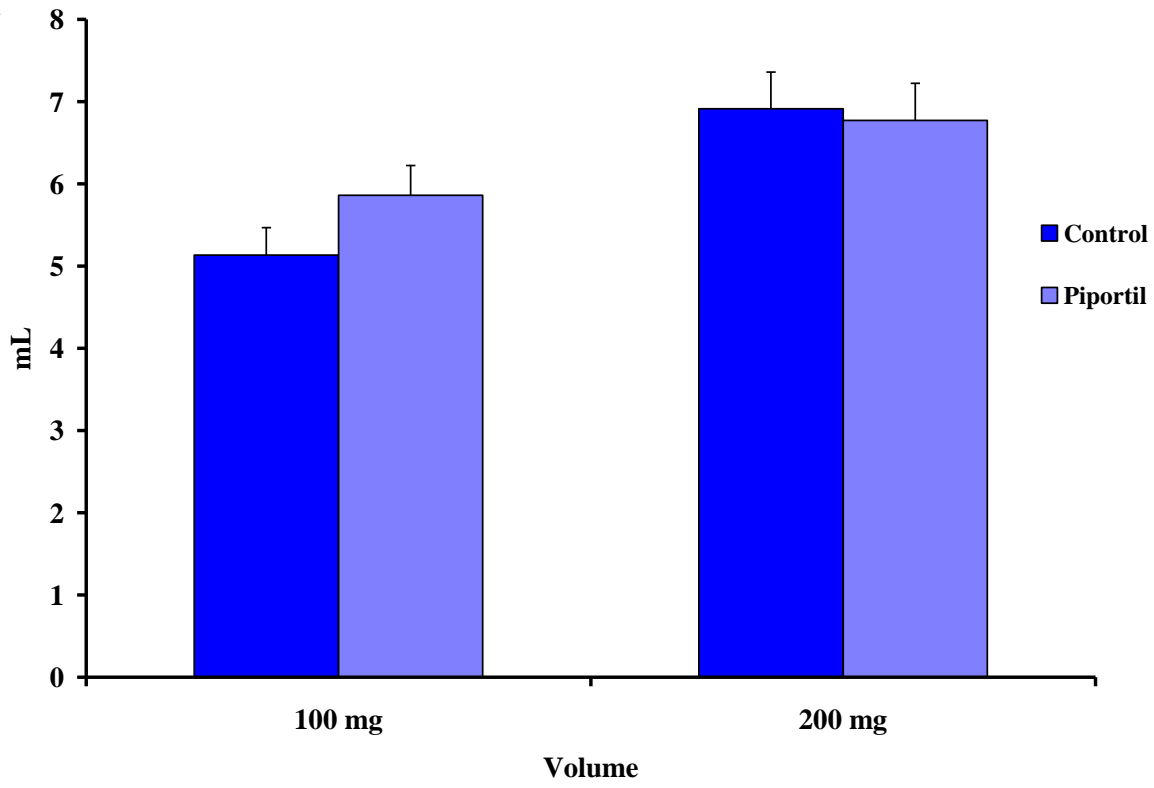


**Figure 3.2:** Endocrine response (A) testosterone, (B) cortisol, and (C) corticosterone serum concentrations of bison bulls treated with or without Piportil<sup>®</sup> (100 and 200 mg dose) to handling procedures A) testosterone, B) cortisol and C) corticosterone (Significant differences in relation to control  $P < 0.05$  are represented with an asterisk\*)

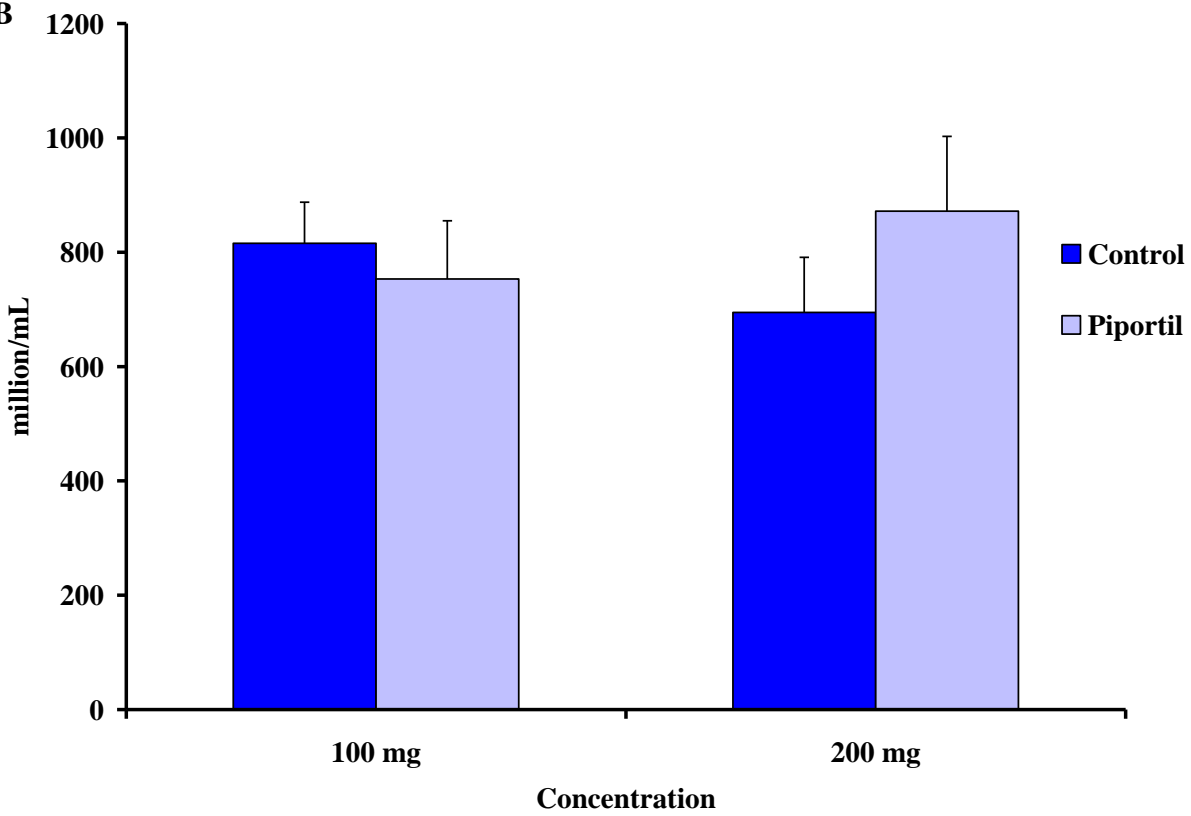
### 3.4.3. Semen quality in relation treatment with Piportil<sup>®</sup>

The six bulls used for this study had a mean for morphologically normal sperm of  $55 \pm 6.7$  % over 19 ejaculates. The most common morphological aberration of sperm was distal cytoplasmic droplets (data not shown). No significant differences were observed in any of the sperm motility parameters between treatment and control groups when 100 mg/bull of Piportil<sup>®</sup> was administered (Figure 3ABCD,  $P < 0.05$ ). When the Piportil<sup>®</sup> dose was increased to 200 mg/bull, a significance difference was noted between the treatment and control groups for all motility parameters ( $P < 0.05$ ). However, no significant differences were observed for both volume and concentration values between treated and control groups (Figure 3AB,  $P > 0.05$ ). Total motility and progressive motility were, respectively,  $73.1 \pm 10.6$  % and  $70.0 \pm 11.5$  % for treated bulls vs.  $63.6 \pm 17.2$  % and  $59.8 \pm 17.5$  % for control bulls (Figure 3C,  $P < 0.05$ ). The velocity parameters were significantly increased for treated bulls vs. control bulls (VCL  $147.5 \pm 27.5$  vs.  $138.1 \pm 20.9$   $\mu\text{m}/\text{sec}$ ), (VAP  $87.5 \pm 15.6$  vs.  $80.9 \pm 11.1$   $\mu\text{m}/\text{sec}$ ) and (VSL  $73.3 \pm 13.1$  vs.  $67.0 \pm 8.4$   $\mu\text{m}/\text{sec}$ ) respectively (Figure 3D,  $P < 0.05$ ).

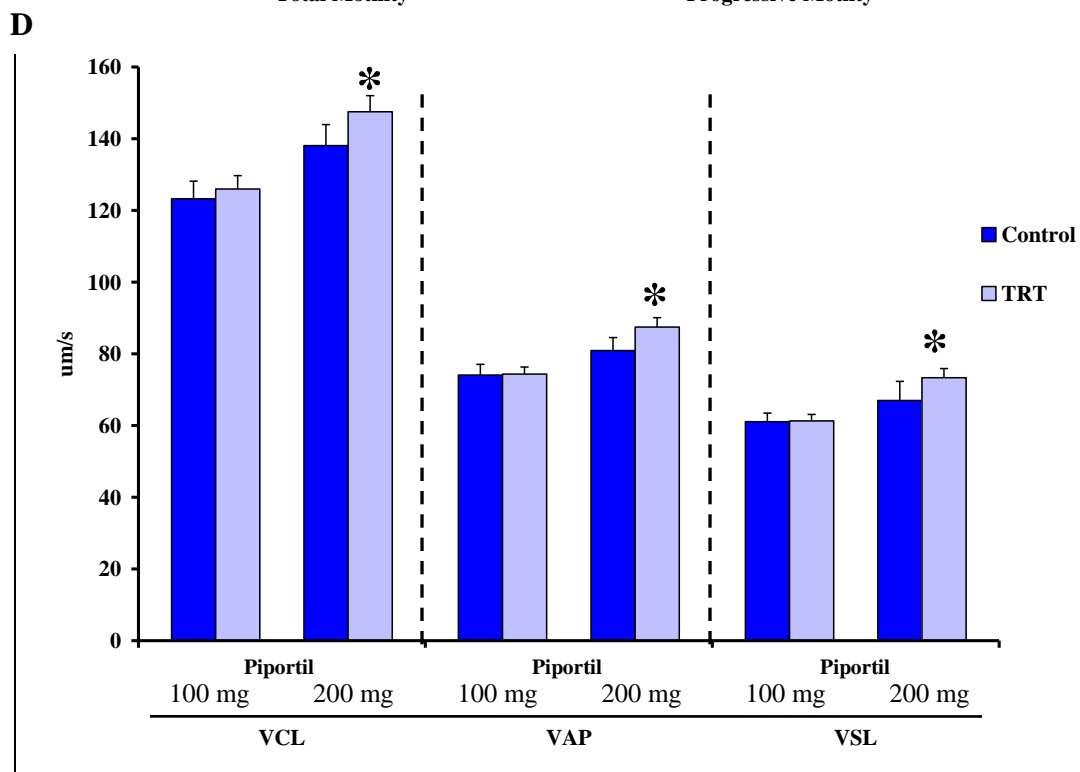
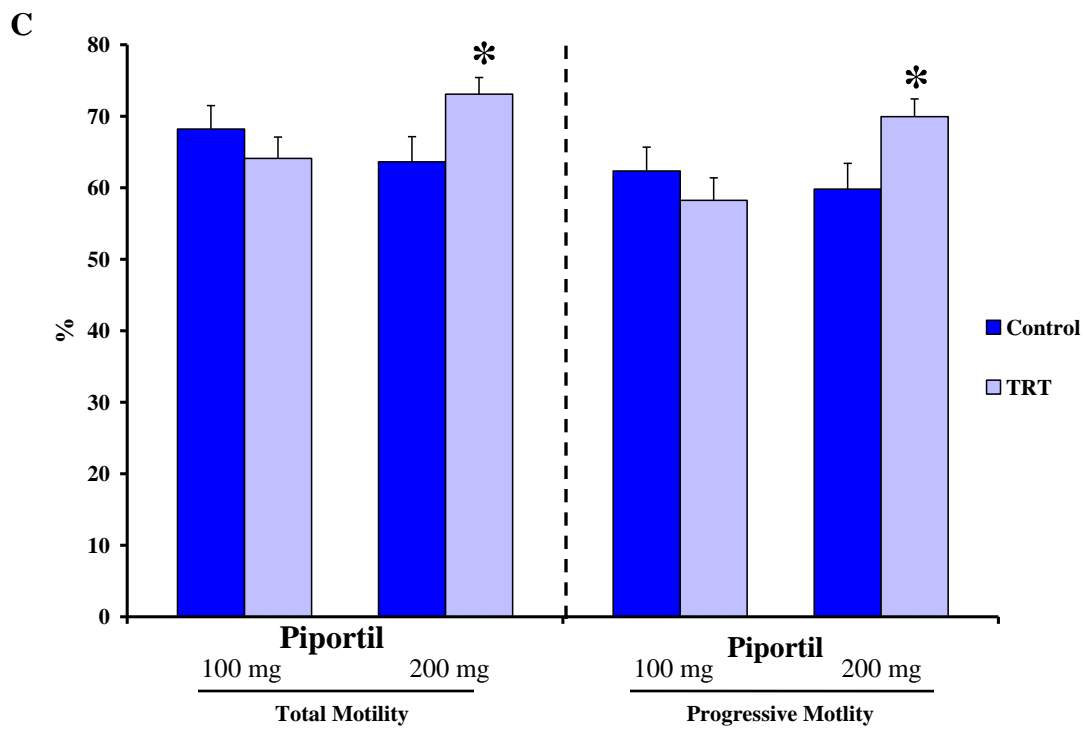
**A**



**B**







**Figure 3.3:** Evaluation of electroejaculated semen parameters for bison bulls treated with or without Piportil® (100 and 200 mg bull). Treated group (TRT). (A) Volume/ejaculate, (B) concentration of sperm/ejaculate, (C) total and progressive motility of sperm and (D) VCL, VAP and VSL. Significant differences in relation to control,  $P < 0.05$ , represented with an asterisk \*

### 3.4 Discussion

Piportil<sup>®</sup> was used to reduce stress levels in bison to allow for an improvement in handling of the animals and an increase of the quality in semen. Interestingly, both injections of 100 and 200 mg of Piportil<sup>®</sup> per bull had measurable effects on behaviour during the handling procedures (Figure 3.1). Piportil<sup>®</sup>-treated animals took less time to be captured within the squeeze chute compared to their respective controls, and the bison injected with 100 mg of Piportil<sup>®</sup> struggled less compared to controls (Figure 3.1AC). The decreased time to be captured within the squeeze chute suggests that the bison were less fearful of the system and the decrease in struggling once squeezed in the system indicates that the bison were less reactive to the stressful situation. However, when the dose was increased to 200 mg no differences in struggling were observed between treated and control bulls. Over the study period, the bison may have become habituated to the system which could account for the lack of differences in the amount of struggling for bison treated with the larger dose of Piportil<sup>®</sup>. Habituation appeared to lower stress; however, it may not be practical to train animals prior to handling and the use of a LAN could be beneficial [12, 28].

Handling stresses have been lowered with the use of LANs in a variety of wild animals including impalas, ibexes and red deer [40, 44]. Furthermore, a LAN used on long term captive impalas appeared to decrease their flight zone allowing handlers to come into close contact with them [40]. After a stressful event the heart rates of ibexes and farmed red deer were observed to return to equilibrium quicker for animals injected with a LAN; however, this result was not observed with treated bison [48, 126]. Electroejaculation, due to physical exertion raised the heart rate for all the bison, so in this regard Piportil<sup>®</sup> was not efficacious (Figure not shown). It could be speculated that the sedative effects of Piportil<sup>®</sup> would be more significant in wild-captured bison when observing handling and other procedures such as semen collection.

Increased blood glucocorticoids have been associated with stress levels in mammals [32]. Bison bulls that are housed with females during the breeding season showed increase cortisol and testosterone serum concentrations [33, 35]. Additional interactions with other males to establish dominance and keep females for breeding have been correlated to the increased of cortisol levels

[33]. Increase of testosterone serum concentrations was associated with bulls producing higher quality semen during the breeding season thus increasing their chances of successfully breeding females [35, 56]. Interestingly, bison bulls injected with 200 mg of Piportil<sup>®</sup> had reduced serum concentrations of corticosterone and increased testosterone compared to the control groups, but there was no difference observed in cortisol serum concentrations (Figure 3.2). A rise in corticosterone serum concentrations has been associated with increased stress in bovine [127]. Our results indicate that the bison were less stressed during handling procedures indicated by lower corticosterone serum concentrations. The bison bulls in this study were housed separately from females so they did not have the group stresses incurred while fighting for the right to breed with the females during the breeding season. It appears that rises in corticosterone observed in the bulls were due to handling stresses and not aggressive bull interactions.

Increased testosterone could be attributed to the fact that the study took place during the rut which is associated with higher testosterone serum concentrations in bison bulls [33, 35]. The bulls involved in our study were able to see and smell females in adjacent pens. This may have increased biological signals in addition to photoperiodic and nutritional signals which would increase testosterone production in anticipation of breeding. However, since blood was only taken once at the time of collection, the testosterone serum concentrations could not be an accurate evaluation due to the fluctuating levels of testosterone throughout the day.

Another objective of this study was to determine if treatment with a LAN would improve the quality of semen collected by electroejaculation from bison bulls. Such improvements in the quality of semen collected would have a direct application on reproductive technologies including cryopreservation, AI and IVF in the bison industry. Higher quality sperm that has been cryopreserved can be exported and used in AI to improve genetic gain in bison herds. In our study, the quality of semen collected from bulls treated with 200 mg of Piportil<sup>®</sup> was superior to that of control bulls for every semen parameter tested (Figure 3.3CD). The effect of Piportil<sup>®</sup> may have been to lower blood catecholamines and glucocorticoids, which have been associated with impaired spermatogenesis [31].

Increased cortisol serum concentrations have a negative feedback for production of luteinizing hormone by the pituitary gland, thus reducing testosterone synthesis by the Leydig cells [128, 129]. In addition, cortisol may affect androgen production by competing for enzymes that are needed for androgen synthesis or it may affect the production of steroid synthesising enzymes via the glucocorticoid receptor, hindering the first wave of spermatogenesis [128, 129]. Our results indicate that cortisol serum concentrations were not influenced by the 200 mg dose of Piportil<sup>®</sup>, but testosterone serum concentrations increased (Figure 3.2AB). This suggests that spermatogenesis was affected by higher levels of testosterone [35].

Another explanation for the better semen quality could be related to the amount of accessory sex gland secretions during ejaculation. The process of electroejaculation is an invasive method of semen collection which involves inducing muscle contractions using electric current; it is believed to be painful for the animal [130]. Electrical stimulation might cause an increase in the amount of accessory sex gland fluids (including from the prostate and seminal vesicles) resulting in lower concentrations of sperm in collected samples [131, 132]. Too much prostate fluid has been shown to have a detrimental effect on semen quality in canine [131]. Moreover, secretions from bovine vesicular glands cause hyperactivation and acrosome reactions in epididymal sperm and in higher doses may increase these actions in ejaculated sperm [133]. On the other hand insufficient vesicular gland reaction may hinder the development of sperm motility. Interestingly, bulls treated with 200 mg of Piportil<sup>®</sup> had a shorter collection time than the control group (Figure 3.1B); resulting in decreased electroejaculation stimulation time. Unfortunately, the proportion of accessory gland fluid to spermatozoa from the bison ejaculations could not be determined. Furthermore, the volume of ejaculate between treated and control bison in the study were not seen to be different (Figure 3.3A) and the concentrations were too variable between animals to find statistical differences (Figure 3.3B). Moreover, bison that are more highly stressed by the electroejaculation procedure may release urine into their urethra during ejaculation, but no testing of urine content was conducted during the study. In sufficiently high concentrations, urine reduces motility and viability of spermatozoa [134]. One or more factors could be contributing to the increased quality of semen observed with the use of Piportil<sup>®</sup>, but it is difficult to determine the contribution of various factors.

This study showed that a LAN can provide advantages when handling bison for semen collection. Treated bison were easier to handle in the chute system, their endocrine stress indicators were lower and the overall semen quality was improved when collected by electroejaculation. The use of Piportil<sup>®</sup> would aid in collecting higher quality semen from genetically superior bulls that could then be used in for a variety of reproductive technologies advantageous to the industry. However, the drug Piportil<sup>®</sup> does have a few disadvantages the foremost being the cost of the drug. Piportil<sup>®</sup> may not be economically feasible to use in large scale ranches. Additionally, the withdrawal period of Piportil<sup>®</sup> in bison is unknown and if the drug persists in the bison's body it could affect the ability to use the animals for human consumption. Nonetheless, the beneficial effects of Piportil<sup>®</sup> to increase the ease of bison handling and allow for better quality semen to be collected would be advantageous to ranchers wanting to expand their herds and their revenue.

### **3.5. Acknowledgments**

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## **CHAPTER 4: IDENTIFICATION OF SEMINAL PLASMA PROTEINS CAPTURED BY LOW DENSITY LIPOPROTEIN (LDL) IN TRILADYL<sup>®</sup> EXTENDER**

G. Gratton, MF. Lusignan, P. Manjunath, M. Anzar and C. Lessard

### **4.1. Abstract**

Bison semen has been difficult to cryopreserve and there is evidence that seminal plasma proteins may adversely affect sperm survival during cryopreservation. The goal of this study was to identify unique proteins found in bison seminal plasma captured by low density lipoprotein (LDL) fraction of Triladyl<sup>®</sup> extender. The LDL fraction found in an egg yolk based extender Triladyl<sup>®</sup> provided protection for sperm during the cryopreservation procedures; however, this protective effect was not evident when a plant based extender was used Andromed<sup>®</sup>. The protective effects of Triladyl<sup>®</sup> extender might be due to LDL capturing deleterious seminal plasma factors. Semen was collected by electroejaculation from captive bison bulls during their breeding season and the spermatozoa were removed from the seminal plasma. The seminal plasma was then mixed with the commercial extenders Triladyl<sup>®</sup> and Andromed<sup>®</sup>. The lipoprotein fraction of the Triladyl<sup>®</sup> + seminal plasma or the phospholipid fraction of Andromed<sup>®</sup> + seminal plasma was isolated using ultracentrifugation. Protein profiles were compared from these fractions to identify which seminal plasma proteins were being captured by the Triladyl<sup>®</sup> LDL and Andromed<sup>®</sup> phospholipids. A total of 116 protein spots were captured in Triladyl<sup>®</sup> and a total of 21 protein spots in Andromed<sup>®</sup>; 47 proteins were unique to Triladyl<sup>®</sup>. Using MALDI-TOF, twelve proteins of interest were identified from the Triladyl<sup>®</sup> LDL + seminal plasma along with five proteins from Andromed<sup>®</sup> phospholipid + seminal plasma. These proteins include but are not limited to binder sperm protein and platelet activating factor proteins, both of which are known to influence lipid efflux from the sperm plasma membrane, along with spermadhesin, which is involved in sperm egg interactions. One or more of these proteins may be the factors that suppress the survival of bison sperm during cryopreservation.

## 4.2. Introduction

Cryopreservation is an important technique that can aid in the protection of valuable bison genetics. However, bison semen that has been collected by electroejaculation does not survive cryopreservation procedures to the same high standards as bovine sperm [12, 49]. Bovine can have post thaw total motility as high as 70 % [106]; bison have post thaw total motility of less than 50 % [12, 49, 106]. Interestingly, after thawing bison semen shows different total and progressive motility depending on which extender was used for cryopreservation, e.g. Triladyl<sup>®</sup>, 35 % and 14 % respectively vs. Andromed<sup>®</sup>, 9 % and 0 % respectively) [12]. However, cryopreserved epididymal sperm with Triladyl<sup>®</sup> or Andromed<sup>®</sup> had post a thaw total motility of 54 % and 45 % respectively [13]. This significant difference in total motility between epididymal and electroejaculated sperm may be attributed to the absence of accessory gland secretions for epididymal sperm, suggesting that there may be a factor in bison seminal plasma that is suppressing sperm survival during the cryopreservation. This factor appears to be captured by the Triladyl<sup>®</sup> extender, but not with Andromed<sup>®</sup> extender [12]. The low density lipoproteins (LDLs) found within egg yolk appears to be able capture certain seminal plasma proteins [135].

Triladyl<sup>®</sup> is a commercial Tris based extender that has egg yolk added at the time of preparation. A fraction of egg yolk known as LDLs have been demonstrated to increase post thaw sperm survival in many species including bovine [72]. The LDLs are able to bind certain seminal plasma proteins minimizing the action on the sperm after ejaculation [89]. If these proteins were not captured by the LDL, they would induce the capacitation process by binding to the sperm membrane creating a cholesterol efflux and remodelling the sperm plasma membrane making it more susceptible to cryodamage [136]. The frozen semen industry is moving away from animal based extenders (i.e. egg yolk or milk) because of fear of transmission of pathogens [116]. Extenders like Andromed<sup>®</sup> are now being developed that contain plant instead of animal proteins. Andromed<sup>®</sup> is made with soybean lecithin; it has been proposed that the phospholipids from the plants are able to provide similar protection as yolk derived LDLs for sperm during cryopreservation [119]. The exact mechanism of protection of Andromed<sup>®</sup> on sperm during cryopreservation is not completely understood. Nonetheless, Andromed<sup>®</sup> has been used successfully to cryopreserve bovine sperm providing similar post thaw results to Triladyl<sup>®</sup>

extenders [117]. However, it has been observed that Andromed<sup>®</sup> is unable to provide the same protection during cryopreservation procedures that Triladyl<sup>®</sup> does for bison semen [12]. This suggests that Triladyl<sup>®</sup> LDLs are capturing deleterious factors in the seminal plasma that are not captured by Andromed<sup>®</sup>.

The objective of this study was to identify bison seminal proteins that are being captured in the LDL fraction of Triladyl<sup>®</sup>. Several unique proteins have been identified in the LDL fraction of Triladyl<sup>®</sup> + bison seminal plasma.

### **4.3. Methods and Materials**

This study was approved by the animal care committee at the University of Saskatchewan. All chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated.

#### ***4.3.1 Animals***

Bison bulls were housed at native hoof stock center, near Saskatoon, SK, Canada (52°02'N, 106°28'W). Bison were maintained on an alfalfa/brome pasture with free access to fresh water and hay. Six bison bulls were used from June to November (2009 and 2010). Semen was collected using standard bovine electroejaculation methods with a Pulsator IV electroejaculator (Lane Manufacturing Denver, CO, USA). Collected semen was immediately placed in an incubator at 37°C and carried to the laboratory within 2 hours. Spermatozoa were removed from seminal plasma through a series of centrifugations, 2 times at 300 g for 10 min and 2 times at 500 g for 10 min. After each centrifugation the supernatant was removed from the pellet so it could be centrifuged again and the pellet was disposed of. The seminal plasma was then observed with a light microscope to insure that all spermatozoa were removed. Seminal plasma was stored at -20°C for further use.

#### ***4.3.2. Extenders***



#### **4.3.2.1. Triladyl<sup>®</sup>**

Large white chicken eggs were obtained (Harman poultry farm, Prince Alberta, SK, Canada). The shell was broken the albumen was disposed of and the yolk was rolled on a 1 # Whatman filter paper to remove any excess albumen and chalaza. The yolk was then pierced with a scissor and carefully poured into a clean beaker making sure to keep out the vitelline membrane from the yolk. The yolk was then centrifuged for 20 min at 10,000 x g to remove any excess debris. The Triladyl<sup>®</sup> extender was then made according to instructions (Minitube, Ingersoll, ON, Canada). The extender was divided into aliquot of 15 mL and kept at -20°C for future use.

#### **4.3.2.2. Andromed<sup>®</sup>**

This extender was prepared according to instruction (Minitube, Canada). Once prepared, the extender was divided into 15 mL aliquots and kept at -20°C for future use.

#### **4.3.3. Isolation of LDL and Phospholipid Fractions by Centrifugation**

The treatment groups: Triladyl<sup>®</sup>, Triladyl<sup>®</sup> + bison seminal plasma, Andromed<sup>®</sup>, and Andromed<sup>®</sup> + bison seminal plasma were used to obtain LDL and lipid fractions. Bison seminal plasma, Andromed<sup>®</sup> and Triladyl<sup>®</sup> were warmed to 37°C. Andromed<sup>®</sup> or Triladyl<sup>®</sup> were added into the seminal plasma with a 1:3 ratio of seminal plasma to extender. The mixtures were left at 37°C for 30 min to allow the equilibration of mixtures. The mixtures were then placed at 4°C and allowed cool for 2 hours to mimic the first steps of cryopreservation procedures.

The procedure used for the LDL extraction was the same as previously described by Manjunath et al. [135] . Briefly, each treatment group was diluted 10 times with 10mM Tris HCL (pH 7.4). Solid potassium bromide was used to allow for the creation of a gradient. Each solution was placed in a 5/8 x 3 inch Beckman polyallomer Quick-seal centrifuge tube (Beckman Coulter, Mississauga, ON, Canada). Tubes were sealed before being placed into a vertical rotor Sorvall 65 V13 and 13 mL UC tube holders (Thermo Fisher Scientific, Ottawa, ON, Canada).

The rotor was then placed into Sorvall WX Ultra series ultracentrifuge (Thermo Electron Corporation, Thermo Fisher Scientific, Canada). The centrifuge was run at 60,000 rpm for 20 hours at 20°C. After completion of the run the tubes were removed and the liquid LDL fraction which had moved to the top of the tubes was collected. The solid LDL (Triladyl<sup>®</sup>) and phospholipid (Andromed<sup>®</sup>) fraction was found vertically along the sides of the tubes and was also collected. The fractions were then dialysed against 10mM Tris HCL (pH 7.4) to remove any excess potassium bromide. The protein concentrations from all samples were determined using the Bradford method [137].

#### ***4.3.4. IEF Focusing***

For each group a two dimensional electrophoresis protein profile was created. The first dimension was run using a PROTEAN IEF cell (Bio-Rad, Canada), no more than 100 µg of proteins were mixed with rehydration buffer (Bio-Rad, Canada) and placed in a focusing tray. On top a 7 cm IPG ReadyStrip broad range pH 3-10 (Bio-Rad, Canada) was laid gel side down, and then mineral oil was overlaid to prevent evaporation. Rehydration was passively done in the PROTEAN IEF cell over a 12 hour period. Once complete the voltage was increased to 250 V for 15 min to allow for desalting. Next, the voltage was linearly increased to 4000 V over 2 hours with a limit of 50 µA/gel. Once the run was complete strips were held at 500 V until removed from the PROTEIN IEF cell. Immediately after removal the strips were washed in Equilibration buffer I (Bio-Rad, Canada) for 10 min followed by Equilibration buffer II (Bio-Rad, Canada) for another 10 min, then a final wash in electrophoresis buffer (25 mM Tris / 192 mM Glycine / 0.1% SDS, pH 8.3) to prepare for second dimension.

#### ***4.3.5. SDS-PAGE***

The second dimension was run on hand cast mini format polyacrylamide gels (8.3 x 7.3 x 0.1cm), with 10 % resolving and 5 % stacking gels. The IEF strip was sealed to the gel with overlay agrose gel (0.5 % in 1 x TGS with BPB). Gels were run in a Mini-PROTEIN Tetra cell (Bio-Rad, Canada) at room temperature in a (25 mM Tris / 192 mM Glycine / 0.1 % SDS, pH

8.3) electrophoresis buffer (Bio-Rad, Canada). Gels were run at a constant voltage of 75 V for 15 min followed by 100 V for 1 hour or until proteins were near the bottom of the gel.

#### ***4.3.6. Staining of Gels***

Gels were stained using silver stain protocol (Bio-Rad, Canada). Gels were fixed in (Methanol 50 % v/v, Acetic Acid 10 % v/v, Fixative Enhancer Concentrate 10 % v/v and distilled water 30 % v/v) followed by rinsing in distilled water. Development was done using development solution (deionized water 35 % v/v, Silver Complex Solution 5 % v/v, Reaction Moderator Solution 5 % v/v, Image Development Reagent 5 % v/v and Development Accelerator Solution 50 % v/v). Development reaction was stopped by placing gel into a 5 % acetic acid solution.

A one dimensional SDS-PAGE gel was stained with Sypro<sup>®</sup> Red protein gel (Molecular Probes inc., Sigma-Aldrich, Eugene, OR, USA) staining procedures. Gel was placed into dark container with stain and agitated for 40-60 min. Once removed from stain the gel was rinsed for 1 min in a 7.5 % acetic acid solution, before being rinsed in distilled water. Gel was analysed using the Typhoon Trio fluorescence scanner (GE Healthcare, Canada) with the red fluorescence in a 633 nm range.

Gels that were used for spot picking were stained with coomassie brilliant blue staining procedures. Briefly, gels were rinsed once in distilled water and stained with coomassie staining solution (0.5 g coomassie brilliant blue, methanol 50 % v/v, deionized water 40 % v/v and acetic acid 10 % v/v.); gels were left to agitate for 15 min. Gels were destained (methanol 10 % v/v, acetic acid 50 % v/v and deionized water 50 % v/v) for approximately 15 min before being rinsed in 2-3 times in distilled water. This stain is compatible with the MALDI-TOF spectrometry used to identify the protein spots.

#### ***4.3.7. Analysis of Two Dimensional Electrophoresis (2DE) Gels***

Gels were imaged using the GS-8000 scanner (Bio-Rad, Canada) and loaded into PDQuest™ Basic software (Bio-Rad, Canada). Master gels were created for each treatment group using 3 replicate gels to insure all protein spots were accounted for. Treatment groups for Triladyl® LDL + bison seminal plasma and Andromed® phospholipid + bison seminal plasma were compared and specific spots were hand chosen for analysis. Spots were removed by hand from the gel and sent for analysis using MALDI-TOF spectrometry (done at the CNRC) and results were run though the MASCOT database to identify proteins

(<http://www.matrixscience.com/home.html> retrieved December 12, 2011).

#### ***4.3.8. MALDI-TOF Spectrometry***

##### ***4.3.8.1. In-gel digestion procedures***

Coomassie-stained protein gel spots were cut into 1 mm<sup>3</sup> size pieces and in-gel digestion was processed on the MassPrep II Proteomics Workstation (Micromass, UK) as described by Sheran et al. [138]. Briefly, protein spots were destained twice with 100 µl 1:1 ammonium bicarbonate:acetonitrile, 10 min incubation each. Protein reduction was performed with the addition of 10 mM DTT prepared in 0.1 M ammonium bicarbonate, for 30 min at 37°C, and followed by alkylation with the addition IAA prepared in 0.1 M ammonium bicarbonate at the final concentration of 27.5 mM. Reaction was allowed for 20 min at the same temperature. Gels were washed and dehydrated before saturated with 25 µl of trypsin prepared in 50 mM ammonium bicarbonate, and digestion was performed at 37°C for 5 hours. Peptides were extracted with 30 uL 0.1 % trifluoroacetic acid/ 3 % acetonitrile for 30 min, and then twice with 24 uL, 0.1 % trifluoroacetic acid/ 50 % acetonitrile for 30 min. The combined extracts were dried in vacuum. Samples were reconstituted in 40 µl of 0.1 % trifluoroacetic acid/ 3 % acetonitrile for LC-ESI MS analysis.

##### ***4.3.8.2. LC-ESI MS Spectra collection and Data analysis***

All analyses were performed on a Quadrupole Time-Of-Flight (Q-TOF) Ultima Global hybrid tandem mass spectrometer (Micromass, Manchester, UK) equipped with a

nanoACQUITY UPLC solvent delivery system (Waters, Milford, MA, USA). The mobile phase was composed from a binary solvent system of **A**, 0.2 % aqueous formic acid and 3 % acetonitrile and **B**, 95 % acetonitrile with 0.2 % formic acid. Peptides were desalted with an in-line solid-phase trap column (180  $\mu\text{m}$   $\times$  20 mm) packed with the 5  $\mu\text{m}$  resin (Symmetry C18, Waters) and separated on a capillary column (100  $\mu\text{m}$   $\times$  100 mm, Waters) packed with BEH130 C18 resin (1.7  $\mu\text{m}$ , Waters) with column temperature kept at 37°C. An injection volume of 2  $\mu\text{L}$  was loaded on the trapping column for desalting at a flow rate of 15  $\mu\text{L}/\text{min}$  for 3 min at initial conditions, with 99:1 of solvents **A:B**; flow was diverted to waste. After desalting, flow is diverted through the trap column to the analytical column with an isocratic condition of 99:1 **A:B** at 400 nL/min for 16 min, followed by a linear gradient of 10-45 % solvent **B** delivered with a flow rate of 400 nL/min over 30 min. A fast gradient of 45-80 % solvent **B** in 6 minutes with flow rate of 800 nL/min was used to clean out the column for subsequent injections followed by equilibrating to initial conditions. Typical Q-TOF parameter settings consist of a capillary voltage of 3850 V, cone voltage 120 V and source temperature 80 °C.

Data Acquisition mode used for sample analysis is called Data Dependant Acquisition (DDA) and consists of the detection of multiply charged positive ions of  $z=2, 3, 4$  from the MS survey scan. The mass scan range was 400 to 1900 m/z with scan time 1 second. Three MS/MS scans were triggered from each MS scan event with a peak detection window 4 m/z (signal intensity threshold 16 counts/sec) and the continuum data were recorded. In MS/MS experiments, dynamic exclusion of previously detected precursors was set at 2 min; peptides from trypsin and keratin were also excluded from MS/MS data collection.

In MS survey scan, the collision cell potential remained 7.5 eV, the collision cell pressure remains constant, typically at 5.2e-5 mBar with argon gas. When the MS/MS spectrum was collected, collision energy varied ranging from 20 to 80 eV depending on the charge state and m/z of the precursor.

To obtain high mass accuracy, a synthetic peptide, LeuEnk (Environmental Resource Associates (ERA), Arvada, CO) with m/z 556.2771, prepared at concentration of 80 fmol/ $\mu\text{L}$  in 1:1 acetonitrile:water with 0.1 % formic acid, was sampled every 20 sec with a flow rate of 0.2

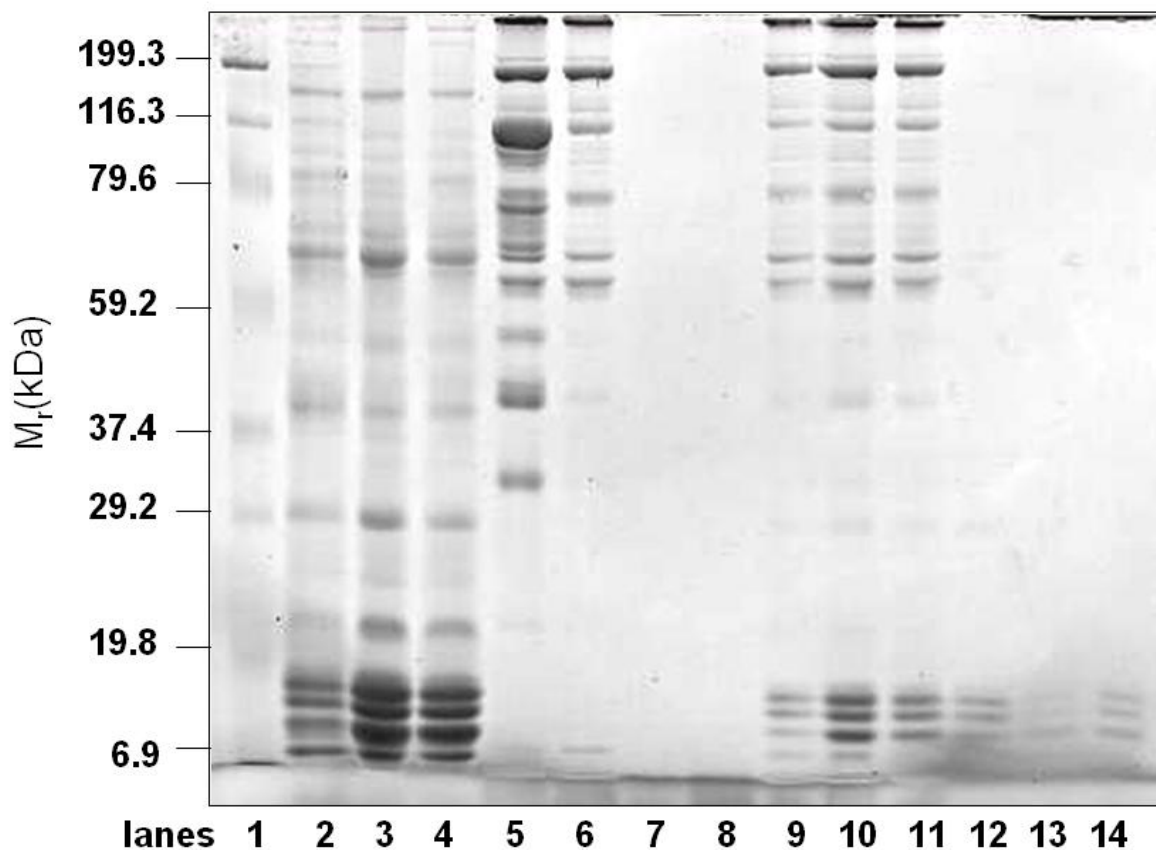
$\mu\text{l}/\text{min}$ . The Leucine Enkephalin mass was used later during data processing as a lock mass for small adjustments to the mass calibration and thus improving mass accuracy.

Data was processed with ProteinLynx Global Server 2.4 ( PLGS 2.4, Waters) using .RAW files from LC-ESI MS and MS/MS, and .PKL files were generated, these files were then subsequently submitted to Mascot (Matrix Science Ltd., London, UK) for peptide search against the NCBI database hosted by National Research Council of Canada (NRC, Ottawa). For database searching parameters, a maximum of 1 miscleavage was allowed for tryptic digestion, the peptide tolerance for precursor ions was  $\pm 50$  ppm and  $\pm 0.4$  Da for fragment ions. Carbamidomethylation of cysteine was selected as fixed modification and oxidation of methionine as variable modification. A minimum of two unique peptides was required for confident protein identification

## 4.4. Results

### 4.4.1. Replications for isolation of bison seminal plasma proteins in LDL

The protein profiles from 3 different pooled bison seminal plasma collections were similar (lanes 2-4, figure 4.1); suggesting the variation of profiles is minimal. A majority of the proteins in the profile had low molecular weights. On the other hand, Triladyl<sup>®</sup> extender alone contains mostly higher density proteins which range between 30 – 200 kDa (lane 5 figure 4.1). Higher molecular weight proteins ranging from 60 – 200 kDa were observed to be associated with the LDL found in Triladyl<sup>®</sup>; hardly any LDL proteins were detected in lower molecular weights (lane 6, figure 4.1). For the other extender, Andromed<sup>®</sup> is a plant based extender and no animal proteins were detected (lane 7 and 8, figure 4.1). When bison seminal plasma was mixed with Triladyl<sup>®</sup>, the LDL fraction contained seminal plasma proteins ranging from high to low molecular weights 6 – 20 kDa (lanes 9-11, figure 4.1). Very little variation was noted between protein profiles of results, indicating that similar seminal plasma proteins are being captured by the LDL (lanes 9-11, figure 4.1). Interestingly, when bison seminal plasma was added to Andromed<sup>®</sup> prior to the phospholipids being isolated, low molecular weight proteins were observed, ranging from 6 – 20 kDa (lanes 12-14, figure 4.1). Little variation was observed between the different phospholipids isolation experiments suggesting that the same seminal plasma proteins were associating with the phospholipids.

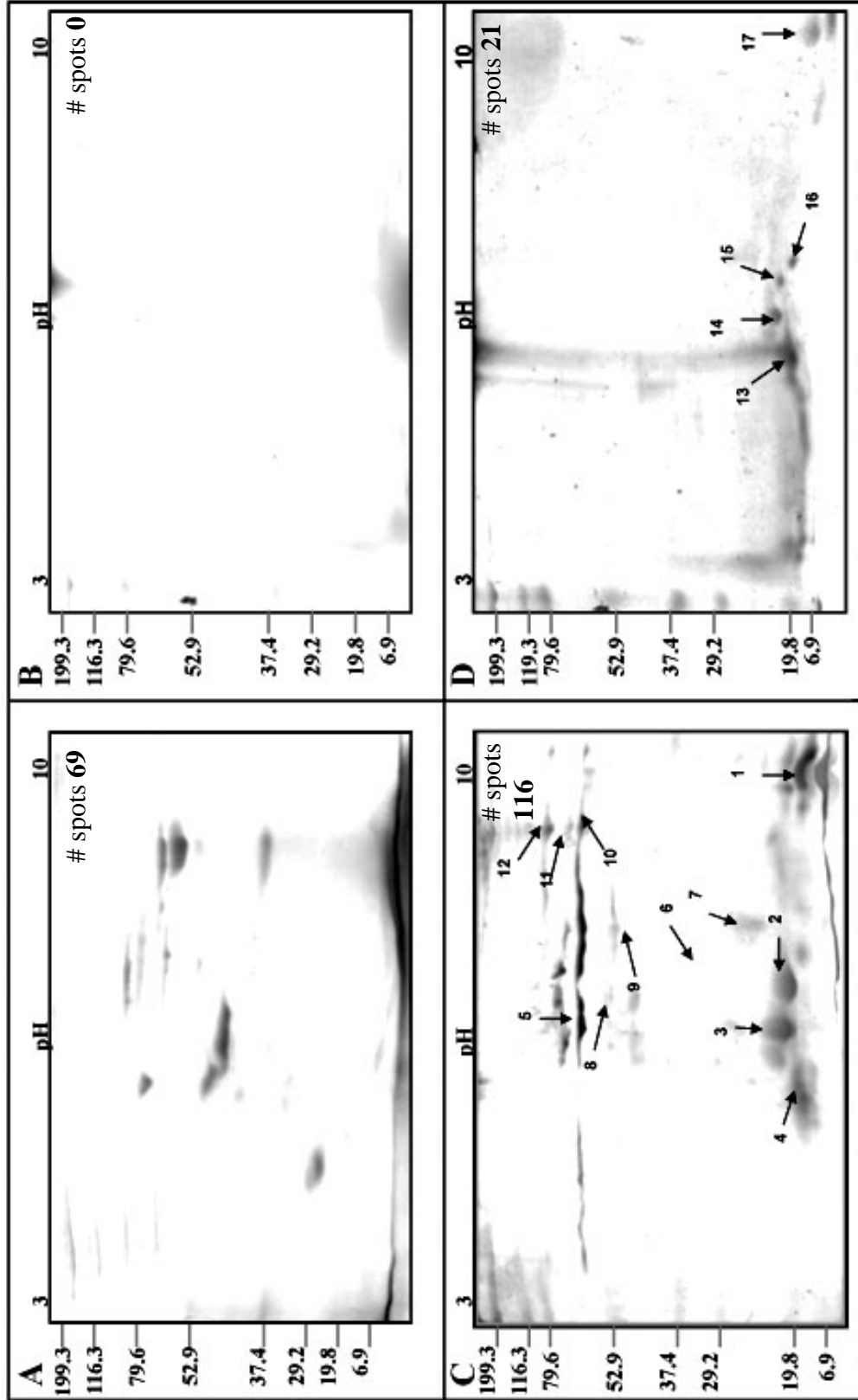


**Figure 4.1:** A SDS-PAGE gel stained with sypro red protein stain. Lanes: **1** broad range marker, **2** bison SP (seminal plasma) from October 16/09, **3** bison SP from September 14/09, **4** bison SP from August 22/09, **5** Triladyl<sup>®</sup>, **6** Triladyl<sup>®</sup> LDL, **7** Andromed<sup>®</sup>, **8** Andromed<sup>®</sup> phospholipids, **9** Triladyl<sup>®</sup> + SP (oct 16) LDL, **10** Triladyl<sup>®</sup> + SP (Sept 14) LDL, **11** Triladyl<sup>®</sup> + SP (Aug 22) LDL, **12** Andromed<sup>®</sup> + SP (Oct 16) phospholipids, **13** Andromed<sup>®</sup> + SP (Sept 14) phospholipids, **14** Andromed<sup>®</sup> + SP (Aug 22) phospholipids



#### **4.4.2. Protein profiles from two dimensional electrophoresis (2DE) of proteins associated with LDL**

The controls for this experiment were Triladyl<sup>®</sup> LDL and Andromed<sup>®</sup> phospholipids; these protein profiles were observed to have 69 and 0 protein spots respectively (figure 4.2 a, b). An overloaded gel of LDL isolated from bison seminal plasma with Triladyl<sup>®</sup> was used to pick spots for identification; the number of protein spots on this gel increased to 116 compared to Triladyl<sup>®</sup> LDL alone (figure 4.2c). Of the 116 protein spots 47 were unique from seminal plasma. The majority of the bison seminal plasma spots were observed to be of low molecular weight; some unique spots were also observed to have mid range molecular weights. Of the protein spots observed from both the Triladyl<sup>®</sup> LDL and bison seminal plasma most had basic pIs ranging from 5 - 9. For the other extender, when the lipoproteins were isolated after bison seminal plasma had been added to Andromed<sup>®</sup>, 21 protein spots were observed from an overloaded gel (figure 4.2d). For this gel all proteins had low molecular weights and were tending toward basic pIs. It was noted that precipitation was common at a pH of approximately 6. The spots picked for identification by MALDI-TOF are labelled 1- 17; the gels used for picking were overloaded with protein and not all the spots can be clearly identified in figure 4.2.

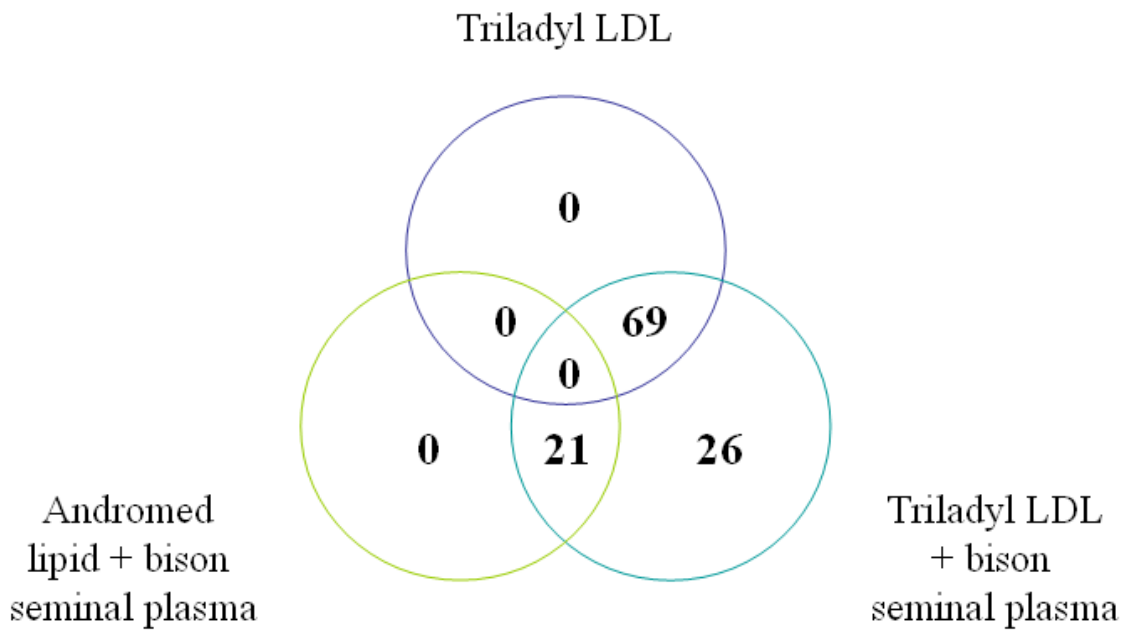


**Figure 4.2:** Two dimensional electrophoresis gels of protein profiles developed from: A) Triladyl® LDL B) Andromed® phospholipid C) Triladyl® LDL + bison seminal plasma D) Andromed® phospholipid + bison seminal plasma. Gels were stained with silver stain. Numbered protein spots were identified using MALDI-TOF.

$M_r$

**4.4.3. Venn diagram representing the protein associations between Triladyl<sup>®</sup> LDL, Triladyl<sup>®</sup> LDL + bison seminal plasma and Andromed<sup>®</sup> phospholipid + bison seminal plasma**

Triladyl LDL alone and Triladyl LDL with bison seminal plasma were found to have 69 proteins in common; while LDL isolated from Triladyl and seminal plasma was observed to have 47 proteins unique to the seminal plasma (Figure 4.3). There were 21 seminal plasma proteins that were isolated by both the lipoprotein fraction of Andromed with seminal plasma and the LDL isolated from Triladyl with seminal plasma.



**Figure 4.3:** A venn diagram relating the association of shared and unique proteins between Triladyl<sup>®</sup> LDL, Triladyl<sup>®</sup> LDL + bison seminal plasma and Andromed<sup>®</sup> phospholipid + bison seminal plasma.

#### **4.4.4. Identification of seminal plasma proteins associated with LDL and phospholipids from extenders**

For identification 12 protein spots were chosen from the overloaded 2DE of LDL isolated from Triladyl<sup>®</sup> with bison seminal plasma and, five protein spots were selected from the overloaded 2DE of phospholipids from Andromed<sup>®</sup> with bison seminal plasma (Table 4.1 and 4.2). These specific spots were chosen because they were large enough to be handpicked without contamination from neighbouring spots. These specific spots were also saturated with enough protein to be able to identify them using MALDI-TOF photometry. All identified spots matched with proteins already found to play roles in proper function of sperm during reproduction. A group of proteins were found to be important for the capacitation process; this group includes BSPs, PAFs, ASFP, ART5. Other proteins have a role in modifying the female reproductive tract to enhance the sperms survival; these proteins include PAF, ASFP, TIMP2.

**Table 4.1:** Protein spots of interest from two dimensional electrophoresis gels Triladyl<sup>®</sup> LDL+ bison seminal plasma.

Triladyl <sup>®</sup> LDL + bison seminal plasma			
Protein Spot #	Identification	Acronym	Protein Function
Spot 1	Seminal fluid protein A3	BSP A3	Release of lipids from sperm plasma membrane
Spot 2	Chain A, bull seminal plasma Pdc-109	BSP A1/A2	Trigger release of phosphatidyl choline and cholesterol from plasma membrane of sperm
Spot 3	Chain A, bull seminal plasma Pdc-109	BSP A1/A2	Trigger release of phosphatidyl choline and cholesterol from plasma membrane of sperm
Spot 4	Spermadhesin-1 precursor/ acidic seminal fluid protein	ASFP	Precursor peptide having diuretic and vasodilating activity
Spot 5	Seminal fluid protein A3	BSP A3	Release of lipids from sperm plasma membrane
Spot 6	Spermadhesin Z13		Role in capacitation and zona binding process
Spot 7	Tissue inhibitor of Metalloproteinase, Type 2	TIMP2	Bind to and inactivate metalloproteinases
Spot 8	ADP-ribosyltransferase 5	ART 5	Transfer ADP-ribose into target proteins
Spot 9	Tissue inhibitor of Metalloproteinase, Type 2	TIMP2	Bind to and inactivate metalloproteinases
Spot 10	Tissue inhibitor of Metalloproteinase, Type 2	TIMP2	Bind to and inactivate metalloproteinases
Spot 11	Platelet activating factor acetylhydrolase	PAF-AH	Immune stimulation and a lipid mediator
Spot 12	Platelet activating factor acetylhydrolase	PAF-AH	Immune stimulation and a lipid mediator

**Table 4.2:** Protein spots of interest from two dimensional electrophoresis gels Andromed<sup>®</sup> phospholipid + bison seminal plasma.

Andromed <sup>®</sup> phospholipid + bison seminal plasma			
Protein spot #	Identification	Acronym	Protein Function
Spot 13	Chain A, bull seminal plasma Pdc-109	BSP A1/A2	Trigger release of phosphatidyl choline and cholesterol from plasma membrane of sperm
Spot 14	Spermadhesin-1 precursor/ acidic seminal fluid protein or Chain A, bull seminal plasma Pdc-109	ASFP or BSP A1/A2	Trigger release of phosphatidyl choline and cholesterol from plasma membrane of sperm Precursor peptide having diuretic and vasodilating activity
Spot 15	Spermadhesin-1 precursor/ acidic seminal fluid protein	ASFP	Precursor peptide having diuretic and vasodilating activity
Spot 16	Chain A, bull seminal plasma Pdc-109	BSP A1/A2	Trigger release of phosphatidyl choline and cholesterol from plasma membrane of sperm
Spot 17	Seminal fluid protein A3	BSP A3	Release of lipids from sperm plasma membrane

## 4.5. Discussion

Unique bison seminal plasma proteins were found to be captured by the LDL from Triladyl<sup>®</sup> extender. Interestingly, the phospholipids fractionated from Andromed<sup>®</sup> extender also had evidence of bison seminal plasma proteins. Bison seminal plasma proteins associated with Triladyl<sup>®</sup> LDL and phospholipids from Andromed<sup>®</sup> had 116 and 21 proteins spots respectively (Figure 4.2). From these protein spots, 17 were chosen to be analysed based on the following criteria: they were relatively large sized, they were far enough from neighbouring protein spots to avoid contamination and they appeared to have enough protein saturation to be able to perform mass spectrophotometer analysis for identification. Of the 17 identified proteins six had functions that were associated with sperm viability (Table 4.1 and 4.2). The functions of these proteins include; initiating capacitation and hyperactivation of sperm, providing protection for sperm from oxidation damage and actual binding of the sperm to the oocyte [68, 74, 81, 139, 140]. Of these proteins, two can possibly affect the bison sperms ability to survive the cryopreservation procedures: Binder of Sperm (BSP) and Platelet Activating Factor Acetylhydrolase (PAF-AH).

The protein PAF-AH is captured by Triladyl<sup>®</sup> LDL, but not by the phospholipids from Andromed<sup>®</sup> (Table 4.1 and 4.2). The family that includes PAF proteins is large and the proteins have a wide array of functions including platelet aggregation, lipid mediators and roles in inflammation responses [91, 92]. If PAF-AH is captured by LDL then the PAF proteins are available to initiate sperm capacitation and hyperactivation [94, 95]. The PAF proteins help to initiate sperm capacitation by assisting in the movement of lipids in the sperm plasma membrane [95]. Hyperactivation of sperm also can be initiated by PAF proteins when they bind to a specific G-protein receptor, initiating an inositol triphosphate-diacylglycerol mediated pathway that leads intracellular calcium levels to increase [140]. Therefore, the role of PAF-AH is to act as a decapacitation factor, regulating the activities of PAF proteins by hydrolyzing acetate from position 2 of the individual PAF proteins, changing them back to the inactive lyso-PAF form; stopping the capacitation actions of the PAF protein [74, 92]. Moreover, PAF-AH has been observed to play a role in removing phospholipids from sperm that have been damaged by oxidation; this keeps the sperm alive and functioning so it can fertilize the oocyte [141]. Studies

have suggested that PAF-AH in bovine seminal plasma strongly associates with LDLs [142]. In the Triladyl<sup>®</sup> extender the PAH-AH is captured by the LDL, allowing the PAF protein to initiate sperm capacitation [74]. During capacitation the bison sperm plasma membrane releases lipids and becomes more vulnerable to ice damage during the cryopreservation procedures.

Andromed does not capture PAF-AH, suggesting that bison sperm should be better protected in Andromed<sup>®</sup> than Triladyl<sup>®</sup>; however, this has not been observed in studies. In fact bison sperm cryopreserved in Triladyl<sup>®</sup> had higher post thaw total motility when compared to Andromed<sup>®</sup> [12]. In summary, we could speculate that PAF-AH is probably not the interfering factor present in bison seminal plasma.

The other proteins identified as possible interfering factors are from the BSP family of proteins. The BSP proteins are secreted from the seminal vesicles of many different mammals including bovine, boar, stallion, bison, ram, and goats [143, 144]. These small acidic proteins contain two homologous type II domains similar to the gelatin-binding domains of fibronectin and have variable amino-terminal extension [72]. The main functions of BSP proteins are to initiate sperm capacitation and to establish a sperm reservoir within the female oviduct [83, 145]. At ejaculation, the BSP proteins bind to the sperm membrane via choline phospholipids, where they cause an efflux of phospholipids and cholesterol [139]. By remodelling the composition of the sperm plasma membrane, BSPs initiate the capacitation process [145]. In fact, the removal of lipids from the bison sperm plasma membrane could destabilize the membrane allowing for increased ice damage during the freezing and thawing procedures [87]. The LDL in egg yolk binds BSP stopping the initiation of sperm capacitation [72]. Interestingly, the phospholipids from Andromed<sup>®</sup> were also associated with BSP proteins (Figure 4.2). However, the composition of the phospholipids in Andromed<sup>®</sup> was not the basis of this study and is currently unknown. The amount of BSPs captured by Triladyl<sup>®</sup> LDL was subjectively observed to be more intense than BSPs captured by Andromed<sup>®</sup> phospholipids (Figure 4.2). This indicates that Triladyl<sup>®</sup> is able to provide a greater degree of protection for bison sperm compared to Andromed<sup>®</sup> during the cryopreservation procedures. However, Triladyl<sup>®</sup> cannot completely stop the effects of BSP on bison sperm because the BSPs bind within seconds of ejaculation so not all



BSP will bind to the LDL; allowing the BSP proteins to continue to interact with the sperm plasma membrane [72].

There were four other proteins identified in this study that had different actions on the bison sperm. They are; Tissue Inhibitor of Metalloproteinase, type 2 (TIMP2), ADP-ribosyltransferase 5 (ART5), spermadhesin-1 (ASFP) and spermadhesin Z13 (Table 4.1 and 4.2). These proteins are not thought to be the interfering factor after observing the ways they interact with sperm. The ASFP protein protects sperm from oxidative damage [82]. It was noted that the Triladyl<sup>®</sup> LDL did not capture significant concentrations of the proteins; leaving many ASFP proteins with the sperm where it would counteract the effect of oxidative damage (Figure 4.2). The exact role of spermadhesin Z13 is still being studied but it plays a role in binding of sperm to the zona pellucida [74], so it should only affect the fertilization ability of sperm and not the ability of the sperm to survive cryopreservation. The TIMP2 proteins are associated with increased fertility in dairy cattle [146]; however, TIMP2 defective mice were found to still be viable and still fertile indicating that TIMP2 does not have an essential role in the reproductive physiology of the sperm [147]. Finally, ART5 is a relatively new discovered protein and its role in the seminal plasma is still unknown [74]. Only small amounts of it were captured by the LDL from Triladyl<sup>®</sup> suggesting that proteins would still be interacting with the sperm (figure 4.2). In conclusion, while each of these proteins may not individually be the interfering factor but, when working together they may play a significant role in the bison's sperm survival during cryopreservation.

In summary, this study showed that specific unique bison seminal plasma proteins can be captured by the LDL and phospholipid fractions of Triladyl<sup>®</sup> and Andromed<sup>®</sup> extenders respectively. Among the 17 proteins chosen to be identified, only BSP could be a candidate for the interfering factor that is affecting the bison sperm ability to survive cryopreservation. In-depth study of the exact mechanisms BSP uses to interact with bison sperm will be needed to confirm its potential role. The knowledge that LDL is able to capture the BSPs allows for procedures to be developed to neutralize the adverse effect of BSPs prior to cryopreserving bison semen.

#### **4.6. Acknowledgments**

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## CHAPTER 5: FINAL CONCLUSION AND FUTURE DIRECTIONS

North American bison (*Bison bison*) are a relatively new animal to be raised as domestic livestock [1] and act like wild animals even when raised in captivity. Bison can become highly stressed if forced to be in close contact with humans, causing them to act unpredictably and aggressively [148]. High stress levels may also negatively influence the quality of semen collected from bison bulls. These issues led us to consider the use of a long acting neuroleptic (LAN) when working with bison bulls. It was predicted that the effect of the LAN would calm the bison during handling and improve semen collection procedures. Our studies revealed that behavioural and endocrine stress markers were lower in the bulls and better quality semen was collected when 200 mg of Piportil<sup>®</sup> was injected (Figure 3.1, 3.2 and 3.3, Chapter 3). This result was different from what was observed when only 100 mg of Piportil<sup>®</sup> was injected; this dose had no effect on endocrine responses to stress and did not improve semen quality (figure 3.2 and 3.3, Chapter 3). However, behavioural modifications were observed with the 100 mg dose of Piportil<sup>®</sup> including bison bulls being captured quicker and struggling less once captured (figure 3.1, Chapter 3). However, because few behavioural modifications were observed when the 100 mg dose of Piportil<sup>®</sup> was used it could be suggested that 100 mg dose of Piportil<sup>®</sup> was too little to provide significant calming effects in bison bulls but, a higher dose calmed the bulls significantly and allow for higher quality semen to be collected. The 100 and 200 mg doses of Piportil<sup>®</sup> were chosen because of previous work done with female bison which suggested that 150 mg/bison dose of Piportil<sup>®</sup> causes significant calming effects with no side effects for the females (Dr. McCorkell, personal communication). The reduced stress and higher quality semen observed when Piportil<sup>®</sup> is used on bison bulls would be a valuable asset for commercial bison ranchers, especially when working with newer animals that have not been trained to work near humans. An example would be training bison to move through a squeeze chute system, which is a stress filled activity for these relatively wild animals. With the introduction of Piportil<sup>®</sup>, the animals would have less stress, allowing them to easily adjust to the situation [28]. The use of Piportil<sup>®</sup> could also ease stress of older breeding bulls that are known to become increasingly aggressive and difficult to handle as their age increases [51]. This would allow ranchers to keep valuable bulls for longer periods of time instead of being forced to cull them as they become too difficult and dangerous to handle. Even though Piportil<sup>®</sup> would be an important resource for

bison ranchers to help streamline production; they may be unlikely to make use of it because of the high cost of the drug (over \$100 per bull) and the fact that it must be bought and administered by a veterinarian which also adds cost to total production.

The use of a LAN could also be very valuable when working with wild bison herds, especially the threatened wood bison in Canada. Organizations such as the National Wood Bison Recovery Team have been investigating ways to salvage important genetics from herds endemically infected with brucellosis and tuberculosis [13, 125, 149]. At this time infected bison are managed by culling to remove them from the populations but, this results in loss of unique genotypes from the population [125]. The Wood Bison Advisory Program, founded in 1985, suggested that all infected wood bison or crossbred wood bison be culled and their gametes should be collected to establish disease free herds without losing valuable genetics [3, 15]. This theory works on paper, but at this point in time, poor results have been obtained when trying to cryopreserve bison gametes. This means that not all of the gametes may survive cryopreservation and there is a chance that important genetics could be lost when the animals are killed. The LAN could be used in wild bison by first capturing bison bulls in temporary corrals then injecting them with Piportil<sup>®</sup> approximately a week prior to collection. The effect of Piportil<sup>®</sup> will cause the stress levels in the bison bulls to be reduced allowing for higher quality semen to be collected, increasing the chances of successful artificial insemination (AI) and *in vitro* fertilization (IVF) for a disease free herd. Once a population of disease free pure wood bison is established in a location separate from the original herds the infected animals could be culled with no risk of losing the valuable genetic pool.

Piportil<sup>®</sup> could also be a useful tool in the handling of bison housed in zoo environments. The handling of these animals to ensure proper health and well-being would be made easier and safer if animal stress levels were managed with a LAN. The lower stress levels may even assist in training these animals to enter a chute system to perform routine health checks performed. To ensure genetic diversity within zoo's bison populations, breeding programmes often need animals to be moved between different locations. Transportation of bison can be a very stressful situation, and an injection of Piportil<sup>®</sup> would help to relieve some of this stress. Moreover, introducing new animals into established herds can be a dangerous and highly stressful situation,

established animals will often attack a new comer and do not allow them to eat or drink [33]. The use of a LAN with the established animals may reduce the aggression towards the new comer allowing for quicker introductions. Finally, because semen collected would be of better quality after a LAN is injected, it would survive movement to other locations better.

The results of this study suggested that the use of Piportil<sup>®</sup> in bison bulls increased the quality of semen collected through electroejaculation (Figure 3.3, Chapter 3). This higher quality semen would enhance bison ranching in a number of ways including; improvement of genetic diversity between animals and allow for increased income for the producers. For instance, AI can be used to inseminate many more bison cows than traditional breeding; one ejaculate from a high quality bovine bull can be used to inseminate 300-1000 cows once extended [150]. It has been calculated that cost per live calf born to a bovine cow bred by AI using chilled semen is \$4 - 19 USD while the cost per calf born to a cow bred by a herd bull kept and raised by the rancher is \$73 - 600 USD [151]. This indicates it would be more economical for bison ranchers to use AI to breed their cows than keeping bulls and allowing them to serve the cows naturally.

Moreover, cryopreserved bison sperm would enhance the movement of genetics between different ranches through AI, and increase the genetic diversity with the world bison herd. Currently, fresh semen collected from bison through electroejaculation has total motility of only approximately 70 % and once it is frozen it loses more than 50 % of its total motility [12, 56]. Treating bulls with Piportil resulted in semen collections of a higher quality with higher total motility (Figure 3.3, Chapter 3). Once cryopreserved this semen could maintain higher quality and motility, even if 50 % motility is lost, compared to a typical electroejaculated sample. The survival rate of post thaw semen would allow for the sperm to be used in a variety of reproductive technologies such as AI or IVF with much higher success rates.

Cryopreserved bison semen is valuable for banking of animal genetics. These banks allow for preservation of genetics that may be at risk through breeding practices, disease or other unforeseen loss of animals. Canada has set up the Canadian Animal Genetic Resource (CAGR) program for exactly this purpose [7]. Cryopreservation of bison semen would also be a valuable

asset for ranchers so they do not have to keep as many live bulls, but are still able to access the genetics from these bulls at a later date.

The second part of this thesis was designed to examine why bison sperm do not survive cryopreservation procedures. It has been observed that electroejaculated bison semen has post thaw total sperm motility of 35 % and 9 % when extended with Triladyl<sup>®</sup> and Andromed<sup>®</sup> respectively [12]. However, if epididymal sperm is used for cryopreservation the post thaw total motility is approximately 50 % regardless of extender used [13]. The major difference with the two studies is that when sperm is collected by electroejaculation it comes in contact with seminal plasma. This suggests that there may be a factor in seminal plasma that is affecting the ability of bison sperm to survive cryopreservation. Moreover, it was hypothesised that the low density lipoprotein (LDL) fraction of Triladyl<sup>®</sup> was able to capture this factor while the phospholipids in Andromed<sup>®</sup> did not, resulting in much lower post thaw motility using extender containing no egg yolk. The LDL fraction from Triladyl<sup>®</sup> with bison seminal plasma was examined using two dimensional electrophoresis (2DE) and it was determined that several seminal plasma proteins were being captured (47 protein spots from Triladyl<sup>®</sup> LDL; Figure 4.2, Chapter 4). Interestingly, seminal plasma proteins were also observed associating with the phospholipid fraction of Andromed<sup>®</sup> (5 protein spots from Andromed phospholipid<sup>®</sup>; Figure 4.2, Chapter 4). From these unique bison seminal plasma proteins, 17 were chosen to be identified by MALDI-TOF (Table 4.1 and 4.2, Chapter 4). The functions of the 17 proteins were discovered, and 6 were observed to directly influence sperm; they are: Binder of Sperm (BSP), Platelet Activating Factor Acetylhydrolase (PAF-AH), Tissue Inhibitor of Metalloproteinase, type 2 (TIMP2), ADP-ribosyltransferase 5 (ART5), spermadhesin-1 (ASFP) and spermadehesin Z13 (Table 4.1 and 4.2, Chapter 4). One of these proteins, BSP has a role that could be detrimental to sperm that is being cryopreserved. The BSP proteins cause a cholesterol efflux from the sperm plasma membrane and initiate capacitation; however, this action could leave sperm more susceptible to ice damages during cryopreservation [72]. Of the other proteins identified it is hypothesised that individually they may not cause significant damages to the sperm but, when working together the different roles may all contribute to the low survival of bison semen when cryopreserved. Further studies need to be conducted to confirm if BSPs alone or by acting with other seminal plasma proteins

are affecting the bison sperm during cryopreservation and to discover methods to counteract these actions.

One possible method to prove if BSP are the interfering factor would involve isolating BSP from seminal plasma with the use of a gelatin-agarose column (Figure 1, Appendix A). Once isolated, BSPs would be exposed to epididymal sperm which has not come into contact with seminal plasma and then be cryopreserved. When compared with control epididymal sperm, the BSP exposed sperm should have very low survival rates (see Appendix A). Our preliminary results indicate that BSP is causing lower motility of sperm immediately after addition (Figure 2, Appendix A).

The other 5 proteins of interest should also be isolated from seminal plasma, and their actions on the sperm membrane should be examined in order to establish if they too may be contributing to the low post thaw survival of bison semen. An electron microscope could be used to examine the plasma membrane of the post thawed bison sperm and determine the type of damage sustained. A confocal microscope could discover which proteins are associating with the plasma membrane and how they are affecting the sperms lipid bilayer. Also, a tool like flow cytometry could be used to examine plasma and acrosome membrane integrity to evaluate if these parts of the sperm are affected by the interfering proteins. Establishing the type of damage would give the researcher some insights about how the interfering factor may be damaging the sperm allowing damage during cryopreservation; allowing for procedures to be designed that could counteract the effects.

If the interfering factor is proven to be BSP, protocols can be developed to counteract the effects on the sperm so that bison semen can be cryopreserved with more success. One method to counteract the effect of BSP proteins is to add cholesterol back into the sperm plasma membrane prior to cryopreservation. Cholesterol can be replaced back into plasma membrane by adding cholesterol-loaded cyclodextrin (CLC) into the extender being used for cryopreservation; the added cholesterol helps to stabilize the plasma membrane. This technique has been successfully used to cryopreserved semen from a variety of animal including bovine, caprine, leporidae, equine and even in a study involving bison sperm [152-156] (bison study has not been

published). Interestingly, it was observed that post thaw semen quality was increased when CLCs were added to extenders with or without egg yolk, but the quality was best when both CLCs and egg yolk was used for Markhoz bucks semen [154]. If these results were observed with bison semen, it could be suggested that the CLCs may increase the cholesterol content in the plasma membrane while, the LDL from the egg yolk helped to capture the BSPs; both helping to improve bison's sperm post thaw survival.

Another action that could help improve bison semen survival during cryopreservation would be to add increased amounts of LDL into an extender. This LDL could be added in addition to egg yolk. For studies where animal products are to be avoided, a synthetic LDL could be developed that would still be able to capture the BSPs from the seminal plasma. An additional strategy to counteract the effects of BSP on sperm would be to find a substance that BSP is more highly attracted to than the lipids in the sperm plasma membrane. This substance could then be added to the extender to bind BSPs before they can cause too much damage to the sperm plasma membrane. Another possible solution that does not rely on identification of the interfering factor would be to centrifuge the semen to remove all seminal plasma prior to cryopreservation. Seminal plasma is removed from the spermatozoa in a number of species at this time including; equine, caprine and porcine; its removal allows for better survival of the sperm from cryopreservation [157-159]. However, bison semen would have to be centrifuged as quickly as possible to remove seminal plasma because; the actions of the interfering factor may start as soon as the seminal plasma comes in contact with the spermatozoa [68].

The most promising theories at this time however, involve the identification of this interfering factor. Once accomplished, improved protocols for cryopreserving bison semen can be developed. The bison industry will benefit by being able to make use of reproductive technologies that are common in the bovine industry including AI and IVF which all use cryopreserved semen. Bison producers will be able to access genetics from valuable bulls without dealing with the animals directly and the ease of shipping cryopreserved semen would allow for increased genetic diversity for ranches. Perhaps the greatest advantage would be the ability to streamline production practices through the use of AI, increasing the number of animals being produced exponentially and increasing rancher's income.



The study presented in this thesis established that a LAN can be used to streamline bison production by allowing ranchers to more easily handle their animals and collect higher quality semen. Moreover, it was observed that seminal plasma proteins are being captured by the LDL in Triladyl<sup>®</sup>. These proteins need to be further investigated to prove if they are the factor interfering with bison semen being able to survive cryopreservation. The knowledge gained from this study will lead to further investigations and advances in bison bull reproduction. Increased knowledge in bison reproduction will allow an expansion of growth in the bison industry in Canada and the USA.

### **III. APPENDIX A**

## **ISOLATION OF BINDER OF SPERM (BSP) PROTEINS AND ITS EFFECT ON EPIDIDYMAL SEMEN FROM BISON BULLS**

### **Introduction**

The bison industry has been growing exponentially since the early 1990's [4]. However, one problem that producers face is that little research has been done in bison reproduction technologies. Bison semen does not cryopreserve to the same standards as bovine semen. Ejaculated bison semen has a greater than 50 % loss in total motility after cryopreservation [12]. Interestingly, the low density lipoprotein (LDL) fraction of Triladyl<sup>®</sup> extender is able to capture a factor that maybe interfering with the ability of sperm to survive cryopreservation. A previous study was able to show that unique bison seminal plasma proteins are captured by LDL and of these proteins binder of sperm proteins (BSPs) were suggested to be the possible interfering factor (Chapter 4).

The BSP proteins are secreted from the vesicular glands of bovine bulls [72, 160]. Three major BSP proteins have been identified in bovine they are: BSP A1/A2 (PDC-109), BSP A3 and BSP 30kDa [72]. However, the BSP family of proteins has grown significantly in the last two decades and BSP proteins, or homologous BSP proteins, have been found in the seminal plasma of a large number of mammals including bovine, caprine, equine, human and bison [144]. The BSP proteins play an important role in fertilization; they participate in sperm membrane lipid modifications by binding and removing cholesterol and phospholipids in the lipid membrane of the sperm, which initiates capacitation [145]. Some BSPs stay bound to the sperm as they move through female reproductive tract; these BSPs help to form a sperm reservoir that holds the sperm until GAG's from the female interact with the BSP's, causing the second stage of capacitation in sperm [72]. The loss of lipids and subsequent destabilization of the sperm membrane caused by the BSP proteins maybe a factor in low sperm survival observed when bison semen is cryopreserved. With fewer lipids holding the sperm membrane stable there is a

higher likelihood of ice and osmotic damage occurring to the membrane during cryopreservation [72].

This study intends to prove whether BSPs are the interfering factor affecting the ability of bison sperm to survive cryopreservation. This will be done by isolating BSP from seminal plasma, then adding them to epididymal sperm that has not been exposed to seminal plasma. This sperm will then be cryopreserved and the BSP should cause the same loss of motility that is seen when ejaculated bison semen is cryopreserved.

The objective of this study was to isolate and intensify BSP proteins; followed by evaluating their effects on cryopreserved epididymal bison sperm. A gelatine-agarose column can be used to isolate BSP proteins.

## **Methods and Materials**

This study was approved by the animal care committee at the University of Saskatchewan. All chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated. The BSP proteins were isolated with the use of Affi-Gel® 15 matrix (Bio-Rad, Canada) in a 1.5 x 30 cm glass column and Saphadex G-25 medium matrix (GE Healthcare, Canada) built in a 1.5 x 30 cm glass column for desalting of the proteins.

### ***Animals***

Bison bulls were located at native hoof stock center, near Saskatoon, Saskatchewan, Canada (52°02'N, 106°28'W). Bison were maintained on an alfalfa/brome pasture with free access to fresh water and hay. Six bison bulls were used for semen collections from June to November 2009 and 2010 (3=N) *Bison bison athabascae* and (3=N) *Bison bison bison*. Semen was collected following standard bovine electroejaculation methods, using a probe and manual ejaculator (Pulsator IV, Lane Manufacturing Denver, CO, USA). Collected semen was

immediately placed in an incubator at 37°C until it could be returned to the laboratory (approximately 2 hours).

### ***Seminal plasma preparation***

Spermatozoa were removed from seminal plasma through a series of centrifugations, two times at 300 g for 10 min and 2 times at 500 g for 10 min. After each centrifugation the supernatant was removed from the pellet so it could be centrifuged again and the pellet was disposed of. Seminal plasma was stored at -20°C for future use.

### ***Isolation of BSP proteins***

#### ***Binding of BSP proteins to affinity column***

Both columns were washed completely through at least 10 x with Tris buffer to completely equilibrate. Columns were kept at 4°C during the whole procedure.

Seminal plasma was slowly poured onto the top of the matrix and allowed to move through the column until it reached the bottom. Once seminal plasma reached the bottom of the column it was closed off and the solution was left to bind for 1 hour. Once binding was completed the column was once again hooked up to the pump, fraction collector and large reservoir of Tris buffer. It was noted that each tube should be collecting approximately 3 mL of liquid before the next tube begins to fill. As fractions were being collected they were being analysed with a NanoDrop 1000 (Thermo Fisher Scientific, Ottawa, ON, Canada) to determine the protein concentration. Once protein concentrations in the fractions had returned to zero all the unbound proteins had been removed from the column. All fractions containing protein were pooled together and kept at -20°C for future use. The column was then changed from the Tris buffer reservoir to a Tris + Urea buffer reservoir. Fractions were collected and analyzed again as previously described. All fractions containing protein were pooled together. If more than one peak of proteins was found then the peaks were pooled separately.

### ***Removal of Urea from BSP proteins***

The pooled sample of proteins collected from the eluting process was loaded into the top of the equilibrated Saphadex G-25 column. This column was connected to Tris buffer reservoir. The fractions were again collected and evaluated as described previously. Once all fractions were run through the column the ones containing protein were pooled together and the protein concentration was analysed.

### ***Concentration of proteins***

The BSP proteins will not be concentrated enough for use in the rest of the experiment after being purified in the columns. A centrifugable vivaspin 15 filter with a membrane of 5,000 HWC0 PES (Sartorius Stedim biotech GmbH, Goettingen, Germany) was used to concentrate the proteins to the desired amount. Briefly proteins were placed into the filter and centrifuged at 3000 x g, the concentration of the protein was checked approximately every 10min until it had reached the desired concentration of about 9 mg/mL.

### ***Western blot***

Briefly, each of the fractions from the gelatine-Agrose column along with bison seminal plasma (control) were run on an SDS-PAGE, following basic procedures. Once the SDS-PAGE was completed the gel was transferred onto PVDF membrane (Bio-Rad, Canada) using a semi-dry electrophoretic transfer cell (Bio-Rad, Canada). Next, the membrane was placed into a blocking buffer (PBS 1X 10% v/v, deionized water 90 % v/v, and BSA 1 % v/v) and gently agitated for 1 hour. Gels were then removed from blocking buffer and rinsed 3 x for 10 min in TTBS (20 mM Tris, 500 mM NaCl, 0.05 % Tween-20, pH 7.5) (Bio-Rad, Canada) before being placed into primary antibody (either Anti BSP1, 3 or 5) (provided by Dr. Manjunath). Gels were kept at 4°C and gently agitated overnight. In the morning the gels were rinsed 3 x 10 min with TTBS before being placed into the secondary antibody FITC- Goat anti-rabbit IgG (H+L) (Zymed, Invitrogen USA) and kept in the dark while being agitated for 2 hours. Finally, the gel

was again rinsed 3 x for 10 min in TTBS. The gel was analysed by fluorescence using the Typhoon Trio (GE Healthcare, Canada).

### ***Recovery of epididymal sperm from abattoir samples***

Testes were received from the abattoir and taken back to laboratory within 3 hours of animals being slaughtered. Once at the laboratory, the testes were removed from the scrotal sac and all tunicate layers were removed. The caput and cauda regions of the epididymis were identified. The vas deferens, cauda and half the corpus regions of the epididymis were removed from the rest of the testicle. A syringe that was filled with air was inserted into the lumen of the vas deferens and air was pushed into the seminiferous tubules. A small incision was made through some seminiferous tubules in the cauda section of the epididymis. As the sperm seeped out, it was collected and placed into a small centrifuge tube. Sperm was then placed in a beaker containing a 1:3 volume ratio of sperm to TCM-199 (Invitrogen, USA). This mixture was placed in a 37°C water bath for 1 hour to allow the epididymal sperm to mature and shed their cytoplasmic droplets. Once matured for an hour the sperm was analysed using CASA. The sperm concentration was adjusted to 150 million/mL.

Semen smears were made at this time using eosin-nigrosin live dead stain. Epididymal sperm from each bull was analysed for morphology using a light microscope. If normal morphology of at least 50 % was not observed the sperm from that particular bull was not used for the experiment.

### ***Preparation of treatments and evaluation***

The extenders Triladyl<sup>®</sup> (Bio-Rad, Canada) and Andromed<sup>®</sup> (Bio-Rad, Canada) were prepared in advance following directions in the product manual. PBSx1 (Cellgro, Mediatech Inc., USA) was prepared and autoclaved to ensure that no contaminants were present. Extenders, seminal plasma that was obtained from ejaculates, PBS 1X and concentrated BSP proteins were all warmed to 37°C in a water bath before being used.

Six treatment groups were tested: for each treatment 1 mL of epididymal sperm was placed into a centrifuge tube, it was then centrifuged at 300 g for 10 min and excess TCM199 was removed from pellet. 1 mL of treatment (PBS, SP, BSP) was added to the pellet. The pellet was resuspended and left to equilibrate for 30 min before 3 mL of extender (Triladyl or Andromed) was added.

- A) Epididymal sperm + PBS 1X + Triladyl<sup>®</sup>
- B) Epididymal sperm + PBS 1X + Andromed<sup>®</sup>
- C) Epididymal sperm + Seminal plasma + Triladyl<sup>®</sup>
- D) Epididymal sperm + Seminal plasma + Andromed<sup>®</sup>
- E) Epididymal sperm + BSP proteins + Triladyl<sup>®</sup>
- F) Epididymal sperm + BSP proteins + Andromed<sup>®</sup>

Once extender was added to the tubes the solutions were left for 30 min in a 37°C water bath to equilibrate.

***Cryopreservation procedure for treatments***

Once all treatments were equilibrated they were placed into a beaker filled with 37°C water which was moved into the cold room at 4°C. The beaker was left to cool down to 4°C. While cooling 0.5 mL French straws (IMV Technologies, France) were printed using an EasyCoder Automatic straw printer (Mini Tube, Canada). Straws were labelled with treatment group and bull number. Once all treatments were cooled to 4°C they were loaded into their corresponding straws and placed onto a freezing rack. The IceCube 14 S-B automatic freezer (MiniTube, Canada) was programmed to a -40°C freezing curve.

	Temp (°C)	Slope (°C/min)	Time (min)
0	4.00		0.00
1	4.00	0.00	1.00
2	-15.00	-3.00	6.33
3	-80.00	-40.21	1.63
4	-80.00	0.00	5.00

When primed the straws on racks were moved into the IceCube to be cooled to  $-80^{\circ}\text{C}$ . Once freezing was complete the straws were immediately placed into liquid nitrogen and moved into storage tanks.

### ***Thawing procedure for treatments and evaluation***

For each treatment group 3 straws were recovered from liquid nitrogen storage and thawed in a  $37^{\circ}\text{C}$  water bath. Semen samples were immediately analysed using CASA, then placed in a  $37^{\circ}\text{C}$  water bath for 2 hours before being analysed once more using CASA.

### ***Data analysis***

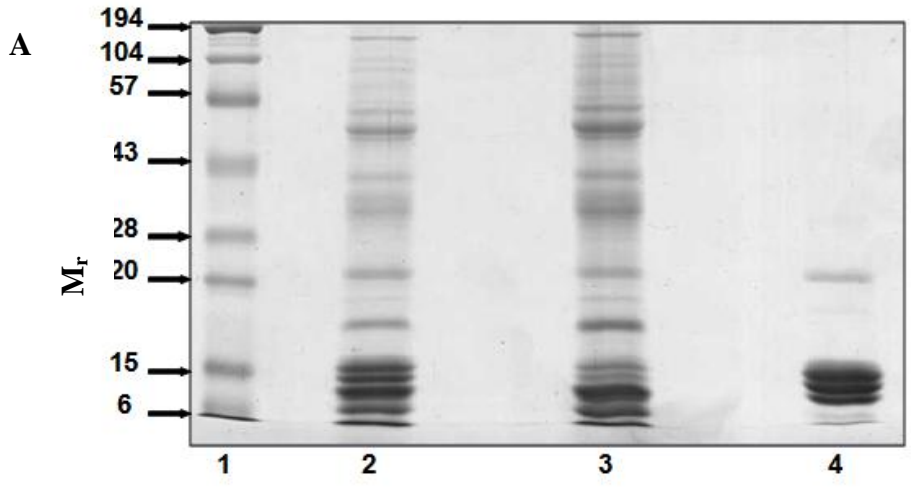
No statistical analysis was done on the post thaw results because too few bison testes were able to be obtained for this experiment to make the results statistically significant.



## **Results**

### **Isolation of BSP proteins**

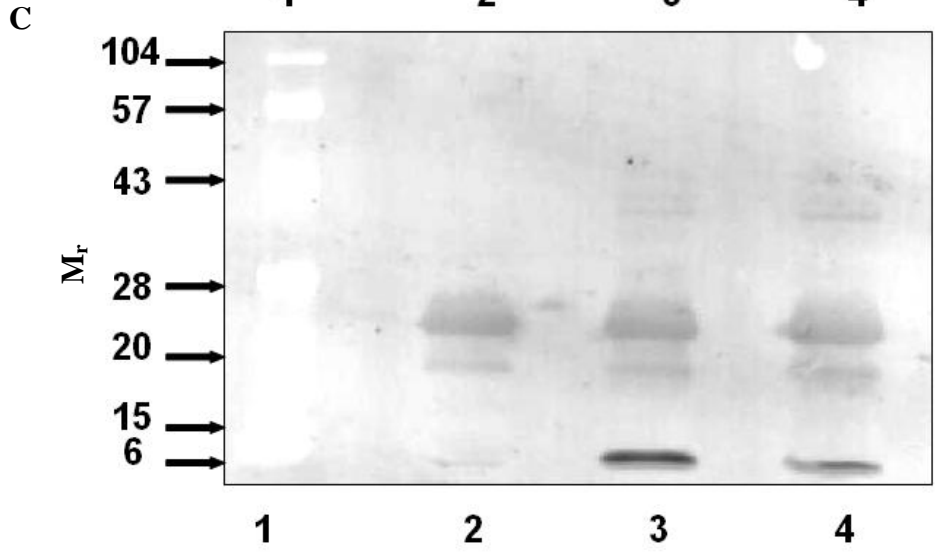
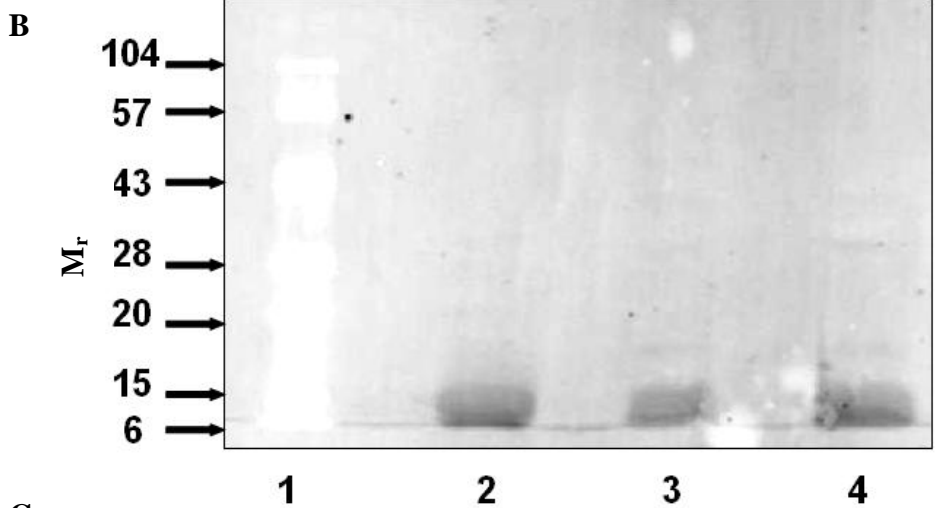
The gelatin agrose column was able to fraction out BSP proteins from complex bison seminal plasma. SDS-PAGE gel was performed in order to check for proteins in the different fractions. Proteins were found in all fractions with the least proteins being found in the last fraction which contained the proteins that bound to the column. The protein profiles suggest that BSPs were found in all the different fractions at approximately 15 kDa and 30 kDa, with the greatest amount BSP proteins being found in the final fraction which consisted of proteins bound to the column (Figure 6.1). This indicates that we were able to isolate and intensify the BSP proteins found in bison seminal plasma.



**Figure 1:** Presence of BSP proteins in different fractions of a gelatine-agarose column **1**- broad range molecular weight marker **2**- bison seminal plasma **3**- unbound protein fraction from column **4**-bound protein fraction from column

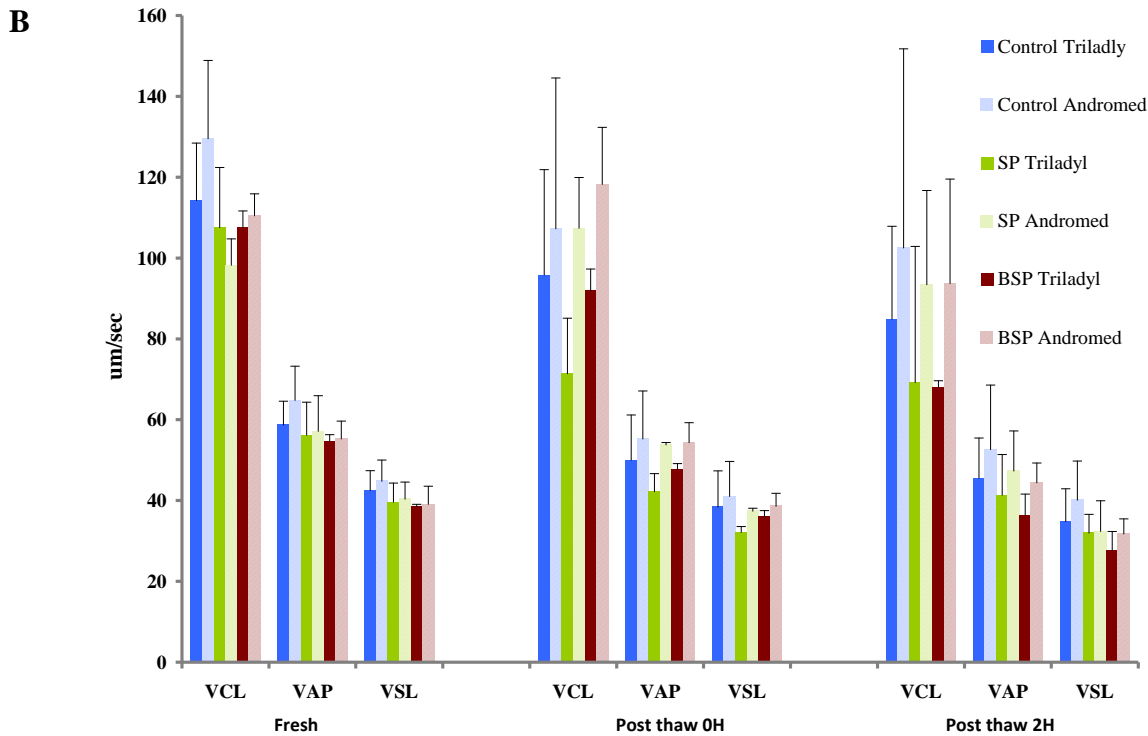
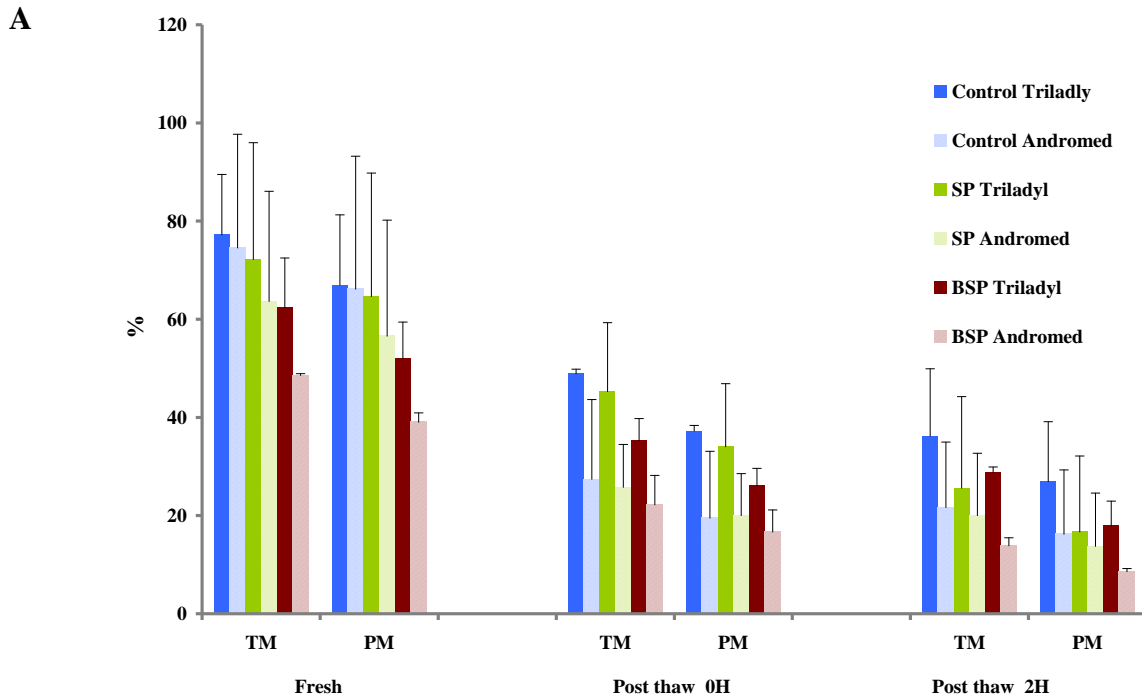
**A)** SDS-PAGE fractions from affinity column  
**B)** Western blot done with anti BSP3 antibodies. Antibody was positive for BSP3 in all fractions at approximately 15 kDa.

**C)** Western blot done with anti BSP5 antibodies. Antibody was positive for BSP5 in all fractions at approximately 30 kDa.



## **The effect of BSP on cryopreserved bison epididymal semen**

Inadequate numbers of testicles were received therefore statistical analysis could not be performed on the results. The semen analysis results from the 2 bison testicles indicate lower sperm post thaw motility for all treatment groups. Andromed<sup>®</sup> groups consistently showed lower sperm motility values than Trilady1<sup>®</sup> groups for both 0 hour and 2 hour post thaw. The BSP treatment groups all showed the lowest post thaw motility. Seminal plasma groups dropped considerably in sperm motility over the 2 hour post thaw waiting period. The velocity results did not suggest any significant trends for any of the groups.



**Figure 2:** Evaluation of epididymal sperm recovered from 2 culled bison bulls. Sperm was treated with 3 treatments then extended in Triladyl<sup>®</sup> or Andromed<sup>®</sup>. Post thaw analysis of semen was done at 0 hour then again 2 hours. A) Total motility and progressive motility B) VSL, VAP and VCL

**Discussion:**

With the use of an affinity column BSP proteins were able to be isolated and intensified from bison seminal plasma (Figure 1) indicating that BSPs are present in significant volumes within bison semen. These isolated BSP proteins were then used to evaluate their effects on bison epididymal sperm during cryopreservation. However, only 2 bison testicles were obtained up to this point in the study, because of the N number no statistical analysis could be completed. No assumptions can be made from the data at this point (Figure 2).

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