STRATEGIES FOR OOCYTE COLLECTION AND *IN VITRO* EMBRYO PRODUCTION IN FREE-ROAMING BISON HERDS

A Thesis Submitted to the College of
Graduate Studies and Postdoctoral Studies
In Partial Fulfillment of the Requirements
For the Degree of Doctor of Philosophy
Department of Veterinary Biomedical Sciences
University of Saskatchewan
Saskatoon, Saskatchewan, Canada

By

MIRANDA LYNN ZWIEFELHOFER

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ABSTRACT

Wood bison (*Bison bison athabascae*) are a threatened subspecies native to North America. Endemic disease in the largest and most genetically diverse wood bison population in Wood Buffalo National Park has resulted in strict monitoring of populations and restricted the transfer of genetics to outside the park. To safeguard the future of wood bison, we are establishing a germplasm biobank through the use of advanced reproductive techniques. Recovery of embryos and semen collected from at-risk populations would reunite bison herds separated by time and space to produce healthy offspring and restore the genetic diversity of wood bison. The recovery of cumulus oocyte complexes (COC) from wild, free-roaming bison herds requires the use of minimal handling COC collection protocols. The following studies (Chapters 2 – 6) investigated novel approaches to improve *in vitro* embryo production (IVP) through a field-friendly approach.

Chapter 2 investigated the effects of *in vitro* maturation, conventional morphologic COC grading and minimal-handling ovarian superstimulation (sustained-release follicle stimulating hormone [FSH]) on nuclear and cytoplasmic characteristics of bison COC and IVP through a 3D computer-assisted quantitative assessment. A greater proportion of active mitochondria was located in the central region of the ooplasm and greater mitochondrial clustering was present in low quality oocytes than high quality oocytes. However, additional analysis is required to characterize mitochondria distributions without quantitative software analysis. The bison oocytes had a high incidence of large lipid droplets similar to extended FSH starvation groups in cattle. Unfortunately, the minimal-handling ovarian superstimulation treatments implemented in the present study did not increase the number of COC collected or improve embryo production rates.

Chapter 3 investigated the effects of ovarian synchronization and a single dose superstimulation protocol using equine chorionic gonadotrophin (eCG) for COC collection and IVP in wood bison. A single dose of 5,000 IU eCG increased the number and size of follicles available for COC collection, more than doubled the number of COC collected for IVP and resulted in the production of more embryos than other groups (approximately 2 embryos per bison). Non-superstimulated COC collections done after follicle wave synchronization resulted in greater embryo production than collections done at random stages of the follicular wave. Repeated COC collections after successive wave synchronization resulted in similar follicular counts and embryo

production rates within individuals. The greatest number of follicles aspirated, COC collected, and embryos produced occured in the anovulatory season.

Chapter 4 investigated the effects of restraint (lateral recumbency after chemical immobilization vs standing position in a hydraulic chute) and follicular wave status (random vs synchronized) on COC collection efficiency in non-superstimulated bison. It examined the effects of superstimulation treatment (single dose of eCG vs multiple doses of FSH plus human chorionic gonadotrophin [hCG]) and method of administration (field dart vs manual injection) on COC collection efficiency and IVP. The COC collections were done as effectively on sedated recumbent bison as those restrained in a standing position in a hydraulic chute. Ovarian superstimulation using the single-dose eCG protocol was as effective as a multiple-dose FSH protocol and field darting was an effective method of administering superstimulation treatments. The use of ovarian superstimulation resulted in greater IVP efficiencies than random, non-stimulated collections (3.1 vs 1.1 embryos per bison).

Chapter 5 investigated the effects of reproductive status (non-pregnant, pregnant, and pre-pubertal), advancing gestation (pregnant bison, 90- vs 120- days) and follicular wave status (random vs synchronized) on ovarian follicles, COC collection and IVP. Oocytes were collected and embryos were produced from bison at both 90- and 120- days of gestation, but the position of the ovaries at the more advanced stage reduced COC collection efficiency. Yearling pre-pubertal bison provided the most promise as COC donors in the field. They had the highest number of follicles available for aspiration and produced a similar number of embryos (approximately 1 freezable embryo per collection) compared to non-pregnant mature bison. Yearling pre-pubertal bison are available for collection throughout the winter season without the limitations imposed by advanced stages of pregnancy or unknown pregnancy status.

Chapter 6 investigated the effect of *in vitro* wood bison embryo characteristics (stage of development, grade, and freeze day) on the post-thaw viability of pre-implantation IVP embryos characterized by microscopic morphology and establishment of pregnancy after embryo transfer. Morphologic grading was positively related to quantitative viability characteristics, with higher quality embryos having less cryo-damage and higher cell counts than low quality embryos. Morulae survived cryopreservation with less damage than blastocysts, and subsequent transfer resulted in a higher pregnancy rate at 30-days and lower pregnancy loss between 30 to 60 days than blastocysts. The vitrification method of cryopreserving embryos resulted in a pregnancy rate

similar to the transfer of fresh embryos. The study produced nine pregnancies at 30 days post-ovulation, of which 6 made it to term and the birth of five calves.

In conclusion, COC collection and IVP methods and procedures investigated throughout this thesis have made COC collection from wild, free-roaming herds a feasible task. Bison stakeholders may now implement these techniques to access inaccessible bison genetics in and around Wood Buffalo National Park.

ACKNOWLEDGMENTS

I want to thank Dr. Gregg Adams for accepting me as a graduate student in his lab. I am grateful for his guidance and critical assessment of my ideas and data to make me a stronger researcher.

I would also like to acknowledge my PhD committee's present and past members. Each of you has impacted my research experience and has pushed me to be the reproductive physiologist I am today. Thank you Dr. Jaswant Singh, for your kindness and always being available and willing to assist with general, laboratory and experimental design questions. A special thank you to Dr. Gabriela Mastromonaco, I appreciate the effort you put into my research and answering questions. Thank you for being a strong female role model in the reproduction world. Thank you to Dr. Murray Woodbury for your effort in initiating the bison research program and for your guidance and advice. I want to thank Dr. Daniel MacPhee for your guidance and support. Thank you to Drs. Todd Shury and Suraj Unniappan for your positive outlooks and advice.

A special thank you to Dr. Barbara Durrant for accepting the role as my external examiner. I appreciate your kind and detailed insight toward reproduction and wildlife.

I would also like to acknowledge the other people around the University that have been helpful and caring during my PhD experience. A special thank you to Cindy Pollard for taking care of Eric and me during our time here in Canada. I appreciate all that you did to make sure our programs went smoothly. Thank you to Krista Larson and Stacy Trainor for always being available to help with the little things. I want to thank Dr. Brent Bobick for his patience, compassion and motivating conversations. A huge thank you to Brad Blackmore for your management of the bison research herd, your friendship and all of the assistance you have provided Eric and me. Thank you to the LFCE staff, including Trent, Gary, Roger and Dan, for the countless hours of caring for and helping us with the bison. I would also like to thank Kris Ringwall for your advice and kind words during your time in Saskatchewan. Finally, I want to thank all of the graduate, summer and visiting students for your camaraderie, engaging discussions and for helping me at the farm and in the laboratory.

Thank you to all of the wonderful Saskatchewan people we have met during our time here that have made us feel at home and allowed us to have a life outside the University. A special thank you to Jen Seaborn for introducing Colby and I to the sport of herding and such friendly

people. With that in mind, I would also like to thank my dog Colby for being the best companion. Thank you to Darald and Vanesa Mischkolz for welcoming us into your home and treating us like family.

I would like to thank my parents, Nancy and Skip Christenson for supporting me during my educational career and nurturing my love of animals and nature. I would also like to thank all my family and friends back home for your distant support and encouragement.

The biggest thank you to my husband Eric for being my strongest supporter. Thank you for being brave enough to start our Canadian journey and believing in me.

I also take this opportunity to acknowledge funding from the Western College of Veterinary Medicine and the Department of Veterinary Biomedical Sciences, the Saskatchewan Innovation & Opportunity Scholarship from the Government of Saskatchewan and the Throlson American Bison Foundation Scholarship from the National Bison Association.

DEDICATION

I dedicate this thesis to my husband Eric. You are my best friend and favorite colleague. Thank you for pushing me to be the best I can be. I can't wait to see what life brings and what challenges we will tackle together next.

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LIST OF ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
AI	Artificial insemination
ATP	Adenosine triphosphate
BNP	Buffalo National Park
Ca	Calcium
cc	Cubic centimeter
CL	Corpus luteum
CO2	Carbon dioxide
COC	Cumulus oocyte complex
COSEWIC	Committee on the Status of Endangered Wildlife in Canada
D-PBS	Dulbecco's phosphate buffered saline
DAPI	4′,6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
eCG	Equine chorionic gonadotropin
EINP	Elk Island National Park
F1	First filial generation
FITC	Fluorescein isothiocyanate
FSH	Follicle stimulating hormone
ga	Gauge
GLM	General linear model
GnRH	Gonadotrophin-releasing hormone
GPS	Global Positioning System
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
h	Hour
hCG	Human chorionic gonadotrophin
ICUN	International Union for Conservation of Nature
ID	Identification
im	Intramuscular
IU	International unit
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* embryo production
LH	luteinizing hormone
kg	Kilogram
MI	Metaphase I

MII	Metaphase II
Mg	Magnesium
mg	Miligram
min	Minute
mL	Mililiter
mm	Milimeter
µg	Microgram
µl	Microliter
µm	Micrometer
N2	Nitrogen
nM	Nanomolar
nm	Nanometer
PBS	Phosphate buffered saline
pH	Potential of hydrogen
PGF	Prostaglandin F2α
PNA	Peanut agglutinin
PVP	Polyvinylpyrrolidone
SARA	Species at Risk Act
SEM	Standard error of mean
SNP	Single-nucleotide polymorphism
TDT	Terminal deoxynucleotidyl transferase
TUNEL	Terminal deoxinucleotidyl transferase mediated dUTP nick end labeling
USA	United States of America
vol	Volume
vs	Versus
v/v	Volume/volume
WBNP	Wood buffalo National Park
wt	Weight
x g	Relative centrifugal force
YNP	Yellowstone National Park

CHAPTER 1:

1. GENERAL INTRODUCTION, HYPOTHESES, AND OBJECTIVES

Subsections 1.1-1.4 are edited excerpts from the co-authored review paper "GF Mastromonaco, ML Zwiefelhofer, GA Wilson, T Shury, GP Adams. The past and future of genetic conservation of North American bison" which is in preparation.

1.1. Brief history of bison populations in North America

The North American bison population is composed of two subspecies or designatable units: plains bison and wood bison (Committee on the status of endangered wildlife in Canada., 2013). The better known of the two, the smaller plains bison, once thrived on the great plains of North America, from central Saskatchewan to northwestern Mexico (List et al., 2007). Meanwhile, the wood bison ranged from central Alberta to Alaska and the Canadian Arctic Archipelago (Gates et al., 2001). Bison have a significant impact on the ecosystems they inhabit: plains bison are a keystone species in the short- and tall-grass prairies, as are wood bison in the boreal regions (Knapp et al., 1999; Gates et al., 2001; Committee on the status of endangered wildlife in Canada., 2013). Bison create a unique, diversified landscape through grazing, dispersing seeds, wallowing (rolling) and fertilizing, which supports the overall health of the entire ecosystem (Griebel et al., 1998; Powell, 2006; Nickell et al., 2018; Geremia et al., 2019). In addition, bison were a major food source of predators such as wolves, grizzly bears, and Indigenous Peoples. The bison were, and remain to be, an essential component of the material and social livelihood of Indigenous Peoples throughout North America.

It is estimated that between the late 1600s and early 1700s, there were approximately 30 million plains bison and 168,000 wood bison in North America (Gates et al., 2001; Montoya, 2001; Cunfer and Waiser, 2016; Environment and Climate Change Canada., 2018). The population decline began in the 1700s and became more precipitous during the 1800s (Roe, 1951; Cunfer & Waiser, 2016). By the turn of the 20th century, plains and wood bison populations were reduced to near-extinction after a series of events that resulted in a contraction to less than 1% of their historic numbers and geographic range (Freese et al., 2007; Sanderson et al., 2008). The decline has been attributed primarily to a shift in the use of bison from a subsistence resource to a lucrative commodity. However, the cause of the near-extinction event of the North American bison may

more accurately be represented as a 'perfect storm' of environmental and social factors that continue to threaten the species today.

The expansion of the railroad in the United States in the mid- to late-1800s resulted in a sudden increase in trade, settlers, domestic livestock and sport hunters (Roe, 1951). To add to this impact, the implementation of new and efficient methods for tanning hides for the leather industry in 1870 allowed American hide hunters to market flint hides (furless hides) all year long. This, along with free trade with Europe, created an unquenchable demand for bison hides (Taylor, 2011). Since the US government did not attempt to restrict the bison trade, hunters were undeniably wasteful, killing hundreds in a single day while taking only the tongue, hide, or nothing (Roe, 1951). At the same time, the introduction of horses to Indigenous Peoples vastly increased the efficiency of harvesting bison and competed for available grazing space (Brower, 2008).

The arrival of domestic cattle brought diseases to which bison were naive. Texas tick fever, a tick-borne protozoal disease, has been implicated in epidemic outbreaks that spread northward, causing 70-90% mortality rates in previously unexposed cattle herds and bison herds (Koucky, 1983). It has been estimated that the reduction of the bison population from four million in 1874 to 25,000 in 1883 could not be solely attributed to hunters, suggesting that disease may have had an important impact on the decline of the bison during the 1800s (Koucky, 1983; Stoneberg Holt, 2018).

Plains bison herds in Canada were virtually eliminated by 1879 (Roe, 1951; Cunfer & Waiser, 2016). With only small numbers of white settlers on the Canadian plains at that time (Dary, 1974), over-hunting by Canadian Metis (individuals with mixed European and Native American ancestry) and First Nations harvesters has been ascribed as a major reason for the bison decline in Canada (Roe, 1951; Dobak, 1996; Brower, 2008; Taylor, 2011; Cunfer & Waiser, 2016). The main bison market in Canada was not for hides but for pemmican – a concentrated mixture of fat and protein that was resistant to spoilage and used as a critical provisional item for the Hudson's Bay Company boatmen (Cunfer & Waiser, 2016).

Although difficult to substantiate, the anthropogenic causes listed above coincided with periods of drought and harsh winters on the plains, which added to bison mortalities from disease, starvation and exposure (Isenberg, 2001; Kolipinski et al., 2014). By 1888, plains bison were reduced to 85 animals in the wild in the United States and extirpated from the wild in Canada (Roe,

1951; Brower, 2008; Hedrick, 2009). The decline of the bison led quickly to widespread starvation and death of Indigenous Peoples (Roe, 1951).

Unlike the plains bison, specific documentation of the historic decline of the wood bison population is sparse, perhaps because of its more remote habitat and smaller starting numbers. The near-extinction of wood bison coincided with the plains bison decline and has been attributed to much the same factors. Exploitation for trade, changes in habitat, and severe winters reduced their historic numbers of over 160,000 to less than 250 by the turn of the 20th century, surviving in scattered herds around present-day Wood Buffalo National Park, at the border of Alberta and the Northwest Territories (Larter et al., 2000; Gates et al., 2001; Environment and Climate Change Canada., 2018).

1.2. Species conservation status

Both plains and wood bison remain classified as a *near threatened species* according to the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Aune et al., 2017). Importantly, the IUCN states that bison are a conservation-dependent species, such that without conservation efforts, bison as a distinct species would cease to exist. Wood bison are currently designated as threatened nationally in Canada under Schedule 1 of the Species At Risk Act, and plains bison are currently not designated nationally (Committee on the status of endangered wildlife in Canada., 2013). Both Canadian and American governments have designated national parks specifically to preserve bison and their natural habitat. Privately funded conservation herds can also be found on reserves such as the American Prairie Reserve in Montana and the Tallgrass Prairie Reserve in Oklahoma.

With a total world population today of about 500,000, the North American bison appears to be making a comeback, but appearances are deceiving. The growing number of bison in livestock production herds has had a disguising effect on the conservation issue. The recent expansion of commercial bison farming accounts for 95% of the bison population in North America today; however, most are excluded from the IUCN's and COSEWIC's (Committee on the status of endangered wildlife in Canada) accounts of conservation herds. In simplified terminology, both organizations define wild herds as those that are of sufficient size on a sufficiently large landscape, free-ranging and not impacted by artificial selection processes

(Committee on the status of endangered wildlife in Canada., 2013; Aune et al., 2017). As a result, the wild bison population (plains and wood) comprises only approximately 19,000 animals in 20 free-ranging herds (Aune et al., 2017). While the number of commercial (farmed) bison has increased dramatically since 1970, the number of bison in conservation herds has remained stagnant since the 1930s (Freese et al., 2007).

1.3. Current threats affecting North American bison

1.3.1. Disease

Brucellosis was discovered as early as 1917 in YNP bison, and the prevalence over the past 2 decades has remained at 60% (Thompson Hobbs et al., 2015). In Canada, brucellosis and tuberculosis are present in some bison herds in and around WBNP. From 1912 to 1925 the first Canadian appearance and subsequent prevalence of bovine tuberculosis in the BNP herd at Wainwright was documented (Deakin et al., 1943; Fuller, 2002; Brower, 2008). The introduction of infected plains bison into the wild in the 1920s resulted in endemic infection of the wood bison in WBNP that persists to this day. It is unknown how brucellosis was introduced to WBNP, as there is no record of brucellosis being present in BNP previously. Wood bison herds within WBNP have a prevalence of infection of 30-60% for both brucellosis and tuberculosis, depending on age class and tests used, but irrespective of population density (Mccormack, 1992; Tessaro et al., 1993; Mitchell and Cormack Gates, 2002; Joly and Messier, 2004). Authors of one study estimated that the 1971–1999 population decline would have occurred in the absence of disease (Bradley and Wilmshurst, 2005), while others concluded that brucellosis and limited growth of the metapopulation as a result of increased mortality, reduced fecundity, and increased vulnerability to predation in affected animals (Joly and Messier, 2005).

The impact of these diseases on bison conservation is further complicated by the risk of disease transmission to neighboring healthy free-ranging bison herds and cattle ranches (Mitchell and Cormack Gates, 2002; Shury et al., 2015). YNP has developed an Interagency Bison Management Plan to: 1) maintain a viable population of approximately 3,000 bison within the Park, 2) ensure brucellosis transmission from bison to cattle does not occur, and 3) reduce the overall prevalence of brucellosis in bison (White et al., 2015). To address these goals a variety of

tools have been implemented to manage numbers including: tolerance areas outside the park, hazing bison away from non-tolerance areas outside the park, harvesting (public and treaty), capturing, and testing and culling *Brucella* positive bison (White et al., 2015). These methods have successfully prevented transmission outside the park, but have not been effective in reducing the prevalence of the disease in the park. In 1987, the Northwest Territories (Gates et al., 2001) and Alberta instituted similar bison control zones to restrict the movement of free-roaming bison. While effectively preventing the spread of disease, killing bison that enter these "buffer zones" has also prevented the establishment of free-ranging herds throughout the original wood bison range (Hartop et al., 2009), and gene flow among herds separated by control zones. Of the free-ranging wood bison today, approximately half reside within populations affected by bovine tuberculosis and brucellosis (Environment and Climate Change Canada., 2018).

The first confirmed incident of anthrax *(Bacillus anthracis)* in a Canadian bison herd was in July – August, 1962 when 281 bison were found dead just outside the northeast border of WBNP in a region called Hook Lake (Dragon & Elkin, 2001). Efforts to quarantine the area and properly dispose of the carcasses by burial were ineffective, and in 1963 and 1964, anthrax spread to neighboring herds in WBNP. With their first case in 1991, the Mackenzie herd has been affected by anthrax on a large scale (Dragon & Elkin, 2001) with more than half of this largest disease-free herd lost to anthrax during 2012 and 2013 (Committee on the status of endangered wildlife in Canada., 2013). Meanwhile, there have been anthrax outbreaks in Prince Albert National Park (Saskatchewan, Canada) and domestic plains bison herds located in the Greater Yellowstone Area (USA).

1.3.2. Domestic cattle introgression

A devastating side-effect of the initial bison conservation effort was the intentional hybridization of bison with domestic cattle. There is no evidence that natural crosses between domestic cattle and bison could occur (Halbert & Derr, 2006; Hedrick, 2009). However, from 1907 to 1940, several ranchers in the United States and Canada attempted to create cattle x bison hybrids, known as cattalo or beefalo, in a concerted effort to introduce bison traits, such as meat quality and quantity, hardiness, feed efficiency, and disease resistance, into domestic cattle (Deakin et al., 1935, 1943; Derr et al., 2012; Hedrick, 2009). Initial hybridization efforts involved bison bull x

domestic cow because of the relative receptivity of domestic cow to mating with a bison bull and the ease of handling domestic cows during and after birthing. However, live births from bison bull x domestic cow crosses were rare as a result of late-term abortions, stillbirths and mortality of the dam at parturition from complications of hydramnios (Hanna, 1989); of 42 pregnancies that were achieved at BNP, only 6 F1 hybrid calves were born alive (Hedrick, 2009). Live births from the opposite cross (domestic bull x bison cow) were more successful; over a 5-year period, 35 male and 49 female hybrid calves were born alive, and only 4 dead at birth and 1 abortion (Deakin et al., 1943). At the end of 5 years, BNP had produced 156 hybrids ranging from F1 to 31/32 domestic cattle. It is noteworthy that of the male offspring, <15/16 domestic were not fertile. Cross-breeding of domestic cattle and bison continues today and is the basis of the product of the American Beefalo Association (American Beefalo Association, 2021).

Today, the introgression of domestic cattle genes in plains bison herds remains a complicated issue for on-going conservation efforts. In a genetic microsatellite study of the 11 federal conservation herds in the USA, only 3 herds had no documented evidence of cattle introgression (YNP in Wyoming, Henry Mountains in Utah and Wind Cave National Park in South Dakota; Ranglack et al., 2015). However, more recent genetic testing with single-nucleotide polymorphism (SNP) chips revealed that approximately 70% of bison derived from Wind Cave National Park were positive for cattle introgression (American Prairie Reserve, personal communication, November 1, 2017). Only one private herd, owned by Turner Enterprises in New Mexico, has been shown to be free of cattle introgression (Freese et al., 2007). More evidence of hybridization will likely be found as genetic testing becomes more accurate.

The physiologic effects of cattle x bison hybridization have not yet been critically examined, but bison with cattle mitochondrial DNA have the phenotypic effect of being consistently smaller and less fertile (Derr et al., 2012). Some have suggested that the mating between bison and domestic cattle represents one of the greatest threats to the survival of the wild species, and therefore, bison with known or possible cattle ancestry must not be introduced to bison free of cattle ancestry (Hedrick, 2009). To-date, there are no published reports of domestic cattle introgression in Canada's conservation herds of plains and wood bison.

1.3.3. Plains x wood bison hybridization

After the introduction of plains bison into WBNP in the 1920s, there was a fear that the non-hybrid wood bison were disappearing (Lothian, 1981). Based on comparison of microsatellite genotypes (Wilson and Strobeck, 1999; Cronin et al., 2013; Ball and Wilson, 2016) and mitochondrial DNA (Forgacs et al., 2016), it has been suggested that "genetically pure" wood bison may no longer exist, and that all herds fall into a spectrum of genetic admixture. However, both phenotypic and genotypic data document distinct clustering of populations as plains and wood bison. The sharpest phenotypic and genotypic distinctions were evident in comparisons between known plains bison (i.e., EINP and US conservation herds) and wood bison herds (i.e., greater WBNP area; Van Zyll De Jong et al., 1995; Ball and Wilson, 2016). Phenotypic characteristics of plains vs wood bison have been identified based on differences in pelage, hump style, and body conformation (Van Zyll De Jong et al., 1995; W. Olson, personal communication, 2019), which have been used to document retention of taxonomic traits despite prolonged displacement from their historic range (e.g., 16 generations of wood bison in central Alberta, 36 generations of plains bison in Alaska; W. Olson, personal communication, 2019).

Until recently, no genetic markers of purity have been identified, but genomic tools are changing rapidly and hold promise for distinguishing between subspecies and rapid identification of cattle introgression. In 2020, a single-nucleotide polymorphism test was created to identify parentage and subspecies composition in bison based on the genomic sequencing of 13 wood and 26 plains bison (Xu et al., 2018). An important step towards addressing genetic diversity in cattle and bison is the need to link phenotypic characteristics with genotype (D. MacPhee & G. Adams, personal communication, June 27, 2015). Recent quantitative scoring of phenotypic traits of bison in the Ronald Lake herd indicated a strong wood bison type with less variability than that of other conservation herds (W. Olson, personal communication, 2019), consistent with a distinctive genotype based on microsatellite analysis (Ball and Wilson, 2016). Genetic clustering of populations within WBNP was not apparent using the microsatellite approach, but clustering may become apparent by direct correlation with phenotypic traits or other genetic techniques.

1.3.4. Geographic isolation and small effective population size

Currently, bison occupy less than 1% of their original range in North America (Freese et al., 2007; Sanderson et al., 2008; Gates et al., 2010). Population growth is impeded by fragmented or unsuitable habitat that is often managed to exclude bison. For long-term viability, a population of ≥1,000 animals is needed to retain 90% of their allelic diversity for 200 years (Gross & Wang, 2005; Freese et al., 2007; Hedrick, 2009). Of the 21 plains bison herds managed by the U.S Department of Interior, only 3 herds exceed 1,000 bison (National Park Service, 2016). The largest of these is YNP, which maintains a population between 2,400 and 5,000 with a mean of ~3,900 (White et al., 2015). Unfortunately, bovine brucellosis is also present in the YNP herd, limiting the ability to transfer genetic material out. The total population size of Canada's plains bison is around 2,500 (Committee on the status of endangered wildlife in Canada., 2013), but the population is distributed among 6 conservation herds, all of which were derived from the Pablo-Allard herd shipped to EINP over 100 years ago. These herds have remained isolated from each other, without new genetic input since their inception, and only one herd has a population size >500. Also of concern is the Sturgeon River plains bison population in Saskatchewan, which has decreased from ~ 500 to less than 150 in the last 15 years (Cherry et al., 2019).

All of the world's wild wood bison reside in Canada except for two herds. Wood bison from EINP were translocated to Lenski Stolby Nature Park near Yakutsk, Sahka Republic, Russia in 2006, 2011, 2013 and 2020 (n=30 animals each; Parks Canada, 2020). Others were sent from EINP and eventually released into the wild in Alaska in 2015 (n=130; Environment and Climate Change Canada., 2018; MacFarland and Seaton, 2018). The total population of wood bison consists of approximately 9,000 animals distributed among geographically distinct herds. Six of the 12 wood bison conservation herds were derived from a single founder population of 11 bison that originated from EINP. Only two herds are >1,000 animals, one of which is WBNP where brucellosis and tuberculosis are prevalent. This situation is not consistent with the stated long-term population and distribution objectives of the Recovery Strategy for the Wood Bison in Canada (2018) *to ensure the existence of at least five disease-free, genetically diverse, connected, self-sustaining, free-ranging populations distributed throughout their original Canadian range, with a minimum of 1,000 animals per herd*.

1.3.5. Loss of genetic diversity

Both plains and wood bison have undergone a significant loss of genetic diversity due to founder effects, population bottlenecks, genetic drift, and selection (Wilson and Strobeck, 1999; Hartway et al., 2020). The effects of lost genetic diversity on bison populations can be illustrated with real-world examples. The Texas State Bison Herd was composed of 40 plains bison that were direct descendants of 5 animals from the Charles Goodnight herd in the 1880s. Without subsequent introduction of new genetics, the herd had an inbreeding equivalent to that of 2 generations of full sibling matings (Halbert et al., 2005). During 6 years of observation, the herd had a mortality rate 12.5 times higher and natality rate 67% lower than other herds, resulting in a stagnant population size of 31-40 animals (Hedrick, 2009). Analyses showed a 99% chance of population extinction within the following 41 to 51 years if no new genetics were added, but a 100% survival for 100 years if new bison genes were introduced into the herd (Halbert et al., 2005). A recent population viability analysis of plains bison herds in North America has shown that loss of genetic diversity is a concern for most herds, and gene flow is a critical tool to reduce future loss of diversity (Hartway et al., 2020).

Loss of genetic diversity due to the lack of gene flow among populations is also of concern for wood bison. The free-ranging Ronald Lake and Harper Creek/Wabasca wood bison herds in the Greater WBNP region are currently free of bovine brucellosis and tuberculosis, and genetically distinct from the diseased herds in WBNP, showing there is little to no gene flow between them (Ball and Wilson, 2016). The small sizes of these two populations (12 for Harper Creek/Wabasca and 186 for Ronald Lake) suggests genetic diversity will continue to be rapidly lost through genetic drift.

1.4. Bison genome biobank

Genome resource banking includes the collection, management and storage of germplasm (sperm, oocytes, embryos, ovarian and testicular tissue) and other biological samples for the purpose of conservation (Holt and Comizzoli, 2022). The creation and deployment of a bison genome biobank could potentially eliminate live animal translocations between populations, decrease space requirements for breeding herds, increase reproductive efficiency, and support the

effective dissemination of valuable genetics (Wildt, 2000; Thundathil et al., 2007). On-going threats to the long-term sustainability of genetic diversity of bison in North America warrant the integration of *in situ* (i.e., free-ranging herds) and *ex situ* (i.e., captive herds and cryo-stored germplasm) conservation efforts. The use of bio-banked samples for reproductive technologies will readily permit gene flow between *ex situ* and *in situ* bison herds (Comizzoli et al., 2000). Long-term preservation of unique alleles and valuable genetic material may prevent future inbreeding and unintentional selection that can occur in small isolated populations (Holt and Comizzoli, 2022). Most importantly for the bison species, it may enable the production of disease-free bison calves from diseased herds (Thundathil et al., 2007). It is imperative to construct functioning biobanks before endangered and extinction status to eliminate last-ditch efforts of species conservation (Wildt et al., 2010).

1.5. Female bison reproductive physiology

Extreme care must be taken while handling bison as they are wild animals that can be dangerous and cause injury to themselves and personnel (Dorn, 1995). The methods of how research is conducted on bison vary whether the animal is free-roaming or in a captive herd. In the field, non-invasive methods of data collection are commonly done on bison, which include visual or GPS/radio-collar movement observations (Jung and Larter, 2017; McMillan et al., 2021) and the collection of fecal, urine and hair for hormone analysis, which requires no direct contact (Kirkpatrick et al., 1993; Shave et al., 2019). If necessary, bison can be captured by net-gun or sedated in field settings (Caulkett, 2014; Slater, 2020). Bison can also be acclimated to be restrained in manual or hydraulic chute systems in captive settings (Othen et al., 1999; McCorkell et al., 2013).

The first step before applying reproductive technologies and the creation of a germplasm biobank is understanding the basic natural reproductive pattern of the species (Wildt et al., 2010). As bison are bovids, the female reproductive anatomical structures of bison are similar to that in cattle. Dorn (1995) described the reproductive tract of bison to be similar in size to primiparous beef cows with small ovaries and reproductive tracts. However, no comprehensive scientific literature is available specifically on the female bison reproductive anatomy, but dissections of

bison reproductive tracts in our laboratory confirm these similarities (Personal communication, Adams).

Bison females between the ages of 3 and 13 produce calves every year or every other year (reviewed in Huntington, 2018). They can live to around 20-years old in the wild and over 40-years old in captivity (Committee on the status of endangered wildlife in Canada., 2013). Unlike domestic cattle, bison are seasonally polyestrus with a distinct ovulatory (breeding) and anovulatory (non-breeding) season (Goodrowe et al., 2007). They are short-day breeders with strong photoperiodic responses (Mastromonaco and Gonzalez-Grajales, 2020). Therefore, there is variation in data across specific latitudes regarding the initiation of the ovulatory season. Bison in the northern hemisphere exhibit peak calving in May, while bison in the southern hemisphere peak in November (Mastromonaco and Gonzalez-Grajales, 2020). The bison in Yellowstone National Park (~44° N latitude; Gogan et al., 2005b) have an onset of parturition in April while bison in Wood Buffalo National Park (~59° N latitude) historically have not started parturition until mid-May (Soper, 1941). The termination of the ovulatory season and the start of the non-ovulatory season was thought to be December or January (reviewed by; Goodrowe et al., 2007). Recent transrectal ultrasonography evaluation of mature wood bison at the University of Saskatchewan determined that a corpus luteum (CL) was still present in 31/33 (94%), 32/35 (91%), 13/33 (39%), and 1/35 (3%) bison on December 19th, January 31st, March 4th, and March 29th, respectively (bison with CL / number of bison scanned; unpublished, Zwiefelhofer). Most females become sexually mature at 2 years old (calve as a 3-year old) but reports of 2-year olds calving exist (reviewed in Huntington, 2018). Evaluation of 2-year-old wood bison by transrectal ultrasonography revealed that 44% of them had a CL during the ovulatory season (n=9; unpublished, Zwiefelhofer).

The ovulatory season in female bison is characterized by follicular growth in a wave-like pattern, ovulation and subsequent CL formation similar to domestic non-seasonal cattle (Adams et al., 2020). Each follicular wave starts with synchronous development of small follicles that coincides with increased concentrations of follicle stimulating hormone (FSH; McCorkell *et al.*, 2013). Consistent with cattle, a dominant follicle is selected and continues to grow while FSH concentrations decrease and the remainder of the follicles in the cohort regress and go through atresia (Adams et al., 2020). In the presence of progesterone (produced by the CL), the dominant follicle regresses, and a second follicular wave emerges. Without the presence of progesterone, the

dominant follicle (approximately 14 mm in bison) will ovulate and subsequently release a cumulus oocyte complex (Adams et al., 2020).

The length of an estrous cycle for bison has been reported by transrectal ultrasonography (mean = 20.0 days; Adams et al., 2020), behavioral estrus (mean = 20.8 days; Matsuda et al., 1996), urine (mean = 21.5 days) and fecal (mean = 20.8) progestin monitoring (Kirkpatrick et al., 1991; Matsuda et al., 1996). However, the results across studies range from 16 to 31 days (reviewed by; Goodrowe et al., 2007). Female bison exhibit behavioral estrous during each estrous cycle, including standing for mounting, homosexual mounting, flehmen response and/or tail flagging (Wolff, 1998; Othen et al., 1999; Vervaecke and Schwarzenberger, 2006). The first cycle of the ovulatory season (first ovulation of the year) results in a short lifespan CL (Vervaecke and Schwarzenberger, 2006) that lasts on average 8 days (Adams et al., 2020) and observable lower progesterone levels and no estrus behavior (Vervaecke and Schwarzenberger, 2006). The anovulatory season is characterized by follicular growth in a wave-like pattern with no ovulation (McCorkell et al., 2013). Evaluation of ovarian structures by transrectal ultrasonography determined an inter-wave interval of 7 days during the anovulatory season in 2-year old wood bison (McCorkell et al., 2013). Female bison will not have a CL during the anovulatory season, except those still pregnant from the ovulatory season.

Pregnancy in bison can be diagnosed through rectal palpation, transrectal ultrasonography or serum antigen detection (pregnancy-specific protein B) in locations where bison can be restrained (Haigh et al., 1991; Dorn, 1995; Love et al., 2017). Hormone profile monitoring of urinary and fecal steroids (i.e., pregnanediol glucuronide, estrone conjugates or total estrogens) are less invasive options for bison (Kirkpatrick et al., 1992, 1993; Goodrowe et al., 2007). Historically the gestation length of bison has been variably reported from 262 to 293 days with an average of 275 days (year 1974-2006; reviewed in Huntington, 2018). However, gestation lengths in bison are commonly based on presumed conception dates which can be observed copulations or from secondary signs associated with copulation (i.e., the presence of a tending bull or the female keeping her tail in a horizontal position) which can be unreliable as copulation is not synonymous with conception (Vervaecke and Schwarzenberger, 2006; Huntington, 2018). More recent reviews estimate the gestation length for dietary supplemented bison to average 265 days with no reports of ≥276 days (Gogan et al., 2005a; Vervaecke and Schwarzenberger, 2006). These reports are consistent with the gestation length of wood bison calves derived by artificial insemination or by

embryo transfer where the mean gestation length was 265 days (n=11; Goodrowe et al., 2007), 266.5 days (n=2; Toosi et al., 2013b), and 266 (n=6; Adams et al., 2020). The birth of twins is rare, but triplets have not been reported (reviewed in Huntington, 2018).

1.6. Reproductive technologies in bison

1.6.1. Manipulation of ovarian function

Assisted reproductive technologies, including gamete recovery, artificial insemination, and embryo transfer, are tools for creating and deploying a genome biobank (Wildt, 2000). After understanding the natural reproductive pattern of a species, learning to control the follicular cycle is crucial to applying reproductive techniques in any species (Mastromonaco and Gonzalez-Grajales, 2020). As there are physiological differences between domestic and wild species, cattle are used as an elementary blueprint for applying reproductive techniques in bison (Wildt et al., 2010). Manipulation of ovarian function is beneficial for the timing of AI, embryo and oocyte collection, and embryo transfer. In bison, a new follicular wave can be induced 1 day after follicular ablation of all ≥5 mm follicles in the ovary or 3 days after treatment with 17β-estradiol + progesterone (Palomino et al., 2014a). Progestogens, in the form of ear implants or intravaginal devices, have been used during synchronization to limit the growth of follicles due to their inverse relationship with pulsatile LH release and prevent ovulation (Robison et al., 1998; Toosi et al., 2013b). Prostaglandins are then used to initiate luteolysis to control the timing of estrus and subsequent ovulation (Benham et al., 2021). Ovulation has been induced in bison after treatment with luteinizing hormone (LH), human chorionic gonadotrophin (hCG) and gonadotrophin-releasing hormone (GnRH; Dorn et al., 1990; Palomino et al., 2015, 2016).

1.6.2. Artificial insemination

Artificial insemination (AI) is the earliest advanced reproductive technique applied to domestic and wild species as it is relatively simple, non-invasive and the least expensive (Durrant, 2009). AI is common and has been implemented in most domestic animal species (Durrant, 2009). It has also been used as a breeding management tool for many wild species ranging from the giant panda (*Ailuropoda melanoleuca*) to the bottlenose dolphin (*Tursiops truncates*; Wildt et al., 2010).

Cryopreservation of sperm makes it even more important for conservation, as genetics can be stored for a later date and/or location, allowing genetics to be disseminated without the male needing to be present and even many years after the male's death (Wildt and Wemmer, 1999). The incorporation of AI into the critically endangered black-footed ferret's (*Mustela nigripes*) breeding program has resulted in one of the most successful examples of a deployed genome biobank in a wild species (Wildt et al., 2010). The inclusion of AI using frozen-thawed sperm into the ferret's captive breeding programs increased the population size from seven (three males, four females) to more than 8,000 born in captivity and resulted in the reintroduction of over 4,000 animals back into the wild (Howard et al., 2016). The first bison calf born from AI was born in 1994 (Dorn, 1995). Semen has successfully been collected from post-mortem specimens (epididymal sperm) and electroejaculation, cryopreserved and used for AI in bison (Lessard et al., 2009; Adams et al., 2010; Hussain et al., 2011, 2013; Toosi et al., 2013a; Garcia Vilela et al., 2017; Yang, 2018). The incorporation of AI into the wood bison breeding program at the Toronto Zoo has resulted in calves born from AI with 35-year old frozen sperm, the longest stored sperm to successfully produce offspring in a zoo species (Toronto Zoo, 2015). The first pregnancy after AI with sex-sorted wood bison sperm was recently reported to increase the proportion of females in conservation herds (Zwiefelhofer et al., 2021). Although AI effectively disseminates male genetics, the females' genetics are stationary, and only a single calf can be born per female in a year. Therefore, additional technologies are required to disseminate the female genome and increase the generation interval in bison.

1.6.3. Embryo technology

The use of embryos preserves both the male and female genome, making it genetically superior to sperm preservation and AI which only passes on the male genome (Comizzoli et al., 2000). The commercial cattle industry uses embryos to increase the lifetime reproductive output of genetically valuable donors while using genetically inferior animals as recipients (Pukazhenthi and Wildt, 2004). More than 350,000 *in vivo* and 1,100,000 *in vitro* bovine embryos were produced worldwide in 2020 (Viana, 2021). Although there is enormous potential for the inclusion of embryos into breeding management programs for exotic species recovery, few have been successful (Wildt et al., 2010). A handful of exotic species, primarily ungulates, including the

eland antelope (*Tragelaphus oryx*), had *in vivo* embryos recovered that successfully produced offspring (Dresser, 1988). However, the technology has not reached its full potential as efficiency is low in most species (Comizzoli et al., 2000). A wide range of reproductive techniques have been incorporated into the rescue of the European mouflon (*Ovis orientalis musimon*), another endangered ungulate (Ptak et al., 2002). Four healthy offspring were born after the interspecies surgical transfer of *in vitro* produced mouflon embryos into domestic sheep (Ptak et al., 2002). The group has also established a biobank for sperm, embryos and somatic cells for the future (Ptak et al., 2002). Protocols have been developed to recover *in vivo* derived embryos and collect cumulus oocyte complexes (COC) to produce *in vitro* embryos in bison.

1.6.3.1. In vivo embryo collection

In vivo embryo collection requires ovarian synchronization, superstimulation, induction of ovulation and AI. The first report of superovulation and subsequent non-surgical embryo collection in bison occurred in 1990 using a multi-dose, FSH-based bovine embryo collection protocol (Dorn et al., 1990). In 1995, Dorn reported that Rafter D Genetics had attempted more than 75 superstimulations in bison using a variety of treatment protocols with limited success. As a result of handling, stress was recognized as a limiting factor (Dorn, 1995). Therefore, superstimulation with equine chorionic gonadotrophin (eCG) was suggested to reduce stress by reducing handlings (i.e., single dose; Dorn, 1995), as eCG has a longer half-life (40 hours; Bó and Mapletoft, 2014). Attempts to utilize eCG for embryo collection in bison were done with poor results (Robison et al., 1998; Othen et al., 1999). Since then, multiple administrations of FSH injections have been primary used in bison for embryo collection (Palomino et al., 2017a; b, 2020). The number of transferrable embryos recovered in bison is lower than reported in cattle (Bó and Mapletoft, 2014). In bison, the ovulatory season produced between 0.7 and 2.3 transferable embryos, while the anovulatory season produced between 0.1 and 1.3 (Palomino et al., 2017b; a, 2020). The overall low success rate and lack of feasibility in the field have made *in vivo* embryo collection less viable for bison conservation.

1.6.3.2. In vitro embryo production

Overall *in vitro* embryo production in bison is similar to that in cattle, with no differences in media or the general protocol (Barfield, 2019). Embryo production from post-mortem material is beneficial to salvage genetics after the death of animals (Thundathil et al., 2007). Bison *in vitro* embryo production was first documented by Thundathil et al., (2007) after aspiration of COC from bison ovaries collected post-mortem and has since been replicated by Aurini et al., (2009), Barfield and Seidel (2011), Krishnakumar et al., (2015) and Benham et al., (2021). The collection of COC from live bison allows the donor to remain part of either the wild or captive population. Transvaginal ultrasound-guided COC collection in live bison was first documented by Palomino et al., (2013). This study reported that ovarian superstimulation with 2 doses of 200 mg FSH im resulted in a greater superstimulatory response and the recovery of more COC than a single dose of 2500 IU eCG im (Palomino et al., 2013). Varying protocols utilizing multiple doses of FSH for ovarian superstimulation have since been investigated to increase COC collection and subsequent embryo production (Cervantes et al., 2016, 2017b; a; Palomino et al., 2020). Collection of COC from live bison without ovarian superstimulation has not been documented in bison.

Oocytes have been collected throughout the year with *in vitro* embryos produced in the anovulatory and ovulatory seasons (Krishnakumar et al., 2015; Palomino et al., 2020). Cervantes et al., (2017b) reported no difference in embryo developmental rates between seasons, but the number of COC recovered was greater in the anovulatory season resulting in almost twice as many embryos (Cervantes et al., 2017b). Meanwhile, Krishnakumar et al., (2015) reported greater embryo production rates in the ovulatory (breeding) season. *In vitro* embryo production can be completed using immature or mature COC. Studies utilizing post-mortem samples matured COC *in vitro* and reported 5 to 16% blastocyst rates (Thundathil et al., 2007; Aurini et al., 2009; Barfield and Seidel, 2011; Krishnakumar et al., 2015; Benham et al., 2021). Cervantes et al., (2017a) reported an overall Day 8 blastocyst rate of 28%, while morphologically selected compact good COC resulted in 54% after *in vitro* maturation. Human chorionic gonadotrophin has been utilized to induce *in vivo* maturation in bison (Cervantes et al., 2016, 2017b; Palomino et al., 2020). Oocyte nuclear maturation occurred earlier in *in vitro* matured oocytes than in *in vivo* matured oocytes (24 vs 30 hours; Cervantes et al., 2016). Cervantes et al., (2016) also observed that an extended *in vivo* maturation time of 34 hours (30 *in vivo* + 4 *in vivo*) or an additional 4 hours of *in vitro* maturation

(30 *in vivo* + 4 *in vitro*) had greater blastocyst rates on Day 8 than 30 hours of *in vivo* maturation (Cervantes et al., 2017b). Barfield and Seidel (2011) reported that the addition of 5% fetal calf serum to culture after the 8-cell stage improved blastocyst rates. To date, the most efficient in vitro embryo production protocol requires 5 bison handlings (1 - follicular ablation, 2 – FSH, 3 – FSH, 4 – hCG, and 5 – COC collection; Cervantes et al., 2017a; Palomino et al., 2020) which may be feasible in a captive herd but not in wild bison in free-roaming herds.

1.6.3.3. Cryopreservation and washing of embryos

The long-term storage of embryos through cryopreservation is essential for the creation and implementation of biobanking by replacing the need to translocate stress susceptible wild animals, which can be dangerous and a biosecurity issue with the movement of germplasm (Wildt, 2000). Although cattle embryos are commonly frozen using conventional freezing (Ferré et al., 2020), alternative species have not had the same success with the technique (Pukazhenthi and Wildt, 2004). Vitrification is an alternative method to freeze embryos, which uses increased concentrations of cryoprotectants and rapid cooling rates (liquid nitrogen submersion), forming a glass-like structure in the cells (Bondioli, 2014). However, the vitrification process is complex and requires a trained professional and therefore has not been adapted for use by bovine practitioners. Vitrification of embryos has been reported in bison (Toosi et al., 2013b; Benham et al., 2021) as bison embryos have been reported to be darker and speculated to have higher lipid content making them more difficult to freeze (Thundathil et al., 2007; Barfield, 2019). Cattle studies show *in vitro* embryos of lower quality result in lower survival rates after transfer than *in vivo* embryos (Thompson, 1997).

Embryo washing techniques are recommended before cryopreservation or fresh transfer to ensure no pathogens from donor to recipient (Viana et al., 2020). A 10-step washing procedure was recommended according to the IETS guidelines (Stringfellow and Givens, 2010). Treatment with trypsin (Benham et al., 2021) and antibiotics (Palomino, 2015; Cervantes, 2016) has been used in bison to eliminate viral and bacterial loads. The 10-step washing procedure with 100 IU/mL penicillin and 100 µg/mL streptomycin was successfully eliminated *Brucella* bacteria after *Brucella* exposure in *in vivo* and *in vitro* bison embryos (Palomino, 2015; Cervantes, 2016).

1.6.3.4. Embryo transfer

The first documented embryo transfer occurred after 8 fresh *in vivo* embryos were transferred into 8 bison non-surgically resulting in 3 pregnancies and 1 healthy calf (Dorn, 1995). Fresh (Toosi et al., 2013b) and frozen (Adams et al., 2020) *in vivo* recovered embryos have since been transferred and resulted in the birth of healthy calves. Three bison calves have resulted after the transfer of fresh *in vitro* embryos, and only 2 calves have been born from frozen *in vitro* embryos (Adams et al., 2020). One wood bison calf was born after an in vitro embryo, produced from in vivo matured COC collected by transvaginal ultrasound-guided COC collection, was transferred. The embryo was frozen at the University of Saskatchewan and shipped to the Toronto Zoo, where it was transferred (Adams et al., 2020). The plains bison calf was produced from an *in vitro* embryo derived from postmortem reproductive material (Benham et al., 2021).

1.7. General hypothesis and objectives

The **overall hypothesis** of this thesis is that COC collection and *in vitro* embryo production protocols can be adapted for use in free-roaming bison herds.

The **overall objective** of this thesis was to develop feasible and efficient COC collection and *in vitro* embryo production protocols for use in free-roaming bison herds.

Specific objectives:

1. To determine the effects of *in vitro* maturation, conventional morphologic COC grading and ovarian superstimulation on nuclear and cytoplasmic characteristics of the bison oocyte
2. To determine the effect of follicular wave status (random vs synchronized) on COC collection and *in vitro* embryo production
3. To determine the effect of minimal-handling ovarian superstimulation protocols on COC collection and *in vitro* embryo production
4. To determine the effect of restraint (lateral recumbency after chemical immobilization vs standing position in a hydraulic chute) on COC collection efficiency
5. To determine the effect of the method of administration (field dart vs manual injection) of superstimulation drugs on COC collection and *in vitro* embryo production
6. To determine the effect of reproductive status (non-pregnant, pregnant, and pre-pubertal) on COC collection and *in vitro* embryo production
7. To determine the effects of advancing gestation (90- vs 120- days) on COC collection and *in vitro* embryo production
8. To determine the effect of embryo factors on post-thaw survivability characteristics for pre-implantation in vitro embryos using microscopic morphology and pregnancy rates following the transfer of embryos
9. To determine the effect of cryopreservation method on pregnancy and calving rates following the transfer of embryos

CHAPTER 2:

2. NUCLEAR AND CYTOPLASMIC CHARACTERISTICS OF WOOD BISON (*BISON BISON ATHABASACE*) OOCYTES DURING IN VITRO MATURATION

Miranda L Zwiefelhofer[1], Jaswant Singh[1], Jessie Hellquist[1], Dinesh Dadarwal[2], Fernando Rivera-Acuna[1], Gregg P Adams[1]

[1]Department of Veterinary Biomedical Sciences and [2]Large Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Relationship of this study to the dissertation

To further increase embryo production rates in bison, there is a need to record nuclear and cytoplasmic characteristics of oocytes undergoing various procedures (after maturation, COC morphological classification, synchronization and superstimulation). There is also a need to simplify oocyte collection protocols and reduce handling for use in free-roaming bison herds as chute handling systems are not available in remote locations. This chapter focuses on the morphologic and functional characteristics of cumulus-oocyte complex oocyte during in vitro maturation in bison.

Authors' contributions

Miranda L Zwiefelhofer: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing-Original Draft **Jaswant Singh:** Conceptualization, Methodology, Investigation, Formal analysis, Resources, Writing-Review & Editing **Jessie Hellquist:** Investigation **Dinesh Dadarwal:** Methodology, Investigation, **Fernando Rivera-Acuna:** Methodology, Investigation **Gregg P Adams:** Conceptualization, Methodology, Resources, Writing-Review & Editing, Supervision, Funding acquisition

***Manuscript in preparation**

2.1. Abstract

The present study was designed to facilitate interpretation of maturational changes of the bison oocyte. Exp. 1 was done to determine the effects of *in vitro* maturation (IVM) and conventional morphologic grading of the cumulus oocyte complexes (COC) on nuclear and cytoplasmic characteristics of bison oocyte viability. Exp. 2 and 3 were done to determine the effects of ovarian superstimulation and conventional morphologic grading of the COC on nuclear and cytoplasmic characteristics of bison oocytes (Exp. 2) and embryo development (Exp. 3). In Exp. 1, ultrasound-guided COC collection was done at a random stage of the follicular wave in adult female wood bison (n=24) during the anovulatory season. The COC (n=130) were stained with Mitotracker Deep Red (active mitochondria) and Fluorescein PNA (cortical granules). The oocytes were then classified by a 2D qualitative visual classification (mitochondria were categorized as scattered or clustered, and cortical granules as peripheral or non-peripheral) using epifluorescence wide-field microscopy. A subset of the oocytes (n=41) underwent 3D computer-assisted quantitative assessment of mitochondria using Autoquant and Imaris software to quantify mitochondria distribution. Immediately after the first collection, the bison were assigned to three groups (n=8 bison/group) and given FSH daily for 3 days, a single dose of sustained-release FSH, or a single dose of saline for Exp. 2. A second COC collection was performed 4 days later and COC were stained and classified by the 2D qualitative visual classification method described above (n=101 oocytes). In Experiment 3, a new group of bison (n=23) we given ovarian superstimulation with the timing of treatments the same as in Exp. 2, but the dose of superstimulation treatments was doubled and the COC were processed for *in vitro* embryo production. In Exp. 1, 3D computer-assisted quantitative assessment determined that the central region of the ooplasm had higher counts and total volume occupied by individual mitochondria and mitochondrial clusters ($P \leq 0.02$) and the COC morphologically graded as low quality had greater counts and total volume occupied by mitochondrial clusters than the high quality COC ($P \leq 0.008$). However, the 2D qualitative visual classification in Exp. 2. showed a greater proportion of high quality COC displayed clustered mitochondria than low quality COC ($P=0.008$). The saline treated group tended to have higher embryo production rates than the conventional or sustained-release FSH treatment groups (54% vs 18% vs 14%; $P=0.08$). In conclusion, the present studies showed that active mitochondria was most abundant (higher counts and total volume) in the central

region of the ooplasm and in low quality oocytes. Unfortunately, the minimal-handling ovarian superstimulation treatments implemented in the present study did not increase the number of COC collected or improve embryo production rates.

2.2. Introduction

Although bison were saved from the brink of extinction at the end of the 19th century (Brower, 2008), wood bison (*Bison bison athabascae*) are classified by the Canadian Government Species at Risk Act (SARA) as Schedule 1 *threatened* (Environment and Climate Change Canada., 2018). Wild herds of wood bison have been endemically infected by cattle diseases such as tuberculosis and brucellosis, which restricts gene flow between herds (White et al., 2011; Shury et al., 2015). With the use of advanced reproductive technologies, our bison research group is creating a bison genome biobank to preserve valuable and inaccessible genetics to reconnect geographically separated herds in a bio-secure manner (Adams et al., 2020).

Nuclear and cytoplasmic maturation are essential for fertilization and early embryo development (Combelles et al., 2002; Chian and Cao, 2014). In cattle, ~90% of oocytes will complete nuclear maturation after *in vitro* maturation (IVM), ~80% will fertilize and continue past the two-cell stage, and 30-40% will successfully develop to the blastocyst stage (Lonergan et al., 2001). According to Cervantes et al. (2016), nuclear maturation occurred more rapidly after *in vitro* than *in vivo* maturation in bison. The study showed that 80% of bison oocytes reached metaphase I or II after maturation of 24 hours *in vitro* or 30 hours *in vivo* (i.e., after ovulation induction treatment; Cervantes et al., 2016). In cattle and humans, disruption of cytoplasmic maturation was associated with inhibition of embryo development and lower embryo production (Izadyar et al., 1998; Combelles et al., 2002). Studies in cattle have shown that cytoplasmic maturation represents a transition from a developmentally incompetent cell to a functionally capable cell that can undergo fertilization and early embryo development (Izadyar et al., 1998). This process has been characterized by a redistribution of organelles including mitochondria and cortical granules in the oocyte during the transition from germinal vesicle to metaphase II (Dadarwal et al., 2015a). In general, mitochondria have been shown to synthesize adenosine triphosphate (ATP) and are essential in calcium homeostasis, metabolism, fatty acid oxidation and apoptosis in the oocyte (Babayev and Seli, 2015). Cortical granules have secretory vesicles that,

upon exocytosis, are crucial in blocking polyspermy during fertilization (Ducibella et al., 1990; Liu, 2011). Disruption of these processes hinder oocyte maturation and early embryo development (Liu, 2011; Babayev and Seli, 2015), but maturational changes of the oocyte have not been critically examined in bison.

As bison are a wild species and subject to stress associated with handling, minimal-handling techniques are needed to make cumulus oocyte complex (COC) collection and *in vitro* embryo production feasible for captive and field conditions (Caven et al., 2021). Previous COC collections procedures reported in bison have required at least four handlings as they involve multiple doses of follicle stimulating hormone (FSH; Folltropin-V, Vetoquinol NA Inc., Lavaltrie, Québec, Canada) to induce ovarian superstimulation (Cervantes et al., 2017a; b). Collections at random stages of the follicular wave are not usually incorporated in domestic cattle COC collection protocols because synchronization and subsequent superstimulation increase COC and embryo numbers and competence (Nasser et al., 1993; Adams, 1994; Guerra et al., 2015). However, the collection of COC from bison in the wild, at a random time point and without superstimulation treatment, is the most plausible scenario in the field.

A series of experiments were done to understand intrinsic and extrinsic factors affecting oocyte competence in bison - a threatened species – in an overall effort to design effective germplasm biobanking and dissemination procedures. Experiment 1 was done to determine the dynamic effects of *in vitro* maturation and whether conventional morphologic grading of the COC reflect nuclear and cytoplasmic characteristics of bison oocyte viability. Experiments 2 and 3 were designed to determine the efficacy of two novel FSH formulations from Kawasaki Mitaka Pharmaceutical Co. (Tokyo, Japan), one of which was a sustained-release formula to improve the superstimulatory response in bison while reducing handlings. The experiments were done to determine the effects of ovarian superstimulation and conventional morphologic grading of the COC on nuclear and cytoplasmic characteristics of bison oocytes (Experiment 2) and embryo development (Experiment 3).

2.3. Materials and methods

Experiments involved the use of adult female wood bison (n=24 [Exp. 1 & 2] and n=23 [Exp. 3]) at the Native Hoofstock Centre maintained by the Livestock and Forage Centre of

Excellence at the University of Saskatchewan. The bison ranged in age from 4 to 14 years and were used during the anovulatory season (May-June). During COC collection procedures, the bison were maintained in corrals with free access to water and grass hay, and released to pasture after each synchronized COC collection. Animal use was approved by the University of Saskatchewan's Animal Research Ethics Board (Protocol No. 20090058) and was conducted in accordance with the guidelines of the Canadian Council on Animal Care.

2.3.1. Experiment 1 - Effect of in vitro maturation time and conventional morphologic grading of COC on nuclear and cytoplasmic characteristics of the bison oocyte

2.3.1.1 Oocyte collections and in vitro maturation

Experiment 1 was used to determine the effects of conventional morphologic classification of COC (high vs low quality) and IVM time (0 h vs 25-28 h) on nuclear and cytoplasmic characteristics of bison oocytes. The COC were collected by transvaginal ultrasound-guided follicle aspiration at random stages of ovarian follicular wave development (n=24 bison) as previously described (Palomino et al., 2014). Briefly, the collection procedure involved insertion of a transvaginal 5-8 MHz convex-array ultrasound probe (MyLab Alpha, Esaote, Fishers, IN, USA) fitted to an extended handle after caudal epidural anesthesia 5 mL of 2% lidocaine (Lurocaine; Vetoquniol, Lavaltrie, QC, Canada) and preparation of the perineum with surgical disinfectant. Wood bison COC donors were restrained in a hydraulic chute for the procedure. Follicular aspiration was done with the use of a disposable 18-gauge needle (WTA, Cravinhos, Sao Paulo, Brazil) connected to an aspiration line (WTA) to a 50 mL conical tube 37ºC containing 3 to 5 mL of collection medium (BO-OPU, Catalog # 51001, IVF bioscience, Falmouth, Cornwall, United Kingdom). Using a regulated vacuum pump (BV 003i Digital Vacuum Pump, WTA) the aspiration rate was maintained at 12-16 mL/min. The follicular aspirate was then filtered through an IVF oocyte filter with 75 μm mesh (Partnar Animal Health, Ilderton, ON, Canada). COC collections were performed on two consecutive days (n=12 bison per day); prior to COC collection, bison were assigned randomly (blocked randomization) to a no maturation (0 h) or *in vitro* maturation (IVM; 25-28 h) groups.

The COC were examined by stereomicroscopy (SMZ 1000, Nikon Instrument Inc., Melville, NY, USA) at 10X and morphologically graded as compact good (≥3 layers of unexpanded cumulus cells and homogeneous ooplasm), compact regular (1-3 layers of unexpanded cumulus cells and homogeneous ooplasm), expanded (cumulus cells expanded or partially dissociated), denuded (oocyte without cumulus cells; partially and completely denuded oocytes were grouped in this category) or degenerate (pyknotic granulosa cells or vacuolated ooplasm; Cervantes et al., 2017a). The COC were then washed three times with holding medium [Dulbecco's phosphate buffered saline (D-PBS) with Ca^{2+} and Mg^{+} (Catalog #D8662, Sigma-Aldrich) and 5% calf serum (Gibco newborn calf serum, ThermoFisher Scientific, Waltham, MA, USA), and imaged and graded while maintaining the individual animal identities. The COC were grouped as high quality (compact good, compact regular, expanded) vs low quality (denuded & degenerate) and pooled among animals.

The COC collected for the 0 h IVM group (n=12 bison) were placed in 1.5 mL conical tubes with holding medium and transferred to the laboratory with the temperature not exceeding 37°C, denuded and stained immediately (i.e., within 4 hours of collection). The COC for the 25-28 h IVM group (n=6 bison per day, 12 bison total) were washed three times in oocyte and embryo wash medium (BO-WASH; IVF bioscience) and once in bovine hepes-buffered oocyte maturation medium (BO-HEPES-IVM; IVF bioscience) within 1 hour of collection. COC groups were then placed in individual 1.0 mL tubes (Sarstedt, Nümbrecht, Germany) containing BO-HEPES-IVM and placed in a portable incubator (Lab Mix Portable Incubator, WTA) maintained at 38.8°C in air (i.e., without CO_2) and transported to the laboratory (4 to 21 COC per tube). The tubes were then moved to a large box incubator (38.8°C; Thermo Forma 3130; ThermoFisher Scientific, Waltham, MA, USA) to complete the 25-28 hours of IVM. The 4 treatment groups are shown in Table 2.1.

Table 2.1. Distribution of wood bison oocytes that were used to determine the effect of *in vitro* maturation and conventional COC morphologic grading on oocyte nuclear and cytoplasmic characteristics for Experiment 1.

Maturation time	**O h**		**25-28 h**	
Morphology grade	**High quality[1]**	**Low quality[2]**	**High quality**	**Low quality**
2D qualitative assessment[3]	42	23	37	24
3D quantitative assessment[4]	14	9	9	9

[1]Denuded [no cumulus cells], degenerate [pyknotic granulosa cells or vacuolated ooplasm]
[2]Compact good [≥3 layers of unexpanded cumulus cells and homogeneous ooplasm], compact regular [1-3 layers of unexpanded cumulus cells and homogeneous ooplasm], expanded [cumulus cells expanded or partially dissociated]
[3] The number of oocytes analyzed that were successfully recovered after stained for mitochondria, cortical granules and nuclear status (n=126 total)
[4]The number of oocytes analyzed by quantitative 3D computer-assisted quantitative assessment (n=41)

2.3.1.2 Oocyte staining and imaging

Mitochondrial activity and cortical granules

After 0 h or 25-28 h of *in vitro* maturation (Table 2.1) the oocytes were denuded in a droplet of 0.5% hyaluronidase (Sigma Aldrich, Oakville, ON, Canada) prepared in Ca^{2+} and Mg^{+} free D-PBS. Denuded oocytes were washed three times in holding medium, once in BO-HEPES-IVM and incubated in 20 µl drops of BO-HEPES-IVM overlaid with oil at 38.5ºC in 5% CO_2 for 30 min. Dyes were then added to the drops containing oocytes and co-incubated at 38.5ºC in 5% CO_2 for an additional 30 min to visualize active mitochondria and cortical granules (Mitotracker Deep Red FM, Invitrogen; 1 µl of 10 µm solution to a final concentration of 500 nM, and Fluorescein [FITC] labeled peanut agglutinin [PNA]; EY Laboratories Inc., San Mateo, CA, USA; 1 µl of 100 µg/mL to a final concentration of 5 µg/mL). Oocytes were then washed three times in holding medium, fixed in 0.5% paraformaldehyde in phosphate buffered saline (PBS) for 30 mins and washed three times in a 0.1% polyvinylpyrrolidone (PVP) and PBS solution. Oocytes were then transferred through serial dilutions of vectashield antifade mounting medium with DAPI (4′,6-diamidino-2-

phenylindole, Vector Labs., Burlingame, CA, USA; Catalog # H-2000) in increasing concentrations (1:3, 1:1, 1:0). Finally, oocytes were mounted in the center of reinforcement tags (Avery, Pickering, ON, Canada) on glass slides (4-10 oocytes per 1 µL droplet). A coverslip with paraffin-vaseline on the corners was placed over the oocytes.

Mounted oocytes (n=130) were examined with a confocal laser scanning microscope (Zeiss LSM 710, White plains, NY, USA) equipped with a 63x water immersion objective for assessment of mitochondria (633 nm) and cortical granules (488 nm) in simultaneous scanning mode, and 11 μm-thick 3D image stacks were obtained (x, y and z = 971 x 979 x 10 voxels, each voxel = 0.16 x 0.16 x 1.14 µm, Channel 1 = 488 nm, Channel 2 = 633 nm, Channel 3 = differential interference contrast [DIC]). The maximum intensity projection signals of 10 images of the z-stack from Channels 1 and 2 were used to generate a 2D TIFF image that was subjected to qualitative visual classification assessment (2D qualitative visual classification; next section) while the quantitative assessment was performed using the 3D information (3D computer-assisted quantitative assessment).

Germinal vesicle and nuclear status

After confocal imaging, the oocytes were recovered from the slides and washed three times in 0.1% PVP + PBS solution. Oocytes were incubated overnight with mouse anti-lamin A/C (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in blocking buffer (1:300) for 12 – 16 hours, washed three more times in the PVP + PBS solution and then processed through the serial dilutions of vectasheild mounting medium as described above. The oocytes were then mounted on glass slides with coverslips and assessed using an epifluorescence wide-field microscope excited at 495 nm and 405 nm (Zeiss Axioskop 5 Carl Zeiss Ltd., Toronto, ON, Canada). A total of 126 oocytes were successfully recovered (Fig. 2.1)

2.3.1.3 Morphometric data analyses

2D qualitative visual assessment of active mitochondria and cortical granule distribution pattern

The 2D qualitative visual classification method consisted of identifying patterns of mitochondrial distribution as scattered or clustered as described for cattle oocytes (Dadarwal et al., 2015b) and cortical granule distribution was categorized as peripheral (location within 10 μm of the plasma membrane) or non-peripheral (remaining ooplasm). Visual assessment of each oocyte image (971x979 pixels, 8-bit color TIFF) was done without knowledge of treatment identity (Fig. 2.2).

3D computer-assisted quantitative assessment of mitochondrial distribution pattern

Randomly selected confocal images of viable oocytes (n=53 oocytes) were subjected to 3D computer-assisted quantitative assessment of mitochondrial distribution to quantify the proportion of number of mitochondria present individually (more than 2 μm away from neighbouring mitochondria) or as clusters (mitochondria closer than 2μm from each other), ooplasm volume occupied by individual mitochondria versus clusters, and to examine their distribution pattern (in 10 μm of peripheral ooplasm versus central region). However, only 41 were able to processed through this method due to damage to oocyte and zona pellucida shape (Fig. 2.1). This analysis was labour-intensive (6 to 8 hours per oocyte) and therefore was used to validate the 2D qualitative classification method (described above; <20 minutes per oocyte including conversions from 3D dataset) that is better suited for application to larger number of oocytes in future studies.

Gray-scale values from Channel 1 (mitochondrial activity) and 2 (cortical granules) from 3D datasets were deconvoluted by Autoquant X3 software (Media Cybernetics, Rockville, MD, USA) to correct for image distortion in the Z-axis (Dadarwal et al., 2015b), followed by segmentation in Imaris 8.0 software (Bitplane AG, Zurich, Switzerland) as previously described (Dadarwal et al., 2015b, 2017). Briefly, confocal datasets (*.lsm files) containing the 3 channels were imported into Imaris to crop the Z-dimension to 10 μm, the image format was changed from 16-bit to a 32-bit float, and the Imaris files were exported as *.ids for processing in Autoquant. Terms enclosed in double quotation marks (“ ”) are software specific names. Deconvolution in Autoquant was done using “adaptive point spread function (blind)” in step 1 and “theoretical point spread function” in step 2, followed by 15 iterations with noise level set to “high”. Finally, datasets were exported as *.lsm files for further processing in Imaris. Deconvoluted files were imported into Imaris 8.0 software, and the mitochondrial channel was selected to remove background noise

(thresholding). The differential interference contrast (DIC) images were used to identify the ooplasm (by creating "surface volume") and by masking intensities in the zona pellucida and perivitelline space. Mitochondria were segmented by "spot" creation in Imaris by estimating the size of spots to be 1µm and adjusting the "quality" slider to select the dimmest mitochondria. Mitochondial clusters were identified using "distance transform" to select spots that were closer than 2µm from each other (individual mitochondria were identified as a distance of $\geq 2\mu m^2$ between mitochondria and clustered mitochondria were identified as a distance of $<2\mu m^2$ between mitochondria) and the "merge" function was used to create a single cluster for counting and volume measurements. Finally, the peripheral ooplasm was defined as the region within 10 µm of the plasma membrane, and the remaining ooplasm was designated the central region. Statistical output from the Imaris software allowed us to obtain the number and volume of individual mitochondria and clustered mitochondria in peripheral and central regions of each oocyte. Oocyte identities and classification were not known to the evaluator, and the 3D computer-assisted quantitative assessment allowed us to objectively record the number, size/volume, and distribution (peripheral vs. central) of individual and clustered mitochondria within the oocyte.

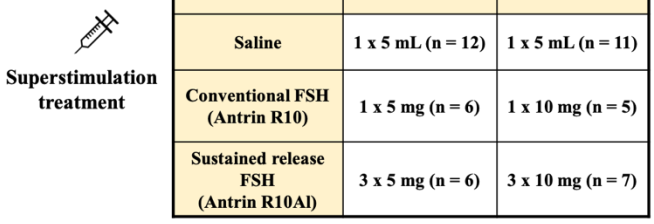
Germinal vesicle breakdown and nuclear status

Images obtained from wide-field fluorescence microscopy after Lamin-DAPI staining were categorized as described previously (Prentice-Biensch et al., 2012) without the knowledge of oocyte identities by one co-author. The nucleus was categorized as viable if they were able to be classified as germinal vesicle stage (GV), germinal vesicle breakdown (GVBD), metaphase I (MI) or metaphase II (MII) or categorized as degenerate if a stage was not determined. Oocytes with degenerate nuclei were excluded from further analysis. Nuclear maturation was categorized as immature (GV) or maturing (GVBD, MI or MII).

2.3.2. Experiment 2 - Effect of ovarian superstimulation (half dose) on nuclear and cytoplasmic characteristics of the bison oocyte

Immediately after the COC collection in Experiment 1, the bison were assigned randomly (blocked randomization) to 3 groups and given either ovarian superstimulatory treatment using conventional FSH (n=6) or sustained-release FSH (n=6) or no superstimulation (saline; n=12; Fig.

2.1). Follicle ablation resulting from the COC collection in Experiment 1 allowed the synchronization of a wave emergence (Palomino et al., 2014a) for Experiment 2. The conventional FSH group was given 5 mg (armour standard units) of Antrin R10 (porcine FSH, Kawasaki Mitaka Pharmaceutical Co., Tokyo, Japan) intramuscularly once per day for three days beginning on the day of the first COC collection (Day -1; Day 0 = expected day of follicular wave emergence). The sustained-release FSH group was given 15 mg of Antrin R10Al (porcine FSH, Kawasaki Mitaka Pharmaceutical Co.) as a single subcutaneous dose on Day -1 (immediately after the COC collection). The non-superstimulation group was given 5 mL saline im on Day -1. Half of the commercially recommended total FSH dose for beef cattle was used (Kawasaki Mitaka Pharmaceutical Co.) to minimize the ambiguity of an overwhelming response (Adams et al., 1993). COC were collected from all groups on Day 3 for second collection (Fig. 2.1). The COC were graded and processed as described in Experiment 1 for the *in vitro* matured group. After 25-28 h of IVM, the COC were stained and evaluated for the 2D qualitative visual classification method as described in Experiment 1, but without 3D computer-assisted quantitative assessment (n=95 oocytes).



Dosage per injection

	Half-dose	Full-dose
Saline	**1 x 5 mL (n = 12)**	**1 x 5 mL (n = 11)**
Conventional FSH (Antrin R10)	**1 x 5 mg (n = 6)**	**1 x 10 mg (n = 5)**
Sustained release FSH (Antrin R10Al)	**3 x 5 mg (n = 6)**	**3 x 10 mg (n = 7)**

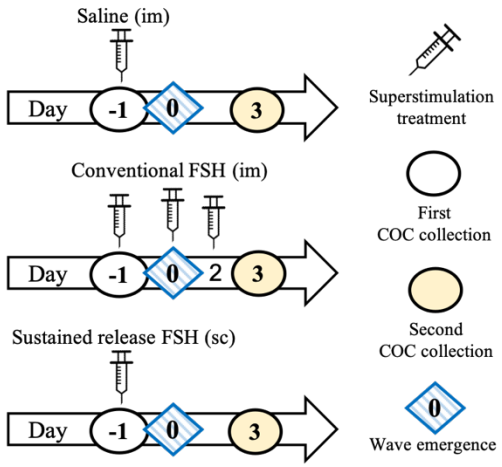


Figure 2.1. Experimental design for ovarian superstimulation and cumulus oocyte complex (COC) collection in wood bison during the anovulatory season. n = the number of bison used per group, ovarian superstimulation products = Kyoritsu Seiyaku Corp, Japan

2.3.3. Experiment 3 - Effect of superstimulation (full dose) on COC collection and embryo production

Follicular wave emergence (Day 0) was synchronized among a new group of bison (n=23) by transvaginal ultrasound-guided follicle ablation on Day -1. Bison were then assigned randomly to three groups: conventional FSH [n=5], or sustained-release FSH [n=7], or no superstimulation [n=11]. A full dose was used, as opposed to the half dose used in Experiment 2, in accordance with the commercially recommended total FSH dose for beef cattle. The conventional FSH group was given 10 mg of Antrin R10 intramuscularly daily for three days beginning on Day -1 (immediately after COC collection) for a total dose of 30 mg. The sustained-release group was given 30 mg of Antrin R10Al as a single subcutaneous dose on Day -1. The non-superstimulation group was given 5 mL saline intramuscularly on Day -1. COC were collected on Day 3 for a synchronized collection. The COC were graded and processed as done in the *in vitro* matured group in Experiment 1. After 25-28 h of *in vitro* maturation, the COC were washed once in a pre-heated and equilibrated 500 µl well of bovine *in vitro* fertilization medium (BO-IVF; IVF bioscience), and transferred quickly into a pre-heated and equilibrated 90 µl drop of BO-IVF with oil (n=10 to 20 COC per drop) in a 35mm petri dish and placed in an incubator (Minc, Cook Medical, Bloomington, IN, USA) at 38.8°C in 5% CO_2 and 95% N_2. Two 0.5 mL wood bison semen straws were taken out of liquid nitrogen and placed in 37.0°C water for 1 minute. The straws were carefully dried and emptied into two 15 mL tubes containing 4 mL of 37.0°C BO-SemenPrep (Catalog #71003, IVF bioscience). Tubes were centrifuged for 5 minutes at 328 x g, and the supernatants were removed, leaving ~300 µl semen pellets. An additional 4 mL of pre-heated SemenPrep was added to each tube and they were centrifuged again for 5 minutes at 328 x g, and the supernatant was removed. Each drop of BO-IVF containing COC was fertilized with the prepared semen (semen pellets combined) at a concentration of 2.0 x 10^6 sperm/mL. Oocytes and sperm were co-incubated at 38.8°C in 5% O_2, 5% CO_2 and 90% N_2 for 18 hours. After fertilization, presumptive zygotes were denuded in BO-WASH, and washed in one 500 µl well of pre-heated and equilibrated bovine *in vitro* culture medium (BO-IVC; IVF bioscience). Each set of presumptive zygotes was then put in a pre-heated and equilibrated 90 µl drop of BO-IVC with oil in a 35 mm petri dish and incubated at 38.8°C in 5% O_2, 5% CO_2 and 90% N_2. Presumptive zygotes

were evaluated 56 hours after fertilization for cleavage. The number of morulae and blastocysts was recorded on Day 7 (Day 0 = day of fertilization).

2.3.4. Statistical analyses

Statistical analyses were performed using SAS 9.4 and Enterprise Guide 4.2 (Statistical Analysis System Institute Inc., Cary, NC, USA). Numerical scale data are represented as mean ± SEM and P-values are considered significant if ≤ 0.05.

The effects of IVM time (0 h vs 25-28 h) and COC morphology grade (high vs low quality) on the proportion of oocytes classified as degenerate nuclear status (oocytes with degenerate nuclear status / total oocytes stained), immature nuclear status (GV stage / oocytes with viable nuclear status), clustered mitochondria (oocytes with a clustered mitochondria distribution / oocytes with viable nuclear status) and peripheral cortical granules (oocytes with a peripheral cortical granule distribution / oocytes with viable nuclear status) were examined by the GLIMMIX procedure using binomial distribution and link logit function. The effects of IVM time (0 h vs 25-28 h), COC morphology grade (high vs low quality) and mitochondria location (central vs peripheral) on total mitochondrial count, size/volume, and mean intensity were examined by ANOVA using a Proc Mixed procedure for repeated measures.

The effect of superstimulation (half recommended dosage; no superstimulation vs regular FSH vs sustained-release FSH) on the number and size of follicles available for aspiration, the number of follicles aspirated and the number and the quality of COC collected were examined by one-way ANOVA and a Tukey post hoc test. The effect of superstimulation on follicle aspiration efficiency (follicles aspirated/follicles available) and COC collection efficiency (COC collected/follicles aspirated) were examined with the GLIMMIX procedure using binomial distribution and link logit function. The effects of superstimulation and COC morphology grade (high vs low quality) on the percent nuclear degenerate, percent nuclear immature, percent of clustered mitochondria and percent of peripheral cortical granules were examined with the GLIMMIX procedure using binomial distribution and link logit function.

The effect of superstimulation treatments (full recommended dosage; no superstimulation vs regular FSH vs sustained-release FSH) on the number and size of follicles available for aspiration, the number of follicles aspirated and the number and the quality of COC collected were examined by a one-way ANOVA and a Tukey post hoc test. The effect of superstimulation on cleavage and

blastocyst rates (based on the total number of COC used in each treatment group), follicle aspiration efficiency and COC collection efficiency were examined with the GLIMMIX procedure using binomial distribution and link logit function.

All superstimulation groups (full and half doses of the regular and sustained-release FSH) were combined to compare the superstimulated vs non-superstimuated collections. The number and size of follicles available for aspiration, the number of follicles aspirated and the number and the quality of COC collected were examined by Student's t-test. Proportional data were accessed by GLIMMIX as described above.

2.4. Results

2.4.1. Experiment 1 - Effect of in vitro maturation time and conventional morphologic grading of COC on nuclear and cytoplasmic characteristics of the bison oocyte

The effects of COC morphology grade and IVM time on the nuclear and cytoplasmic characteristics of bison oocytes are shown in Table 2.2 and Fig. 2.2. Of the 130 oocytes originally stained for mitochondria and cortical granules, 126 oocytes were successfully recovered and stained for nuclear status and were categorized as viable or degenerate nuclear status, and of those with viable nuclear status (n=112), 108 originally had PNA signal, and 104 had the Mitotracker Deep Red signal and were used for analysis. A greater proportion of COC that were morphologically graded as low quality were of degenerate nuclear status than high quality oocytes ($P = 0.04$; Table 2.2). As expected, a greater proportion of oocytes were of immature nuclear status after 0 h vs 25-28 h of IVM (48/59 [81.4%] vs 6/53 [11.3%]; $P = 0.0001$; Table 2.2).

The effects COC morphology grade, IVM time and location on mitochondrial distribution in bison oocytes is shown in Figs. 2.3 & 2.4. Of the 53 oocytes originally selected, 41 were able to be analyzed by the 3D computer-assisted quantitative assessment. The central region of the ooplasm had higher counts and total volume occupied by individual mitochondria and mitochondrial clusters (location, $P \leq 0.02$; Fig. 2.4), and pixel intensity of individual mitochondria and mitochondrial clusters except for the low-grade oocytes at 0 h (IVM*location*grade interaction, $P \leq 0.02$). The COC morphologically graded as low quality had greater counts and total volume occupied by mitochondrial clusters than the high quality COC ($P \leq 0.008$; Fig. 2.4).

There was also higher pixel intensity for individual mitochondria and mitochondrial clusters in low quality COC after maturation than the high quality COC at this time of the culture (IVM*location*grade interaction, $P \leq 0.02$; Fig. 2.4).

Table 2.2. Effect of cumulus oocyte complex (COC) morphology grade and maturation time on nuclear and cytoplasmic characteristics of wood bison oocytes after staining with Mitotracker Deep Red and Fluorescein PNA (Experiment 1, analyzed by GLIMMIX).

Maturation time	**0 h**		**25-28 h**	
Morphology grade	**Low quality[1]**	**High quality[2]**	**Low quality**	**High quality**
Degenerate nuclear status (%)[3,x]	4/23 (17.4%)	2/42 (4.8%)	5/24 (20.8%)	3/37 (8.1%)
Immature nuclear status (%)[4,y]	14/19 (73.7%)	34/40 (85.0%)	3/19 (15.8%)	3/34 (8.8%)
Clustered mitochondria (%)[5]	9/12 (75.0%)	25/40 (62.5%)	13/19 (68.4%)	29/33 (87.9%)
Peripheral cortical granules (%)[6]	14/17 (82.4%)	35/37 (94.6%)	17/19 (89.5%)	31/35 (88.6%)

[1]Denuded [no cumulus cells], degenerate [pyknotic granulosa cells or vacuolated ooplasm]
[2]Compact good [≥3 layers of unexpanded cumulus cells and homogeneous ooplasm], compact regular [1-3 layers of unexpanded cumulus cells and homogeneous ooplasm], expanded [cumulus cells expanded or partially dissociated]
[3]Oocytes with degenerate nuclear status/total oocytes stained; total n=126
[4]Oocytes in germinal vesicle stage/oocytes classified as not degenerate nuclear status; total n=112
[5]Oocytes with a clustered mitochondria distribution/oocytes classified as not degenerate nuclear status; oocytes with no mitochondria signal were excluded; total n=104
[6]Oocytes with a peripheral cortical granule distribution/oocytes classified as not degenerate nuclear status; oocytes with no PNA signal were excluded; total n=108
[x]Effect of morphology grade ($P \leq 0.05$, GLIMMIX)
[y]Effect of maturation time ($P \leq 0.05$, GLIMMIX)

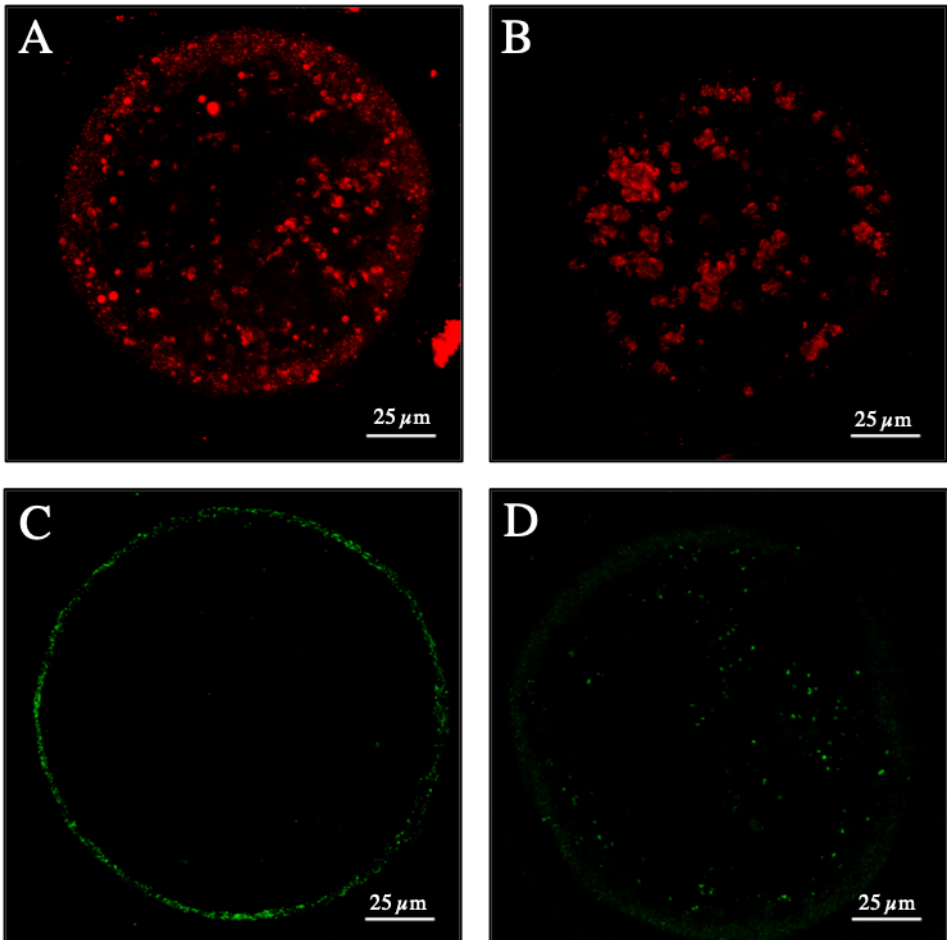


Figure 2.2. Images of bison oocytes representing cytoplasmic distributions of mitochondria and cortical granules after staining with Mitotracker Deep Red and Fluorescein PNA (A) mitochondria: scattered distribution (B) mitochondria: clustered distribution (C) cortical granules: peripheral distribution (D) cortical granules: non-peripheral distribution (Experiment 1).

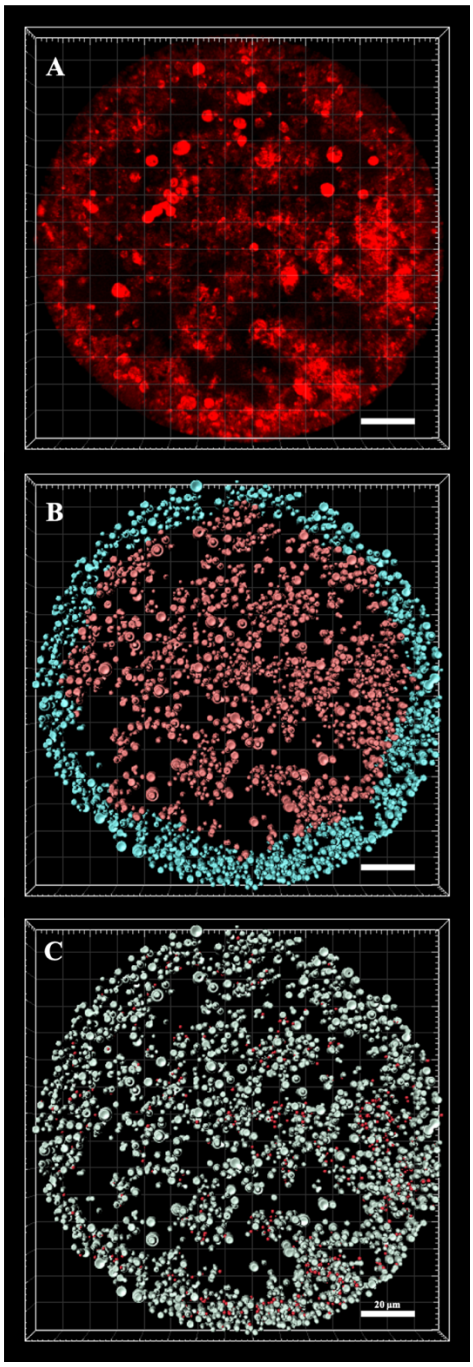


Figure 2.3. Images used for quantitative analysis of mitochondrial distribution in bison oocytes using Autoquant (deconvolution) and Imaris software (thresholding and segmentation). A) Confocal image of a bison oocyte stained with Mitotracker Deep Red, B) A bison oocyte showing a central distribution (pink) vs peripheral distribution of mitochondria (blue), C) A bison oocyte showing individual mitochondria (red) and clustered mitochondria (white, Experiment 1).

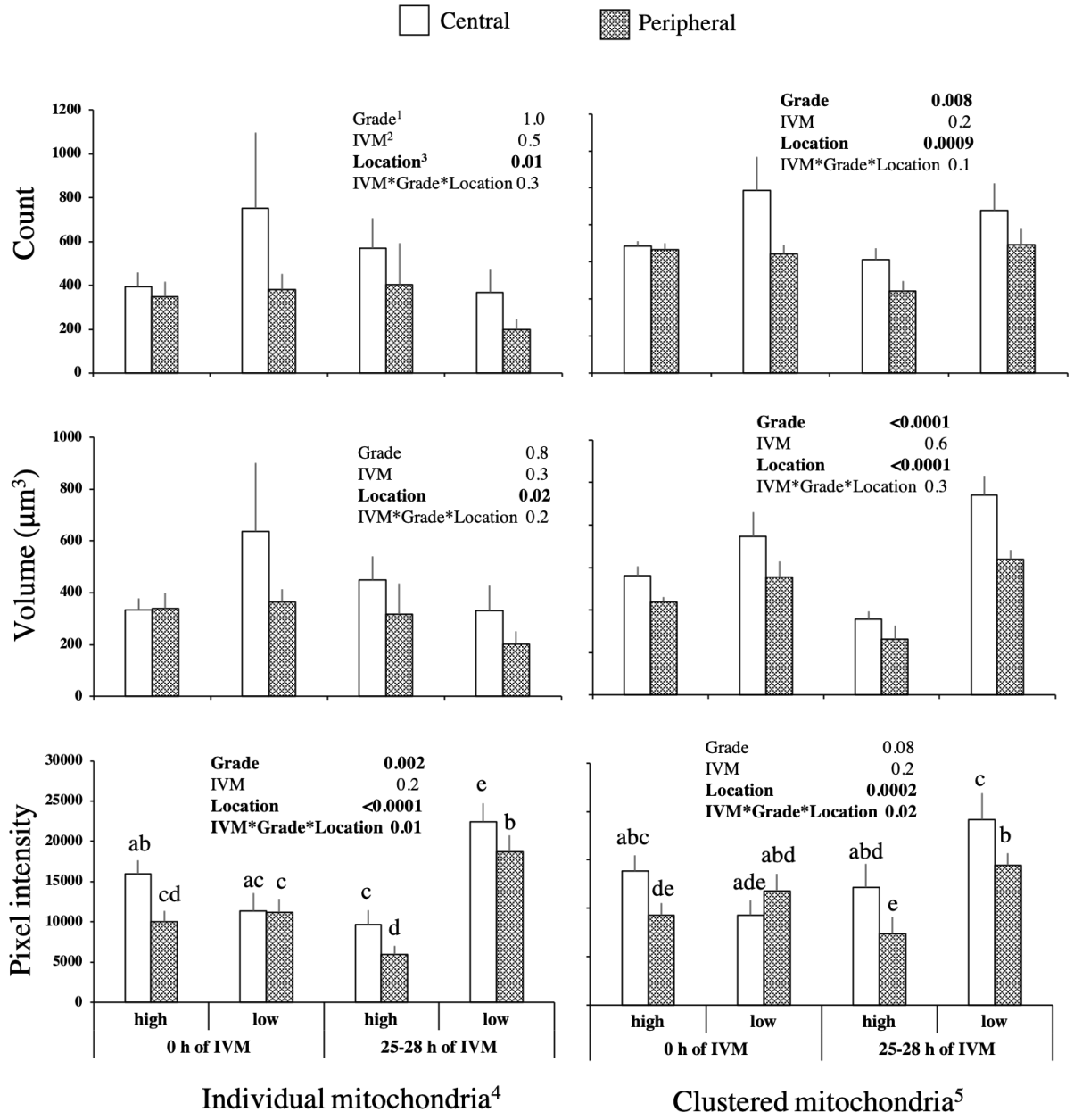


Figure 2.4. The effects of maturation time (0 h vs 25-28 h), COC morphology grade (high vs low quality) and mitochondria location (central vs peripheral) on the number of mitochondria (count), the total volume of mitochondria, and the mitochondrial signal (pixel intensity) in bison oocytes calculated by the computer-assisted morphometry (n=41 oocytes; Experiment 1). Data were analyzed by ANOVA using a Proc Mixed procedure for repeated measures with bolded text indicating ($P \leq 0.05$).

[1]Morphology grade (high vs low quality)

[2]In vitro maturation time prior to staining and fixation (0 h vs 25-28 h)

[3]Location of mitochondria within ooplasm (central vs peripheral regions)

[4]Individual mitochondria had a distance of ≥2 µm² between mitochondria

[5]Clustered mitochondria had a distance of <2 µm² between mitochondria

2.4.2. Experiment 2 - Effect of ovarian superstimulation (half-dose) on nuclear and cytoplasmic characteristics of the bison oocyte

The total number of follicles ≥3 mm did not differ among treatment groups (P = 0.1; Table 2.3). However, conventional FSH treatment resulted in a greater number of follicles ≥8 mm than the saline and sustained-release FSH groups (P = 0.003). Follicle aspiration efficiency was greatest in the conventional FSH group and lowest in the saline group (P < 0.0001), but there was no effect of treatment on COC collection efficiency (P = 0.3) or the mean number of COC collected (P = 0.8; Table 2.3).

The effects of morphology grade and ovarian superstimulation on nuclear and cytoplasmic characteristics of bison oocytes are shown in Table 2.4. Of the 101 oocytes originally stained for mitochondria and cortical granules, 95 oocytes were successfully recovered and stained for nuclear status and were categorized as viable or degenerate nuclear status, and of those with viable nuclear status (n=90), 89 originally had PNA signal, and 87 had the Mitotracker Deep Red signal and were used for analysis. A greater proportion of oocytes that were morphologically graded as high quality displayed clustered mitochondria than low quality oocytes (P = 0.008; Table 2.4).

Table 2.3. Ovarian follicles and cumulus oocyte-complex (COC) collection (mean ± SEM) in wood bison after superstimulation treatment at half of the recommended dosage during the anovulatory season (Experiment 2). COC collections were done 3 days after wave emergence and data were analyzed by one-way ANOVA and GLIMMIX.

Superstimulation treatment	Saline[1]	Conventional FSH[2]	Sustained-release FSH[3]
Number of bison	12	6	6
Number of follicles			
3-4 mm	13.8 ± 3.7	5.8 ± 1.4	4.8 ± 2.1
4.5-7.5 mm	8.7 ± 2.1	6.0 ± 1.1	6.3 ± 1.8
≥8 mm	0.9 ± 0.1[a]	4.7 ± 1.5[b]	1.7 ± 0.3[a]
Total	23.4 ± 5.6	16.5 ± 2.6	12.8 ± 3.1
Follicles aspirated	10.3 ± 1.6	14.0 ± 2.8	8.7 ± 2.4
Follicle aspiration efficiency[4]	124/281 (44.1%)[a]	84/99 (84.8%)[b]	52/77 (67.5%)[c]
COC collection efficiency[5]	62/124 (50.0%)	33/84 (39.2%)	25/52 (48.1%)
COC recovered			
Compact-good	2.4 ± 0.7	3.2 ± 1.4	1.7 ± 0.9
Compact-regular	0.8 ± 0.3	0.8 ± 0.3	1.3 ± 0.4
Expanded	0.1 ± 0.1	0.2 ± 0.2	0.2 ± 0.2
Denuded	1.8 ± 0.5	1.3 ± 0.4	0.7 ± 0.3
Degenerate	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.3 ± 0.2[b]
Total	5.2 ± 0.9	5.5 ± 1.3	4.2 ± 1.5

[1]COC collection after treatment with saline given intramuscularly on Day -1.
[2]COC collection after treatment with conventional FSH (5 mg [Antrin R10] intramuscularly once per day from Day -1 to Day 1 for a total dose of 15 mg)
[3]COC oocyte collection after treatment with sustained-release FSH (15 mg (Antrin R10Al) as a single subcutaneous dose on Day -1.
[4]Follicles aspirated/follicles ≥3 mm available
[5]COC collected/follicles aspirated
[abc]Within rows, values with no common superscript are different ($P \leq 0.05$)

Table 2.4. Effect of morphology grade and superstimulation treatment at half of the recommended dosage on wood bison oocyte nuclear and cytoplasmic characteristics after staining with Mitotracker Deep Red and Fluorescein PNA (Experiment 2). COC collections were done 3 days after wave emergence and data were analyzed by GLIMMIX.

Superstimulation treatment	**Saline[1]**		**Conventional FSH[2]**		**Sustained-release FSH[3]**	
Morphology grade	**Low quality[4]**	**High quality[5]**	**Low quality**	**High quality**	**Low quality**	**High quality**
Degenerate nuclear status (%)[6]	4/18 (22.2%	0/33 (0.0%)	1/8 (12.5%)	0/22 (0.0%)	0/6 (0.0%)	0/8 (0.0%)
Immature nuclear status (%)[7]	1/14 (7.1%)	9/33 (27.3%)	1/7 (14.3%)	3/22 (13.6%)	2/6 (33.3%)	3/8 (37.5%)
Clustered mitochondria (%)[8,x]	7/13 (53.9%)	27/33 (81.8%)	2/5 (40.0%)	8/9 (88.9%)	2/6 (33.3%)	13/21 (61.9%)
Peripheral cortical granules (%)[9]	5/14 (35.7%)	18/32 (56.3%)	6/7 (85.7%)	12/22 (54.7%)	5/5 (100.0%)	6/9 (66.7%)

[1]Cumulus oocyte collection after treatment with saline given intramuscularly on Day -1.
[2]Cumulus oocyte collection after treatment with conventional FSH (5 mg [Antrin R10] intramuscularly once per day from Day -1 to Day 1 for a total dose of 15 mg)
[3]Cumulus oocyte collection after treatment with sustained-release FSH (15 mg (Antrin R10Al) as a single subcutaneous dose on Day -1
[4]Denuded [no cumulus cells], degenerate [pyknotic granulosa cells or vacuolated ooplasm]
[5]Compact good [≥3 layers of unexpanded cumulus cells and homogeneous ooplasm], compact regular [1-3 layers of unexpanded cumulus cells and homogeneous ooplasm], expanded [cumulus cells expanded or partially dissociated]
[6]Oocytes with degenerate nuclear status/total oocytes stained; total n=95
[7]Oocytes in germinal vesicle stage/oocytes classified as not degenerate nuclear status; total n=90
[8]Oocytes with a clustered mitochondria distribution/oocytes classified as not degenerate nuclear status; oocytes with no Mitotracker Deep Red signal were excluded; total n=87
[9]Oocytes with a peripheral cortical granule distribution/oocytes classified as not degenerate nuclear status; oocytes with no PNA signal were excluded; total n=89
[x]Effect of morphology grade ($P \leq 0.05$, GLIMMIX)

2.4.3. Experiment 3 - Effect of superstimulation (full dose) on COC collection and embryo production

The effect of superstimulation treatments at the full recommended dosage (for cattle) on follicle characteristics, COC collection and *in vitro* embryo production are displayed in Table 2.5. Treatment with conventional FSH resulted in a greater number of follicles ≥8 mm than the saline

and sustained-release FSH groups (2.8 ± 1.0 vs 1.1 ± 0.2 vs 1.9 ± 0.3, respectively; P = 0.04). The total number of COC recovered was similar among groups (P = 0.6; Table 2.5) but the saline treated group tended to have greater cleavage (25/35 [71.4%] vs 7/28 [25.0%] vs 8/35 [22.9%], respectively; P = 0.07) and embryo rates (19/35 [54.3%] vs 5/28 [17.9%] vs 5/35 [14.3%], respectively; P = 0.08) than the conventional or sustained-release FSH treatments.

Table 2.5. Ovarian follicles, cumulus oocyte-complex (COC) collection (mean ± SEM) and in vitro embryo production in wood bison after superstimulation treatment (full recommended dosage for cattle) during the anovulatory season (Experiment 3). COC collections were done 3 days after wave emergence and data were analyzed by one-way ANOVA and GLIMMIX.

Superstimulation treatment	Saline[1]	Conventional FSH[2]	Sustained-release FSH[3]
Number of bison	11	5	7
Number of follicles			
3-4 mm	8.1 ± 1.2	6.6 ± 0.7	7.4 ± 0.8
4.5-7.5 mm	4.4 ± 1.0	6.2 ± 0.8	4.6 ± 1.0
≥8 mm	1.1 ± 0.2[a]	2.8 ± 1.0[b]	1.9 ± 0.3[ab]
Total	13.5 ± 1.7	15.6 ± 1.3	13.9 ± 1.7
Follicles aspirated	10.4 ± 1.4	12.4 ± 0.9	10.9 ± 1.4
Follicle aspiration efficiency[4]	114/149 (76.5%)	62/78 (79.5%)	76/97 (78.4%)
COC collection efficiency[5]	58/114 (50.9%)	36/62 (58.1%)	45/76 (59.2%)
COC recovered			
Compact-good	0.7 ± 0.2	1.2 ± 0.6	0.4 ± 0.2
Compact-regular	2.5 ± 0.6	4.4 ± 1.2	4.6 ± 1.2
Expanded	0.2 ± 0.1	0.6 ± 0.4	0.4 ± 0.2
Denuded	1.5 ± 0.6	0.8 ± 0.8	0.9 ± 0.3
Degenerative	0.4 ± 0.2	0.2 ± 0.2	0.0 ± 0.0
Total	5.3 ± 1.4	7.2 ± 0.9	6.4 ± 1.5
Cleavage rate[6]	25/35[x] (71.4%)	7/28[y] (25.0%)	8/35[y] (22.9%)
Embryo rate[7]	19/35[x] (54.3%)	5/28[y] (17.9%)	5/35[y] (14.3%)

[1]Cumulus oocyte collection after treatment with saline given intramuscularly on Day -1.
[2]Cumulus oocyte collection after treatment with conventional FSH (10 mg [Antrin R10] intramuscularly once per day from Day -1 to Day 1 for a total dose of 30 mg)
[3]Cumulus oocyte collection after treatment with sustained-release FSH (30 mg (Antrin R10Al) as a single subcutaneous dose on Day -1.
[4]Follicles aspirated/follicles ≥3 mm available
[5]COC collected/follicles aspirated
[6]Number of presumptive zygotes cleaved/COC submitted to *in vitro* maturation
[7]Number of embryos produced/COC submitted to *in vitro* maturation
[ab]Within rows, values with no common superscript are different (P ≤ 0.05, ANOVA).
[xy]Within rows, values with no common superscript tended to be different (P ≤ 0.08, GLIMMIX)

2.4.4. Experiments 2 & 3 – Effect of ovarian superstimulation

A secondary analysis of the effects of superstimulation treatment (conventional vs sustained-release FSH) and dosage (half vs full recommended dosage) was done. Superstimulation dosage and treatment had little effect on ovarian follicles and COC collection. Therefore, all superstimulation groups were combined to determine the effect of superstimulation (non-superstimulation vs superstimulation) in Table 2.6. Although the total number of follicles ≥3 mm did not differ between groups (P = 0.2; Table 2.6), non-superstimulated collections had a greater number of 3-4 mm follicles (11.1 ± 2.1 vs 6.2 ± 0.7; P = 0.03) and fewer ≥8 mm follicles (1.0 ± 0.1 vs 2.7 ± 0.5; P = 0.002) than the superstimulated collections. The follicle aspiration efficiency was lower in the non-superstimulated collections than the superstimulated collections (238/430 [55.4%] vs 274/351 [78.1%]; P = 0.0001)

Table 2.6. Ovarian follicles and cumulus oocyte-complex (COC) collection (mean ± SEM) in wood bison with or without superstimulation treatment during the anovulatory season (Experiments 2 & 3 combined). COC collections were done 3 days after wave emergence and data were analyzed by Student's t-test and GLIMMIX.

Treatment	Non-superstimulation[1]	Superstimulation[2]
Number of bison	23	24
Number of follicles		
3-4 mm	11.1 ± 2.1[a]	6.2 ± 0.7[b]
4.5-7.5 mm	6.6 ± 1.3	5.7 ± 0.6
≥8 mm	1.0 ± 0.1[a]	2.7 ± 0.5[b]
Total	18.7 ± 3.1	14.6 ± 1.1
Follicles aspirated	10.3 ± 1.0	11.4 ± 1.0
Follicle aspiration efficiency[3]	238/430[a] (55.4%)	274/351[b] (78.1%)
COC collection efficiency[4]	120/238 (50.4%)	139/274 (50.7%)
COC recovered		
Compact-good	1.6 ± 0.5	1.6 ± 0.5
Compact-regular	2.8 ± 0.5	2.8 ± 0.5
Expanded	0.3 ± 0.1	0.3 ± 0.1
Denuded	0.9 ± 0.2	0.9 ± 0.2
Degenerate	0.1 ± 0.1	0.1 ± 0.1
Total	5.2 ± 0.8	5.8 ± 0.7

[1]COC collection after treatment with saline given intramuscularly on Day -1.
[2]COC collection after treatment with conventional FSH or sustained-release FSH
[3]Follicles aspirated/follicles ≥3 mm available
[4]COC collected/follicles aspirated
[ab]Within rows, values with no common superscript are different ($P \leq 0.05$, Student's t-test)
[xy]Within rows, values with no common superscript are different ($P \leq 0.05$, GLIMMIX)

2.5. Discussion

The current experiments were designed to develop a cytological technique for determining changes in nuclear and cytoplasmic characteristics of bison oocytes. Initial 2D qualitative visual classification of mitochondria distribution in COC collected at a random follicular wave (Experiment 1) did not detect differences in mitochondria or cortical granule distribution in the present study. 3D computer-assisted quantitative assessment was done on a subset of oocytes to investigate the distribution of mitochondria within the bison oocyte at a quantitative level. The quantitative software analysis did not detect any impact of IVM status on any mitochondria endpoint. However, every mitochondria endpoint (count, volume and pixel intensity) had more

activity in the central location compared to the peripheral region. The COC morphologically graded as low quality had more mitochondrial clustering and higher pixel intensity than the high quality COC. However, the 2D qualitative visual classification of mitochondria after superstimulation in the subsequent experiment did not clarify the results. In Experiment 2, COC morphologically classified as high quality had a greater proportion of clustered mitochondria (visually identified) than the low quality COC ($P = 0.008$). No conclusive pattern of mitochondria was detected from the 2D qualitative visual classification method (clustered and scattered) as it was not consistent with the 3D computer-assisted quantitative assessment. The oocytes utilized for quantitative assessment were collected at random stages of the follicular wave, accounting for variation in the results. Studies in cattle have shown that oocyte competence directly relates to the follicular stage at which they are collected. Oocytes collected at random stages of the follicular wave have a higher incidence of follicular atresia than ovarian synchronized and FSH-stimulated collections, reducing embryo production efficiency (Adams, 1994; Vassena et al., 2003). In domestic bovids, the relocation of mitochondria during maturation from the peripheral to central region indicates competent oocytes capable of embryo development (Bavister and Squirrell, 2000). However, mitochondria tend to cluster around lipid droplets in regressing ovarian follicles after distributing evenly throughout the oocyte (Dadarwal et al., 2015a). Therefore, oocytes identified as having mitochondria characteristics which indicated positive future embryo production (i.e., centrally located) could be in the beginning stages of atresia. As the computer analysis identified clusters of mitochondria to be at a distance of <2 μm to each other, the human eye is unable to distinguish individual clusters. Therefore, the 2D qualitative visual classification method without quantitative software analysis is not reliable at the current state. Although the quantitative software analysis was able to detect mitochondrial distribution trends, it is not recommended for everyday use. Limitations of this approach include labour intensiveness (each oocyte took one day of data processing), proprietary and expensive software, and operator expertise.

Mitochondria in bison oocytes appeared similar to an FSH starvation group in cattle (COC collection occurred 84 hours after the last FSH injection) that accumulated large lipid droplets (Dadarwal et al., 2015b). Previous studies have noted that bison oocytes exhibit darker cellular matter and have a higher level of vacuolization than cattle oocytes (Thundathil et al., 2007; Barfield, 2019). Little is known why there has been variation between (Matorras et al., 1998; McEvoy et al., 2000; Dunning et al., 2014) and within species (Sudano et al., 2012; Baldoceda et

al., 2016) regarding oocyte and embryo lipid volumes and profiles. In cattle, lipid droplets were closely associated with the endoplasmic reticulum and mitochondria (Ferguson and Leese, 1999). Abnormal mitochondrial distribution patterns may result from metabolism issues or the physical mechanisms that move organelles within the oocyte (Pasquariello et al., 2019). As oocytes in all groups, including those that did not undergo IVM, appeared to have large lipid droplets, we believe it is a species-specific bison characteristic and not a result of secondary lipid uptake from the culture system during maturation. Additional studies are required to characterize and investigate the relationship between mitochondria and lipid droplets in bison oocytes.

The study also tested two novel FSH formulations with the prospect of improving the ovarian superstimulation response in bison while reducing handlings and subsequent stress in bison. The two formulations of FSH (conventional or sustained FSH) investigated did not increase the number of follicles available or COC recovered. The ovarian superstimulation protocols examined had lower embryo production rates than the saline treated group (5/28 [17.9%] vs 5/35 [14.3%] vs 19/35 [54.3%]). In cattle, selection of follicular size during COC collection impacts fertilization and blastocyst rates, with follicles of larger size having a greater chance of further development (Blondin et al., 2012). In the present study, the saline treated group had higher embryo production rates and a greater proportion of 3-4 mm follicles collected. In comparison, the superstimulated collections had lower embryo production rates and a greater proportion of ≥4.5 mm follicles collected. The use of non-superstimulated COC collections may be a valid option for use in wild and free-roaming bison herds and requires further investigation. Overall, the present study results show that the two formulations of FSH at the given dosages were not successful. Future experiments with alternative pharmaceuticals or increased dosages may be warranted.

In conclusion, 3D quantitative software analysis allowed us to analyze cytoplasmic maturation in bison oocytes. A greater proportion of active mitochondria was located in the central region of the ooplasm and mitochondria clustering was present in low quality oocytes. However, additional analysis is required to characterize mitochondria distributions without quantitative software analysis. The bison oocytes had a high incidence of large lipid droplets similar to extended FSH starvation groups in cattle. Unfortunately, the minimal-handling ovarian superstimulation treatments implemented in the present study did not increase the number of COC collected or improve embryo production rates. However, the use of non-superstimulated COC collections may be a valid option for use in wild and free-roaming bison herds in the future.

2.6. Acknowledgments

The bison project and this study is supported by the Natural Sciences and Engineering Research Council of Canada and Saskatchewan ADF. The authors thank Dr. O Dochi and the Kawasaki Mitaka Pharmaceutical Co. for the donation of superstimulation drugs. Student support was provided by Boehringer Ingelheim Veterinary Scholarship and the Western College of Veterinary Medicine. The authors would also like to thank Eric Zwiefelhofer, Sayuri Maeda, Steve Yang and Miguel L Chirivi for assistance with data collection and the staff of the Livestock and Forage Centre of Excellence for managing the bison at the Native Hoofstock Centre.

CHAPTER 3:

3. INFLUENCE OF OVARIAN FOLLICULAR WAVE SYNCHRONIZATION AND SINGLE-DOSE ECG SUPERSTIMULATION ON OOCYTE COLLECTION AND *IN VITRO* EMBRYO PRODUCTION IN BISON

Miranda L Zwiefelhofer[1], Jaswant Singh[1], Eric M Zwiefelhofer[1], Gabriela Mastromonaco[2], Gregg P Adams[1]

[1]Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine
University of Saskatchewan, Saskatoon, Saskatchewan, Canada
[2]Department of Reproductive Sciences, Toronto Zoo, Toronto, Ontario, Canada

Relationship of this study to the dissertation

There is a need to modify oocyte collection protocols to minimize handling and apply reproductive technologies under field conditions rather than solely for use in captive herds. We determined that a single dose eCG superstimulation protocol increased the number and quality of oocytes collected while reducing the required number of bison handlings.

Miranda L Zwiefelhofer: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing-Original Draft, Visualization **Jaswant Singh:** Conceptualization, Methodology, Formal analysis, Resources, Writing-Review & Editing **Eric M Zwiefelhofer:** Conceptualization, Methodology, Investigation, Writing-Review & Editing **Gabriela F Mastromonaco:** Conceptualization, Writing-Review & Editing **Gregg P Adams:** Conceptualization, Methodology, Resources, Writing-Review & Editing, Supervision, Funding acquisition

Manuscript submitted to Theriogenology in January 2022

3.1. Abstract

In an effort to develop an effective, minimum-handling protocol for the conservation of wood bison, the present study was designed to determine the effects of ovarian synchronization and superstimulation on cumulus oocyte complex (COC) collection and *in vitro* embryo production in wood bison during the ovulatory (Exp. 1) and anovulatory seasons (Exp. 2). We tested the hypotheses that COC collection and *in vitro* embryo production are 1) greater after follicular wave synchronization than at random stages of the follicular wave, 2) repeatable within individuals, 3) greater after ovarian superstimulation with a single dose of equine chorionic gonadotrophin (eCG) than without treatment, and 4) greater during the anovulatory season than the ovulatory season. In Exp. 1, ultrasound-guided COC collection was performed in wood bison to induce follicular wave emergence (Day = 0) the following day. Immediately after the first collection (random start), bison were given a single im dose of 2,500 IU eCG or saline (n = 6 per group). Second and third COC collections were performed 5 and 10 d later. A similar design was used in Exp. 2, with an additional treatment group given 5,000 IU eCG (n = 8 per group). Compared to the saline-treated group, a single dose of 2,500 IU eCG resulted in a greater number of large follicles at the time of COC collection 5 d after treatment (P=0.03), but not at 10 d after treatment. In Exp. 2, treatment with 5,000 IU eCG resulted in a greater number of follicles ≥8 mm than 2,500 IU eCG or the saline treatment (37.5 ± 6.9, 17.5 ± 2.0, 16.9 ± 2.0; P=0.01). Although the number of embryos produced/COC submitted to IVM was not different among groups (mean = 18.6%), treatment with 5,000 IU eCG produced more than twice as many embryos per bison as unstimulated bison (0.8 vs 1.9). In summary, embryo production rates were higher from COC collected subsequent to follicular wave synchronization vs random stages of the wave, and ovarian superstimulation with eCG resulted in a dose-related increase in the number of follicles ≥8 mm, COC collected, and embryos produced. Repeated COC collections after successive wave synchronization resulted in similar follicular counts and embryo production rates within individuals, and the greatest number of follicles aspirated, COC collected, and embryos produced was in the anovulatory season. We conclude that the minimum-handling COC collection protocols in the present study are effective and provide realistic options for embryo production in wild bison.

3.2. Introduction

Wood bison (*Bison bison athabascae*) and plains bison (*Bison bison bison)* are classified as near threatened subspecies native to North America (Aune et al., 2017). As a keystone species in the short- and tall-grass prairies in the south and the boreal ecosystem in the north (Knapp et al., 1999; Gates et al., 2001), bison are also a cultural keystone for many Indigenous Peoples in North America (Cunfer and Waiser, 2016). Of the 500,000 bison, only 5% are maintained in wild or conservation herds and are of known origin (Hedrick, 2009; Committee on the status of endangered wildlife in Canada., 2013). The largest wild populations of wood bison (Wood Buffalo National Park in Canada) and plains bison (Yellowstone National Park in USA) have been strictly monitored and controlled because of endemic infection with cattle diseases - tuberculosis and brucellosis (White et al., 2011; Shury et al., 2015). Assisted reproductive technologies are being developed as a strategy to preserve valuable and inaccessible genetics for the purpose of reconnecting geographically distant herds in a bio-secure manner (Adams et al., 2020).

Substantial progress has been made during the last 10 years in our understanding of the reproductive phenology of bison and in species-specific development of assisted reproductive techniques. Serial ultrasonography and endocrinology were used to elucidate the circannual pattern of ovarian function in bison (McCorkell et al., 2013), and results formed the basis of studies on elective control of ovarian follicle development and ovulation during the ovulatory and anovulatory seasons (Palomino et al., 2015). Subsequently, a variety of protocols were tested to synchronize and superstimulate ovarian follicle development (Palomino et al., 2013, 2014a) for the purpose of *in vivo* embryo collection (Palomino et al., 2017a; b, 2020) and *in vitro* production of embryos (IVP) derived from cumulus oocyte complexes (COC) collected by transvaginal ultrasound-guided follicular aspiration (Cervantes et al., 2017a; b; Palomino et al., 2020) and post-mortem material (Benham et al., 2021). The most efficient embryo production protocol to-date involves synchronization of ovarian follicular wave emergence followed by superstimulation using two doses of follicle stimulating hormone (FSH) and a single dose of human chorionic gonadotrophin (hCG) - a protocol which requires five animal handlings for every COC collection (Cervantes et al., 2017b; Palomino et al., 2020). As bison are a wild species, efforts are being made to modify protocols so that handling can be minimized and reproductive technologies can be applied under field conditions rather than solely for use in intensively managed herds.

Equine chorionic gonadotrophin (eCG) is a glycoprotein with a half-life of 40 hours in cattle which exhibits both FSH and luteinizing hormone (LH) properties (Mapletoft et al., 2002). The use of eCG for ovarian superstimulation has the advantage of fewer treatments than FSH preparations, but has not been used as regularly as FSH in cattle because of lower embryo collection rates and persistent follicles (Mapletoft et al., 2002). Intramuscular injections of 300 to 3,000 IU of eCG have been used to stimulate follicle development in cattle, sheep and pigs (Seidel and Seidel, 1991; Aller et al., 2012); the most common dose used in cattle is 2,500 IU (Mapletoft et al., 2002). In an earlier study in bison we found that multiple doses of FSH resulted in greater ovarian superstimulation and more COC collected than a single dose of 2,500 IU eCG (Palomino et al., 2013, 2014b), but required more handlings, and no comparisons were made with non-superstimulated and random-start (non-synchronized) bison.

The objective of the present study was to determine the effects of ovarian synchronization and superstimulation on oocyte collection and *in vitro* embryo production in bison during the ovulatory (Experiment 1) and anovulatory seasons (Experiment 2). The experimental design permitted test of the hypotheses that COC collection and *in vitro* embryo production are 1) greater after follicular wave synchronization than at random stages of the follicular wave, 2) repeatable within individuals between successive wave synchronizations, 3) greater after ovarian superstimulation with a single dose of eCG than without superstimulatory treatment, and 4) greater during the anovulatory season than the ovulatory season.

3.3. Materials and methods

Adult female wood bison, ranging in age from 3 to 14 years, were selected from a single herd of 35 females during the ovulatory season (November; Experiment 1) and anovulatory season (May; Experiment 2). Bison were maintained in corrals throughout the experiment with free access to water and grass hay. After each replicate they were released to pasture. Animal procedures were approved by the University of Saskatchewan's Animal Care Committee and performed in accordance with the guidelines of the Canadian Council on Animal Care.

3.3.1. Experiment 1 – Ovulatory season

Female bison (n=12) at random stages of the estrous cycle were restrained in a hydraulic chute, the perineum was washed with disinfectant, and caudal epidural anesthesia was induced with 5 mL of 2% lidocaine (Lurocaine; Vetoquniol, Lavaltrie, QC, CAN) in preparation for transvaginal ultrasound-guided oocyte collection (Palomino et al., 2014a). The vagina was lavaged by transrectal manual manipulation after intravaginal infusion of two 60 mL-volumes of saline (pH 5.6) followed by one 60 mL-volume of Dulbecco's phosphate buffered saline, DPBS; pH 6.9 - 7.1; Sigma-Aldrich, Oakville, ON, CAN) through an 18-inch infusion pipette (Continental, Delavan, WI, USA). The perineum was washed again in preparation for transvaginal ultrasound-guided COC collection, as described above. The ovaries were examined using a 5-8 MHz convex-array transvaginal probe (MyLab Alpha, Esaote North America Inc, Fishers, IN, USA) fitted to an extended handle. Cine-loops of ovarian images were recorded for later determination of the number of follicles in small (3-4 mm), medium (4.5-7.5 mm) and large (>8 mm) categories. Aspiration of follicles ≥3 mm was then performed using a disposable 18-gauge short-bevel needle (WTA, Cravinhos, SP, Brazil) connected via aspiration line (WTA) into a single 50 mL Falcon tube (all follicles combined) containing 5 mL of ovum pick-up medium (BO-OPU, Catalog # 51001, IVF bioscience, Falmouth, Cornwall, UK). The aspiration flow-rate was regulated with a vacuum pump (BV 003i Digital Vacuum Pump, WTA) set at 12-16 mL/min and the aspirate was maintained at 37ºC until filtration and COC searching.

Analogous to transvaginal follicle ablation as an ovarian synchronization technique (Palomino et al., 2014a), the random start COC collection procedure was used to induce synchronous emergence of a new follicular wave among bison 1 day later. This effect permitted comparison between a random start (first COC collection) vs a synchronized start (second and third COC collections), and enabled synchronous superstimulatory treatment after the initial collection. Immediately after the first (random start) COC collection, bison were assigned to one of two groups (n=6 per group) and given either 2,500 IU eCG im (Folligon, Merck Animal Health, Kirkland, QC, CAN) or an equivalent volume of normal saline im (control). Day -1 was the day of the first (random start) COC collection, Day 0 was taken as the expected day of wave emergence, and second and third COC collections were conducted on Day 4 and Day 9 (i.e., 4 days after the expected day of successive follicular wave emergence; Fig. 1) without additional

superstimulatory treatments. The third collection (Day 9) was used to assess a residual or prolonged effect of eCG on follicular growth and the repeatability between successive wave synchronizations and within individuals. The experiment was completed in a single replicate.

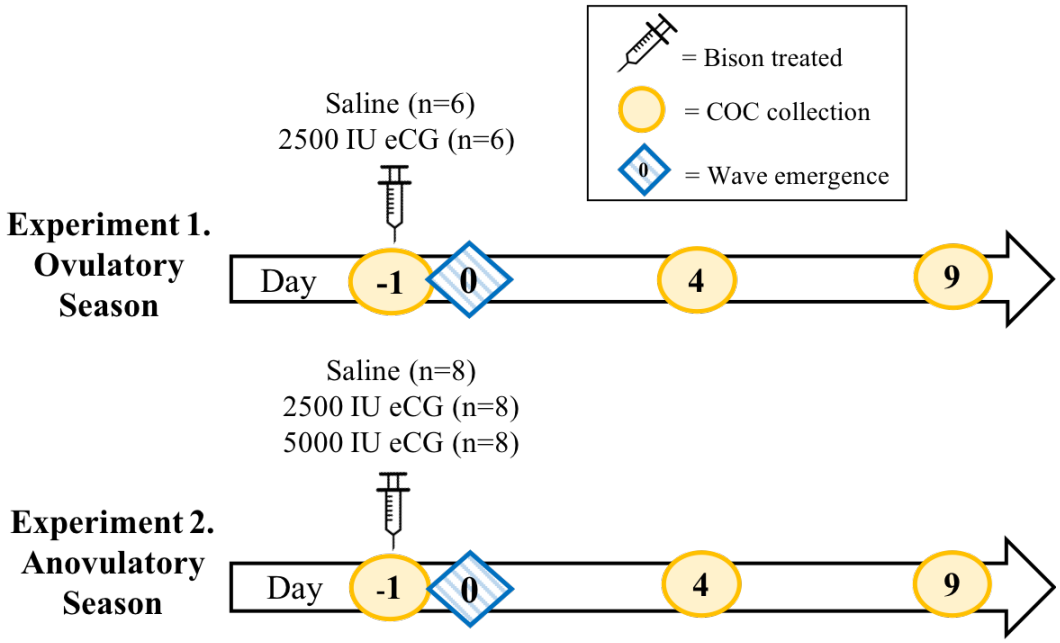


Figure 3.1. Experimental design for the ovulatory (May) and anovulatory (November) seasons in wood bison. n = the number of bison used per group, COC: Cumulus-oocyte complexes, eCG: equine chorionic gonadotrophin

3.3.2. COC morphologic classification

The follicular aspirate was filtered through a 75 µm mesh oocyte filter (Partnar Animal Health, Ilderton, ON, CAN). Stereomicroscopy (M8; Wild Heerbrugg, Heerbrugg, CHE and SMZ800, Nikon Instruments Inc., Americas) at 10x magnification was used to locate and morphologically classify the COC as compact-good (≥3 layers of unexpanded cumulus cells and homogeneous ooplasm), compact-regular (1 to 3 layers of unexpanded cumulus cells and homogeneous ooplasm), compact-poor (≥1 layers of unexpanded cumulus cells and heterogeneous ooplasm), expanded (cumulus cells expanded or partially dissociated), denuded (oocyte without cumulus cells), or degenerate (pyknotic granulosa cells or vacuolated ooplasm). The COC were placed in DPBS with Ca^{2+} and Mg^{+} (Catalog #D8662, Sigma-Aldrich) with 5% calf serum (Catalog # 16010167, Thermo Fisher Scientific, Ottawa, ON, CA) to be classified. The compact-good and

compact-regular COC were combined within treatment group to create a high-quality COC group to be processed further for *in vitro* maturation, fertilization and culture. The COC classified as compact-poor, expanded, denuded and degenerate were combined to create a single low-quality COC group and were not processed further. The high-quality COCs were divided into groups of 10 to 20 to be processed for maturation.

3.3.3. In vitro maturation, fertilization and culture

Immediately after classification, the COC were washed 3 times in oocyte and embryo wash medium (WASH; Catalog #51002, IVF bioscience) and once in bovine hepes-buffered oocyte maturation medium (BO-HEPES-IVM; Catalog # 71001, IVF bioscience). The COC were then placed in 1.5 mL sonification tubes (Active Motif, Carlsbad, CA, USA) containing 1 mL BO-HEPES-IVM and placed in a portable incubator (Lab Mix Portable Incubator, WTA) maintained at 38.8°C in air (i.e., without CO_2) for a maturation time of 25 to 28 hours. After maturation, the COC were removed from the maturation tubes and washed twice in prewarmed and equilibrated 500 µl wells of bovine fertilization medium (BO-IVF, Catalog # 71004, IVF bioscience). Each tube of oocytes was then transferred into a pre-warmed and equilibrated 90 µl drop of BO-IVF covered with oil in a 35 mm petri dish. The dish was placed in an incubator (Miri Benchtop Multi-room Incubator, Esco Medical ApS, Egaa, DNK) at 38.8°C in 5% O_2, 5% CO_2 and 90% N_2. Two 0.5 mL straws of wood bison semen were removed from liquid nitrogen and placed in 37.0°C water for 1 minute. The straws were dried and each emptied into one of the 15 mL tubes containing 4 mL of 37.0°C BO-SemenPrep (Catalog #71003, IVF bioscience). Tubes were centrifuged for 5 minutes at 300 x g. Supernatants were removed leaving semen pellets of ~300 µl. An additional 4 mL of pre-heated BO-SemenPrep were added to each 15 mL tube, and centrifuged again for 5 minutes at 300 x g. Supernatants were removed again, leaving ~300 µl of sperm pellet. The 2 semen pellets were combined and mixed with a pipette. Each 90 µl drop of BO-IVF containing COC was then fertilized with the prepared semen for a final concentration of 2.0 x 10^6 sperm/mL. Oocytes and sperm were co-incubated at 38.8°C in 5% O_2, 5% CO_2 and 90% N_2 for 16 to 20 hours, and presumptive zygotes were denuded in BO-WASH, and transferred through two wells of 500 µl of pre-warmed and equilibrated bovine embryo culture medium (BO-IVC, Catalog #71005, IVF bioscience) prior to being put into a pre-warmed and equilibrated 90 µl drop of BO-IVC covered

with oil in a 35 mm petri dish and incubated at 38.8°C in 5% O_2, 5% CO_2 and 90% N_2. Presumptive zygotes were evaluated for cleavage (≥ 2-cell) 3 days after *in vitro* fertilization. The total number of embryos (morulae, early blastocysts, blastocysts and expanded blastocysts) were monitored daily from 7 to 10 days after fertilization.

3.3.4. Technical control (cattle abattoir ovaries)

A technical control with cattle abattoir ovaries was used on the same day as the second COC collection (Day 4). The ovaries were processed at the same facility, as described above (i.e., bison chute and lab). Ovaries were washed with normal saline after excess tissue was removed and follicles ≥3 mm were aspirated from each ovary manually using an 18 gauge needle attached to a 12 mL syringe, and deposited into 50 mL tubes containing BO-OPU (Prentice et al., 2011). The cattle COC were processed in tandem (in separate plates) with the bison COC, as described above, except that the *in vitro* maturation period was 20-24 hours. Cattle oocytes were co-incubated with the same batch of frozen-thawed bison semen and processed for IVP of hybrid embryos as a technical control.

3.3.5. Experiment 2 – Anovulatory season

Follicular wave emergence (Day 0) was synchronized among bison (n=24) at random stages of the follicular wave by transvaginal ultrasound-guided COC collection (random start, n=24, Day -1), as described in Experiment 1. Bison were then assigned randomly to 3 groups (n=8 per group) and given 2,500 IU eCG im (Folligon; Merck Animal Health), 5,000 IU eCG im, or an equivalent volume of normal saline im (control) immediately after the first COC collection (Day -1). Subsequent COC collections were conducted on Day 4 and Day 9 (i.e., 4 days after expected day of successive follicular wave emergence). No additional treatments were given after the second COC collection on Day 4, as described in Experiment 1 (Fig. 1), to assess the residual effect of eCG and repeatability of collections on Day 9. The experiment was completed in 2 replicates (n=12 bison/replicate) two days apart. COC collection, morphological classification, *in vitro* maturation, fertilization and culture, and technical controls were done as described in Experiment 1.

3.3.6. Statistical analyses

Statistical analyses were performed using SAS 9.4 and Enterprise Guide 4.2 (Statistical Analysis System Institute Inc., Cary, NC, USA). Numerical scale data are represented as mean ± SEM and P-values are considered significant if ≤ 0.05.

The number and size of follicles available for aspiration, the number of follicles aspirated and the number and the quality of COC collected were examined by analysis of variance using a Proc Mixed procedure for repeated measures. Proportional data (cleavage and blastocyst rates [based on the total number of COC used in each treatment group], follicle aspiration efficiency [follicles aspirated/follicles available] and COC collection efficiency [COC collected/follicles aspirated]) were examined with the GLIMMIX procedure using binomial distribution and link logit function. The number of embryos produced per bison was compared using chi-square. The effects of ovarian superstimulatory treatment (non-superstimulated vs superstimulated) on the number and size of follicles and the grade of COC collected was compared between groups after the first and second synchronized waves by analysis of variance using a Proc Mixed procedure for repeated measures. If main effects or interactions were significant, Tukey's *post-hoc* test was used for multiple comparisons. Proportional data (cleavage and blastocyst rates, follicle aspiration efficiency and COC collection efficiency) were examined with the GLIMMIX procedure using binomial distribution and link logit function. The number of embryos produced per bison was compared using chi-square.

The effects of ovarian follicular wave status (random vs synchronized start) and superstimulatory treatment (eCG vs saline) were examined separately in each experiment, but data from Experiments 1 and 2 were subsequently combined to examine the effects of season (presented in Tables 1, 4, and 5). Analysis of variance using a general linear model (GLM) factorial design was used for numerical scale endpoints, and Tukey's *post-hoc* test was used for multiple comparisons. Proportional data were examined with the GLIMMIX procedure using binomial distribution and link logit function. The number of embryos produced per bison was compared using chi-square. To further examine the repeatability of the number of follicles ≥3 mm within individuals in the non-superstimulated group, the bison were grouped into low (0-15 follicles) and high (>16 follicles) number categorizes on the days of first and second COC collection (Days 4 and 9). Pearson's correlation was used to assess the relationship between the number of follicles

≥3 mm on the days of first and second COC collection (Days 4 and Day 9) in the non-superstimulated group for combined anovulatory and ovulatory data.

3.4. Results

3.4.1. Experiment 1 – Ovulatory season

A comparison of the COC collection and embryo production efficiency for non-superstimulated bison collected at random stages of follicular wave development vs those synchronized at Day 4 of wave emergence is provided in Table 1. The comparison was made using data from all bison at the random start (n=12) and bison in the saline-treated group at the first (n=6) and second (n=6) synchronized collections (total n=12). No differences were detected in the number of follicles in small, medium or large size categories, or in the number of follicles aspirated between the random and synchronized collection during the ovulatory season. However, follicle aspiration efficiency, cleavage rate, and embryo production were greater ($P < 0.05$) in the group synchronized for collection on Day 4 of the follicular wave than at random stages of the follicular wave.

A comparison of the ovarian response and embryo production between bison treated with 2,500 IU of eCG vs no superstimulatory treatment is summarized in Table 2. Treatment with 2,500 IU of eCG had no effect on the number of small ovarian follicles present at the time of the first or second synchronized COC collections (4 days after respective follicular wave emergence), but was associated with a greater number of large follicles at the time of the first collection (treatment x collection session interaction; $P = 0.03$; Table 2). No superstimulatory effect was apparent at the time of the second collection. The COC collection efficiency (COC collected/follicles aspirated) was not affected by either superstimulatory treatment or collection session. Therefore, the number of COC collected was greater in the eCG group than the saline group at the time of the first collection ($P \leq 0.05$), in association with the greatest number of large follicles. There tended to be fewer low-quality COC collected in the eCG group than the saline group at the first collection (treatment x collection session interaction; $P \leq 0.08$; Table 2).

Table 3.1. Ovarian follicles, cumulus oocyte-complex (COC) collection, and embryo production (mean ± SEM) in wood bison at random vs synchronized stages of follicular wave development during the ovulatory season (Experiment 1) and anovulatory season (Experiment 2).

	Ovulatory season		Anovulatory season	
Ovarian stage	**Random start[1]**	**Synchronized (Day 4)[2]**	**Random start**	**Synchronized (Day 4)**
Number of collections	12	12	24	16
Number of follicles				
3-4 mm	10.8 ± 1.8	7.4 ± 1.6	13.9 ± 2.0	10.0 ± 1.2
4.5-7.5 mm[x]	2.1 ± 0.7	3.8 ± 0.8	6.5 ± 0.8	5.4 ± 0.6
≥8 mm[xy]	1.0 ± 0.3[ab]	0.8 ± 0.2[a]	1.6 ± 0.2[b]	1.2 ± 0.2[ab]
Total[xy]	13.9 ± 1.9[a]	12.0 ± 2.3[a]	22.0 ± 2.5[b]	17.5 ± 1.6[ab]
Follicles aspirated[xy]	8.4 ± 1.3[a]	9.6 ± 2.0[a]	12.8 ± 1.4[ab]	14.6 ± 1.7[b]
Follicle asp. eff.[3,y]	101/167 (61%)	115/144 (80%)	306/527 (58%)	234/280 (84%)
COC coll. eff.[4,x]	47/101 (47%)	60/115 (52%)	214/306 (70%)	161/234 (69%)
COC recovered				
High quality[x]	2.3 ± 0.6	2.9 ± 0.9	4.5 ± 0.9	4.6 ± 0.7
Low quality[x]	1.7 ± 0.5	1.8 ± 0.7	4.5 ± 0.8	4.5 ± 0.8
Total[x]	3.9 ± 0.9	5.0 ± 1.7	8.9 ± 1.3	10.1 ± 1.4
Cleavage rate[5,y]	11/31 (36%)	20/37 (54%)	34/112 (30%)	41/72 (57%)
Embryo rate[6,y]	0/31 (0%)	7/37 (19%)	9/112 (8%)	15/72 (21%)
Embryos per bison[xy]	0/12[a] (0.0)	7/12[ab] (0.6)	9/24[ab] (0.4)	15/16[b] (0.9)

[1]Random stages of the follicular wave
[2]Synchronized to Day 4 of the follicular wave (Day 0 = wave emergence)
[3]Follicles aspirated/follicles ≥3 mm available
[4]COC collected/follicles aspirated
[5]Number of presumptive zygotes cleaved/COC submitted to *in vitro* maturation
[6]Number of morulae and blastocysts produced/COC submitted to IVM
[ab]Within rows, values with no common superscript are different ($P \leq 0.05$)
[x]Effect of season ($P \leq 0.05$)
[y]Effect of synchronization ($P \leq 0.05$)
[xy]Season x synchronization interaction ($P \leq 0.05$)

Table 3.2. The effect of single-dose superstimulatory treatment on follicle numbers, cumulus oocyte-complex (COC) collection and *in vitro* embryo production (mean ± SEM) in wood bison during the ovulatory season (Experiment 1). Treatment was given on the day of transvaginal follicle ablation, and successive COC collection sessions were done 5 and 10 days later (i.e., 4 days after emergence of successive follicular waves).

	Saline		eCG (2,500 IU)	
Collection session	**1**	**2**	**1**	**2**
Number of bison	6	6	6	6
Number of follicles				
3-4 mm	7.7 ± 2.4	7.2 ± 2.2	4.5 ± 0.6	8.2 ± 1.6
4.5-7.5 mm[xy]	3.3 ± 1.2[ab]	4.3 ± 1.2[a]	4.2 ± 0.9[a]	1.0 ± 0.4[b]
≥8 mm[xy]	0.7 ± 0.3[a]	0.8 ± 0.3[a]	2.5 ± 0.7[b]	0.5 ± 0.2[a]
Total	11.7 ± 3.4	12.3 ± 3.4	11.2 ± 1.2	9.7 ± 1.9
Follicles aspirated	10.2 ± 2.9	9.0 ± 2.9	10.0 ± 2.1	6.3 ± 1.43
Follicle asp. eff.[1]	61/70 (87%)	54/74 (73%)	60/67 (90%)	38/58 (66%)
COC coll. eff.[2]	32/61 (53%)	28/54 (52%)	36/60 (60%)	15/38 (40%)
COC recovered				
High quality	2.8 ± 1.4	3.0 ± 1.4	5.5 ± 1.5	1.5 ± 0.7
Low quality	2.5 ± 1.3	1.7 ± 1.1	0.5 ± 0.3	1.0 ± 0.5
Total	5.3 ± 2.4	4.7 ± 2.5	6.0 ± 1.7	2.5 ± 1.0
Cleavage rate[3]	9/18 (50%)	11/19 (58%)	21/35 (60%)	2/10 (20%)
Embryo rate[4]	3/18 (17%)	4/19 (21%)	5/35 (14%)	0/10 (0%)
Embryos per bison	3/6 (0.5)	4/6 (0.7)	5/6 (0.8)	0/6 (0.0)

[1]Follicles aspirated/follicles ≥3 mm available
[2]COC collected/follicles aspirated
[3]Number of presumptive zygotes cleaved/COC submitted to *in vitro* maturation
[4]Number of embryos produced/COC submitted to IVM
[ab]Within rows, values with no common superscript are different ($P \leq 0.05$)
[xy]Superstimulatory treatment x collection session interaction ($P \leq 0.05$)

3.4.2. Experiment 2 – Anovulatory season

A comparison of the efficiency of COC collection and embryo production between non-superstimulated bison collected at random stages of follicular wave development vs to those synchronized at Day 4 of wave emergence is provided in Table 1. The comparison was made using data from all bison at the random start (n=24) and from bison in the saline-treated group at the first (n=8) and second (n=8) synchronized collections (total n=16). No differences were detected in the

number of follicles in small, medium or large size categories, or in the number of follicles aspirated between the random and synchronized collections in the anovulatory season. However, follicle aspiration efficiency, cleavage rate, and embryo production were greater ($P < 0.05$) in the group synchronized for collection on Day 4 of the follicular wave.

A comparison of the ovarian response and embryo production between bison treated with 2,500 IU eCG, 5,000 IU eCG or saline is summarized in Table 3. The effects of treatment and treatment-by-collection session interaction were attributed to a predominant influence of the high-dose group (5,000 IU eCG) which had a greater number of follicles ≥3 mm at the first COC collection (interaction, $P < 0.0001$; Table 3). Superstimulatory treatment with 5,000 IU of eCG increased the number of large and medium follicles, and the number of follicles aspirated at the time of the first collection compared to the other groups (interaction, $P < 0.0007$; Table 3; Fig. 2). A greater number of small ovarian follicles was present at the time of the first and second COC collections in the non-superstimulated group than in the superstimulated groups ($P = 0.002$; Table 3). The 5,000 IU eCG group at the first collection had the greatest total number of COC and high quality COC collected, (interaction, $P \leq 0.03$; Table 3; Fig. 3). COC collection efficiency was the lowest in the 5,000 IU eCG group at the first collection (interaction, $P = 0.0001$, Table 3). Cleavage and embryo development rates did not differ among groups; however, a greater number of embryos were produced per bison in the 5,000 IU eCG group after the first collection (Table 3; Fig. 3).

Table 3.3. The effect of single-dose superstimulatory treatment on follicle numbers, cumulus oocyte-complex (COC) collection and *in vitro* embryo production (mean ± SEM) in wood bison during the anovulatory season (Experiment 2). Treatment was given on the day of transvaginal follicle ablation, and successive COC collection sessions were done 5 and 10 days later (i.e., 4 days after expected emergence of successive follicular waves).

	Saline		eCG (2,500 IU)		eCG (5,000 IU)	
Collection session	**1**	**2**	**1**	**2**	**1**	**2**
Number of bison	8	8	8	8	8	8
Number of follicles						
3-4 mm[x]	10.3 ± 1.7	9.8 ± 1.9	6.6 ± 1.0	4.6 ± 0.8	3.9 ± 0.6	2.8 ± 0.9
4.5-7.5 mm[xy]	5.8 ± 0.9[ab]	5.1 ± 0.9[abe]	7.6 ± 1.0[b]	2.9 ± 1.0[ac]	12.5 ± 2.1[d]	2.4 ± 0.6[ce]
≥8 mm[xy]	0.9 ± 0.2[a]	1.5 ± 0.2[a]	3.3 ± 1.3[a]	0.8 ± 0.3[a]	21.1 ± 5.4[b]	4.4 ± 1.0[a]
Total[xy]	16.9 ± 2.0[a]	18.1 ± 2.5[a]	17.5+2.0[a]	8.3 ± 1.5[a]	37.5 ± 6.9[b]	9.5 ± 1.7[a]
Follicles aspirated[x,y,xy]	13.1 ± 1.9[a]	16.1 ± 3.0[a]	14.8 ± 1.9[a]	7.4 ± 1.3[a]	31.4 ± 4.8[b]	10.3 ± 1.7[a]
Follicle asp. eff.[1]	105/135 (78%)	129/145 (89%)	118/140 (84%)	59/66 (85%)	251/300 (84%)	76/82 (93%)
COC collect. eff.[2,xy]	75/105[a] (71%)	86/129[a] (67%)	76/118[a] (64%)	40/59[a] (68%)	124/251[b] (49%)	41/76[b] (54%)
COC recovered						
High quality[xy]	4.5 ± 1.0[ab]	4.6 ± 1.1[ab]	4.9 ± 1.7[ab]	1.6 ± 0.6[a]	10.5 ± 3.1[b]	2.6 ± 1.2[a]
Low quality	4.9 ± 1.3	6.1 ± 1.3	4.6 ± 0.5	3.3 ± 0.7	5.1 ± 1.9	2.5 ± 0.7
Total[xy]	9.4 ± 1.9[ab]	10.8+2.1[ab]	9.5 ± 2.1[ab]	5.0 ± 0.7[a]	15.5 ± 2.3[b]	5.1 ± 1.4[a]
Cleavage rate[3]	21/37 (57%)	20/35 (57%)	27/39 (69%)	9/13 (69%)	42/84 (50%)	18/23 (78%)
Embryo rate[4]	7/37 (19%)	8/35 (23%)	10/39 (26%)	0/13 (0%)	15/84 (18%)	3/23 (13%)
Embryos per bison[xy]	7/8[ab] (0.9)	8/8[ab] (1.0)	10/8[ab] (1.3)	0/8[c] (0.0)	15/8[a] (1.9)	3/8[bc] (0.4)

[1]Follicles aspirated/follicles ≥3 mm available
[2]COC collected/follicles aspirated
[3]Number of presumptive zygotes cleaved/COC submitted to *in vitro* maturation
[4]Number of embryos produced/COC submitted to IVM
[abcde]Within rows, values with no common superscript are different ($P \leq 0.05$)
[x]Effect of superstimulatory treatment ($P \leq 0.05$)
[y]Effect of collection session ($P \leq 0.05$)
[xy]Superstimulatory treatment x collection session interaction ($P \leq 0.05$)

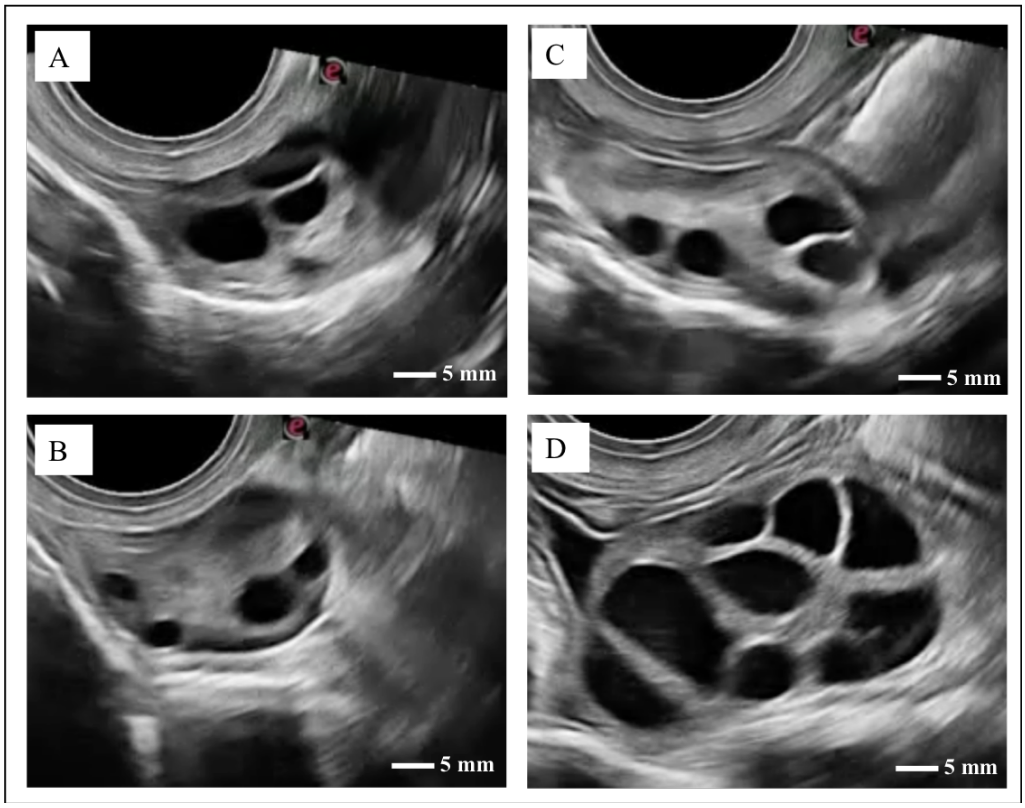


Figure 3.2. Transvaginal ultrasound images of the ovaries in wood bison in the anovulatory season (Experiment 1) immediately prior to cumulus-oocyte-complex collection (A), at Day 4 of wave emergence in a synchronized and non-superstimulated bison (B), and at Day 4 of wave emergence in bison superstimulated with 2,500 IU eCG (C), or 5,000 IU eCG (D); Day 0 = wave emergence.

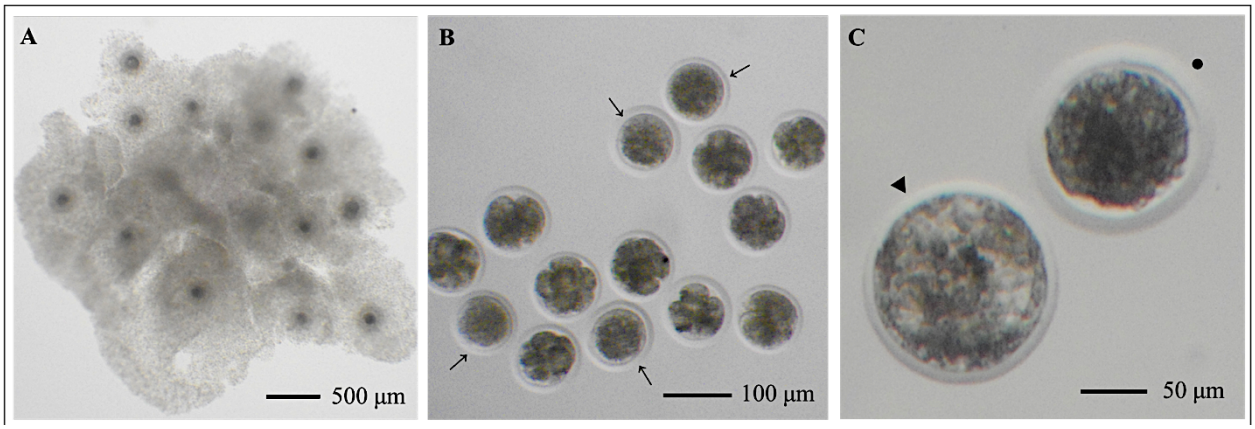


Figure 3.3. Stereomicrographs of (A) expanded cumulus-oocyte complexes (COC) from wood bison after 25-28 hours of *in vitro* maturation. The COC were classified as compact-good at the time of collection by transvaginal ultrasound guided follicle aspiration. (B) *In vitro* produced wood bison embryos 3 days after fertilization. Arrows (→) indicate unfertilized oocytes. (C) *In vitro* produced wood bison embryos 9 days after fertilization, at the blastocyst stage (▶ grade 1) and early blastocyst stage (● grade 1).

3.4.3. Seasonal comparison

A greater number of medium follicles were available for aspiration during the anovulatory season than the ovulatory season (Table 1; $P < 0.05$). COC collection efficiency was greater in the anovulatory season ($P < 0.05$; Table 1) which resulted in collection of a greater number of COC (low and high quality) during the anovulatory season ($P \leq 0.04$; Table 1). Synchronized collections were associated with greater cleavage and embryo development rates than random collections ($P \leq 0.006$; Table 1). Among non-superstimulated groups, embryo production (per COC and per bison) was greatest after synchronized collection in the anovulatory season (interaction; $P = 0.002$).

A seasonal comparison of the ovarian response and embryo production between bison treated with the lower dose of eCG (2,500 IU) vs no superstimulatory treatment is summarized in Table 4. A greater number of total and medium sized follicles were available for aspiration ($P < 0.02$), and more COC were recovered ($P \leq 0.02$) in the anovulatory vs ovulatory season. No seasonal or treatment effect was detected in the cleavage rate, embryo rate or the embryos produced per bison.

In the saline-treated group (season data combined), the number of follicles ≥3 mm at the time of the first and second COC collection (5 and 10 days after superstimulatory treatment; 4 days after emergence of successive follicular waves) was highly correlated within individuals ($r = 0.85$, $P = 0.0001$). Furthermore, when the bison were grouped according to the number of follicles ≥3 mm (low [≤15 follicles] vs high [≥16 follicles]), 12 out of 14 individual bison maintained the same category for the first and second COC collections.

In the technical controls, no seasonal effect was detected in cleavage rate or embryo rate for either homologous (bison oocyte x bison sperm) or hybrid (domestic cattle oocyte x bison sperm) *in vitro* embryo production (Table 5). However, *in vitro* production was higher for hybrid embryos than homologous bison embryos ($P < 0.0001$; Table 5).

Table 3.4. Ovarian response, cumulus oocyte-complex (COC) collection efficiency and in vitro embryo production (mean ± SEM) in the first superstimulated vs non-superstimulated wood bison synchronized collection during the ovulatory season (Experiment 1) and anovulatory season (Experiment 2).

	Ovulatory season		Anovulatory season	
Treatment	**Saline**	**eCG (2,500IU)**	**Saline**	**eCG (2,500 IU)**
Number of collections	12	6	16	8
Number of follicles				
3-4 mm[y]	7.4 ± 1.6	4.5 ± 0.6	10.0 ± 1.2	6.6 ± 1.0
4.5-7.5 mm[x]	3.8 ± 0.8	4.2 ± 0.9	5.4 ± 0.6	7.6 ± 1.0
≥8 mm[y]	0.8 ± 0.2	2.5 ± 0.7	1.19 ± 0.2	3.3 ± 1.3
Total[x]	12.0 ± 2.3	11.2 ± 1.2	17.5 ± 1.6	17.5 ± 2.0
Follicles aspirated[x]	9.6 ± 2.0	10.0 ± 2.1	14.6 ± 1.7	14.8 ± 1.9
Follicle asp. eff.[1]	115/144 (78%)	60/67 (90%)	234/280 (84%)	118/140 (84%)
COC coll. eff.[2]	60/115 (52%)	36/60 (60%)	161/234 (69%)	76/118 (64%)
COC recovered				
High quality	2.9 ± 0.9	5.5 ± 1.5	4.6 ± 0.7	4.9 ± 1.7
Low quality[x]	1.8 ± 0.7	0.5 ± 0.34	4.5 ± 0.8	4.6 ± 0.5
Total[x]	5.0 ± 1.7	6.0 ± 1.7	10.1 ± 1.4	9.5 ± 2.1
Cleavage rate[3]	20/37 (54%)	21/35 (60%)	41/72 (57%)	27/39 (69%)
Embryo rate[4]	7/37 (19%)	5/35 (14%)	15/72 (21%)	10/39 (26%)
Embryos per bison	7/12 (0.6)	5/6 (0.8)	15/16 (0.9)	10/8 (1.3)

[1]Follicles aspirated/follicles ≥3 mm available
[2]COC collected/follicles aspirated
[3]Number of presumptive zygotes cleaved/COC submitted to *in vitro* maturation
[4]Number of embryos produced/COC submitted to IVM
[x]Effect of season ($P \leq 0.05$)
[y]Effect of treatment ($P \leq 0.05$)

Table 3.5. Embryo production rates for wood bison and the technical control group (cattle x bison hybrid) from the first synchronized cumulus oocyte-complex (COC) collections in the ovulatory (Experiment 1) and anovulatory (Experiment 2) seasons.

	Ovulatory season		Anovulatory season	
Species	**Bison[1]**	**Hybrid[2]**	**Bison**	**Hybrid**
Cleavage rate[3,x]	31/68 (46%)	31/37 (84%)	97/233 (42%)	27/36 (75%)
Embryo rate[4,x]	7/68 (10%)	15/37 (41%)	31/233 (13%)	14/36 (39%)

[1]Bison COC fertilized with bison semen
[2]Abattoir-collected cattle COC fertilized with bison semen
[3]Number of presumptive zygotes cleaved/COC submitted to *in vitro* maturation
[4]Number of embryos produced/COC submitted to *in vitro* maturation
[x]Effect of species ($P \leq 0.05$)

3.5. Discussion

The intent of the present study was to develop an effective, minimum-handling protocol for *in vitro* embryo production in bison; studies were designed to determine the effects of ovarian synchronization and single-treatment superstimulation on oocyte collection and embryo production in bison during the ovulatory (Exp. 1) and anovulatory seasons (Exp. 2). Results supported the hypothesis that COC collection and *in vitro* embryo production are greater after follicular wave synchronization than at random stages of the follicular wave. Results are consistent with cattle studies in that synchronization of follicular wave emergence increases the COC competence and embryo production rates by ensuring a lower incidence of follicular atresia compared to COC collected at random stages of the wave (Nasser et al., 1993; Adams, 1994; Guerra et al., 2015).

Ovarian synchronization and superstimulation is common practice to optimize IVP in cattle since repetitive handling is feasible. However, in reference to wild species, access to the animal may be limited to single handling in free-roaming herds as well in zoological and conservation herds where bison are not handled as domestic cattle. Although embryo production was lower in random-start, unstimulated bison in the present study, results demonstrate the feasibility of embryo production in wild free-roaming bison after only a single handling.

Results are also consistent with the concept that the effect of superstimulatory treatment is brought about by preventing atresia and stimulating continued development of subordinate follicles within a wave (Adams et al., 1993; Nasser et al., 1993; Guerra et al., 2015). The effect in bison was more evident in those treated with the higher dose of eCG; COC collection and *in vitro* embryo production was greater after ovarian superstimulation with a single dose of 5,000 IU eCG than without superstimulatory treatment. While a dose of 1,000 to 3,000 IU eCG induced an effective superstimulatory response in cattle (Drion et al., 2001; Mapletoft et al., 2002; Aller et al., 2012; Bó and Mapletoft, 2014), results of the present study demonstrate that the lower dose of 2,500 IU eCG was not as effective for ovarian superstimulation for IVP in bison. Although eCG was one of the first superstimulatory drugs used in cattle, FSH is currently the most commonly utilized superstimulatory drug as the long half-life of eCG resulted in refractoriness after repeated use (Willett et al., 1953; Drion et al., 2001), and because high LH activity was associated with alterations in follicular and oocyte competence and lower *in vivo* embryo production rates (Moor et al., 1984). However, we do not see this as an immediate issue in wild bison herds as it would be unlikely that the same animal would be treated more than once in its lifetime. Repeated use of the eCG protocol in captive herds, however, would require further investigation of eCG sensitivity in bison. Un-ovulated follicles caused by continued ovarian stimulation (reviewed in Bó and Mapletoft, 2014), are also an issue for *in vivo* embryo collection. However, treatment with eCG followed by COC collection (aspiration of follicles ≥3 mm in both ovaries) circumvents the need for inducing ovulation. Although eCG has a relatively long half-life in cattle (i.e., 40 hours; Mapletoft et al., 2002), results of the present study established that the superstimulatory effect of eCG in bison did not extend to the time of a second COC collection 10 days after treatment.

Results support the hypothesis that COC collection and *in vitro* embryo production are greater during the anovulatory season than the ovulatory season which is consistent with a previous study (Palomino et al., 2020). More follicles were available for COC collection and a greater number of embryos were produced per bison during the anovulatory season than the ovulatory season. In contrast, the number of aspirated follicles in goats following laparoscopic COC collection was not different between seasons and the cleavage/embryo rate was higher in the ovulatory season (reviewed in De Souza-Fabjan et al., 2014). Seasonal differences disfavoring the summer months were also noted in water buffalo (reviewed in Gasparrini, 2019) when the number of follicles available and aspirated were lower. Additional water buffalo studies have indicated

lower maturation, cleavage and blastocyst rates as well as altered gene expression which was attributed to high environmental temperatures in the summer months (reviewed in Gasparrini, 2019).

Results also support the hypothesis that the number of follicles available for COC collection and *in vitro* embryo production is repeatable within individual bison. In non-superstimulated bison, the number of follicles counted and COC collected did not differ between the first and second synchronized follicular waves (i.e., after ablation). This is consistent with observations in cattle wherein COC collection was done twice weekly at a 4- day interval (Garcia and Salaheddine, 1998). The number of follicles ≥3 mm for individual bison in subsequent synchronized collections and between seasons were also corelated. This appears to reflect an inherent antral follicle count within individuals; i.e., the antral follicle count varies markedly among individuals but is repeatable within individuals (Singh et al., 2004; Morotti et al., 2017). As in previous studies in cattle, superstimulatory treatment in the present study resulted in continued growth of small follicles within the wave which increased COC competence (Vassena et al., 2003; Guerra et al., 2015). Individual variation for COC collection and IVP in bison was apparent in the present study, similar to that seen in cattle (Singh et al., 2004; Ireland et al., 2007). In the anovulatory season, one bison produced seven embryos from a single collection while another bison in the same group failed to produce any embryos. In the present study, the number of follicles ≥3 mm available for aspiration in individual bison varied from 2 to 77. While repeatability in antral follicle count provides a useful tool for predicting and selecting for fecundity in domestic cattle, it has less relevance in field settings with a wild ungulate captured randomly and only once.

The technical control using abattoir ovaries at the time of COC collection demonstrated that the IVP system performed to cattle standards with domestic cattle oocyte x bison sperm hybrid embryo production rates being >30% (number of embryos produced/COC submitted to *in vitro* maturation). This is consistent with domestic cattle oocyte x wisent (*bison bonasus*) sperm hybrids in which blastocyst rates did not differ from cattle oocyte x cattle sperm embryos (Shedova et al., 2017). In the present study, embryo production was lower for the bison oocyte x bison sperm combination than domestic cattle oocyte x bison sperm hybrids. This may be attributed to either compromised competence of bison COC or an IVF system has not yet been optimized for use in

bison. Future inquiries are required to distinguish factors affecting bison COC competence and *in vitro* culture conditions.

In summary, the present study resulted in the development of minimal-handling protocols for COC collection and IVP in bison using ovarian synchronization and superstimulation in bison. A single dose of 5,000 IU eCG increased the number and size of follicles available for COC collection, more than doubled the number of COC collected for IVP and resulted in the production of more embryos than other groups. Importantly, results also document that non-superstimulated COC collection for the purpose of IVP in bison is a viable option for conservation. Collections done after follicle wave synchronization resulted in greater embryo production than collections done at random stages of the follicular wave. Repeated COC collections after successive wave synchronization resulted in similar follicular counts and embryo production rates within individuals, and the greatest number of follicles aspirated, COC collected, and embryos produced was in the anovulatory season. The ovarian synchronization and superstimulation minimum-handling protocols examined in the present study were effective for COC collection and IVP in bison.

3.6. Acknowledgments

The study was supported by grants from the Natural Sciences and Engineering Research Council of Canada, and Parks Canada. The authors thank Steve Yang, Victoria Wallace and Chris Jermey for assistance with data collection. We also thank the staff of the Livestock and Forage Centre of Excellence for managing the bison at the Native Hoofstock Centre.

CHAPTER 4:

4. FIELD STRATEGIES FOR OOCYTE COLLECTION AND IN VITRO EMBRYO PRODUCTION IN FREE-ROAMING BISON HERDS

Miranda L Zwiefelhofer[1], Todd Shury[2,3], Eric M Zwiefelhofer[1], Jaswant Singh[1], Gabriela F Mastromonaco[4], Gregg P Adams[1]

[1]Departments of Veterinary Biomedical Sciences and [2]Veterinary Pathology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada
[3]Parks Canada Agency, Government of Canada, Saskatoon, Saskatchewan, Canada
[4]Department of Reproductive Sciences, Toronto Zoo, Toronto, Ontario, Canada

Relationship of this study to the dissertation

There is a need to transition oocyte collection protocols for use in free-roaming bison herds as chute handling systems are not available in remote locations. The use of field sedation to immobilize wild bison will be required to collect oocytes.

Authors' contributions

Miranda L Zwiefelhofer: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing-Original Draft, Visualization **Todd Shury:** Conceptualization, Methodology, Investigation, Resources, Writing-Review & Editing **Eric M Zwiefelhofer:** Conceptualization, Methodology, Investigation, Writing-Review & Editing **Jaswant Singh:** Conceptualization, Methodology, Resources, Writing-Review & Editing **Gabriela F Mastromonaco:** Conceptualization, Writing-Review & Editing **Gregg P Adams:** Conceptualization, Methodology, Resources, Writing-Review & Editing, Supervision, Funding acquisition

***Manuscript in preparation**

4.1. Abstract

The study was conducted to test the feasibility of protocols for field-collection of cumulus-oocyte complexes (COC) for *in vitro* embryo production (IVP) in wild bison. The study was done with captive wood bison during the late anovulatory season. In Experiment 1, the efficiency of transvaginal ultrasound-guided COC collection was compared between bison restrained in a squeeze chute without sedation vs in lateral recumbency after chemical immobilization using a dart gun (n=8/group). Initial COC collection was done at a random stage of the ovarian follicular wave and results were compared with a subsequent collection on Day -1 after follicular wave synchronization (wave emergence = Day 0). In Experiment 2, a 2x2 design was used to examine the effects of superstimulation treatment (single dose eCG vs multiple dose FSH) and method of drug administration (manual injection vs field darting) on COC collection and IVP. Initial COC collection (Day -1) served as a non-superstimulated random start (n=17) for comparison with a subsequent synchronized collection after superstimulation. In Experiment 1, no difference was detected between chute-restrained vs chemically immobilized groups in the time required to complete COC collections (6.9 ± 1.0 vs 8.9 ± 1.0 minutes; P = 0.2), the number of follicles aspirated (11.5 ± 1.9 vs 9.3 ± 1.8; P = 0.4) or the COC recovery rate (COC recovered/follicle aspirated; 58/92 [63%] vs 44/69 [64%]; P = 0.9). The number of follicles aspirated and COC recovered was similar between the random start and synchronized COC collection. In Experiment 2, no differences were detected between superstimulation treatments (eCG vs FSH). The total number of follicles available for aspiration did not differ between manual injection and field darting (23.9 ± 2.7 vs 21.6 ± 1.9; P = 0.4), but the number of follicles ≥8 mm was greater (10.1 ± 0.8 vs 7.0 ± 1.2; P = 0.04) in the manual injection group. Compared to the random start unstimulated group, the number of follicles ≥8 mm available for aspiration was greater (3.4 ± 1.5 vs 8.6 ± 0.8; P = 0.004) and the embryo production rate was higher (18/132 [14%] vs 53/189 [28%]; P = 0.04) after wave synchronization and superstimulation. Results suggest that COC collection is equally feasible in a recumbent position after chemical immobilization as those bison restrained in a standing position in a hydraulic chute. Ovarian superstimulation with a single-dose eCG protocol is as effective as a multiple-dose FSH protocol, and field darting is as effective as chute-side administration of superstimulation treatments. The strategies in the present study are ready to be incorporated into field collections in free-roaming bison herds.

4.2. Introduction

A bison genome biobank (repositories of cryopreserved sperm and embryos) is being developed for wood bison (*Bison bison athabascae*) which are classified by the Canadian Government Species at Risk Act (SARA) as Schedule 1 *threatened* (Environment and Climate Change Canada., 2018). The largest and most genetically diverse population of wood bison in the world is located in Wood Buffalo National Park (WBNP), Canada, but is infected by endemic brucellosis and tuberculosis (Joly and Messier, 2005). In 1970, 11 disease-free bison calves were salvaged from a group previously translocated from WBNP to Elk Island National Park (Environment and Climate Change Canada., 2018). These calves were the foundation genetics for a majority of disease-free wood bison conservation herds in the world today (Environment and Climate Change Canada., 2018). The creation and deployment of a bison genome biobank will provide security against future catastrophes (e.g. anthrax outbreak, New et al., 2017) which may result in abrupt population decline and subsequent loss of genetic diversity (Comizzoli et al., 2000). The critically endangered black-footed ferret (*Mustela nigripes*) is a prime example of the deployment a genome biobank for species conservation. Reproductive technologies were used to increase the population of black-footed ferrets from seven to more than 8,000 born in captivity, and enabled reintroduction of over 4,000 animals back to the wild (Howard et al., 2016).

The biobanking and subsequent redistribution of germplasm collected from isolated free-roaming herds will ensure sufficient genetic diversity for the long-term survival of the species, and minimize the risk of disease transmission (Thundathil et al., 2007). In remote locations, helicopters and planes are used to track, monitor and capture wild species such as bison (Caulkett, 2014; Slater, 2020); hence, protocols for the collection of germplasm from bison in wild settings are needed. Cumulus-oocyte complexes (COC) have been collected from bison ovaries recovered post mortem (Benham et al., 2021) and from live bison restrained in a chute (Cervantes et al., 2017a; b; Palomino et al., 2020, Chapter 3) to create *in vitro* produced embryos, but the collection of COC from immobilized bison has yet to be documented. Current COC collection protocols in bison rely primarily on multiple handlings and ovarian superstimulation treatment (Cervantes et al., 2017a; b; Palomino et al., 2020, Chapter 3) to increase follicular size and subsequent COC competence (Vassena et al., 2003). To align with field capture and handling standards to decrease stress in

bison (Slater, 2020), reduced handling techniques (maximum of 2 handlings) were investigated in the present study.

As a step toward establishment of a bison genome biobank, two experiments were done to test COC collection protocols designed for use in captive vs wild settings. Experiment 1 was designed to determine the effects of restraint (lateral recumbency after chemical immobilization vs standing position in a hydraulic chute) and allowed us to compare follicular wave status (random vs synchronized) on COC collection efficiency in non-superstimulated bison. Experiment 2 was designed to determine the effects of superstimulation treatment (single dose of equine chorionic gonodotrophin [eCG] vs multiple doses of follicle stimulating hormone [FSH] plus human chorionic gonadotrophin [hCG]) and method of administration (field dart vs manual injection) on COC collection efficiency and IVP.

4.3. Materials and methods

Experiments involved the use of mature female wood bison (n=33), ranging in age from 3-14 years, during the month of July (anovulatory season). The bison were maintained in corrals at the Native Hoofstock Centre near Saskatoon, Saskatchewan, Canada (52°N, 106°W) for the duration of the study and given free access to water and grass hay. Animal use was approved by the University of Saskatchewan's Animal Research Ethics Board (Protocol No. 20090058) and procedures were done in accordance with the guidelines of the Canadian Council on Animal Care.

4.3.1. Experiment 1 – Effects of restraint and wave synchronization in non-superstimulated bison

At random stages of ovarian follicular wave development, bison were assigned randomly to two groups (n=8 per group) for transvaginal ultrasound-guided COC collection in either a standing position without sedation (chute restraint) or in lateral recumbency after chemical immobilization (sedated; Fig. 4.1). The random start collection induced synchronous ovarian follicular wave emergence among bison on the following day (Day 0; Toosi et al., 2013; Palomino et al., 2014), and a second collection was done on Day 2 (synchronized start). The synchronized COC collections were done in the hydraulic chute without sedation (n=16). The experiment was

designed principally to examine the effect of restraint method, but also provided an opportunity to examine the effect of random vs synchronized follicular wave status.

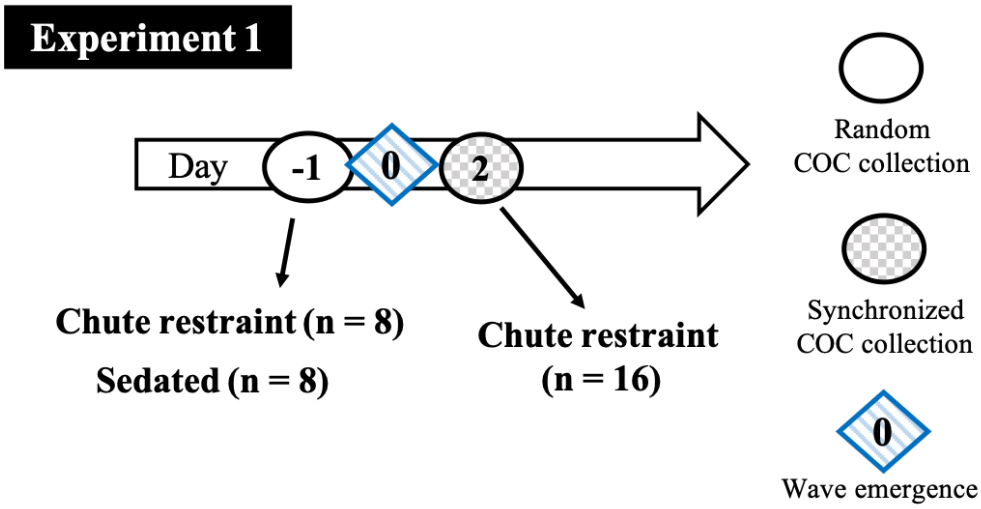


Figure 4.1. Experimental design for examining the effects of method of restraint and follicular wave synchronization on cumulus oocyte complex (COC) collection in non-superstimulated bison (Experiment 1). Chute restraint = bison collected in standing position without sedation, sedated = bison collected in recumbent position after chemical immobilization, n = number of bison per group, and random COC collection = collection at random stage of follicular wave.

In the chute restraint group, transvaginal ultrasound-guided COC collection was done in the standing position without sedation in a hydraulic chute (Fig 4.2). Caudal epidural anesthesia was induced with 5 mL of 2% lidocaine (Lurocaine; Vetoquniol, Lavaltrie, QC, Canada), as previously described (Palomino et al., 2014, Chapter 3). In the sedated group, COC collection was done in lateral recumbency with their left side down, after immobilization using a pre-mixed combination (BAM, 3.5-5.7 mL intramuscularly; Chiron Compounding Pharmacy, Guelph, ON, Canada) of butorphanol tartrate (27.3 mg/mL), azaperone tartrate (9.1 mg/ml), and medetomidine hydrochloride (10.9 mg/mL) delivered by a Dan-Inject CO_2 (Dan-Inject Canada, St. Albert, AB, Canada) dart rifle using 5 mL Dan-Inject 13 mm polycarbonate darts (Fig 4.3). Sedated bison were given supplemental oxygen intranasally via a portable oxygen cylinder during immobilization if deemed necessary (5-8 L/minute flow) and were blindfolded to reduce stimulation. Sedation was reversed with intramuscular naltrexone hydrochloride (50 mg/mL, 1cc) and atipamezole hydrochloride (25 mg/mL, twice the volume of BAM administered). Bison were released back to pasture after regaining normal ambulation.

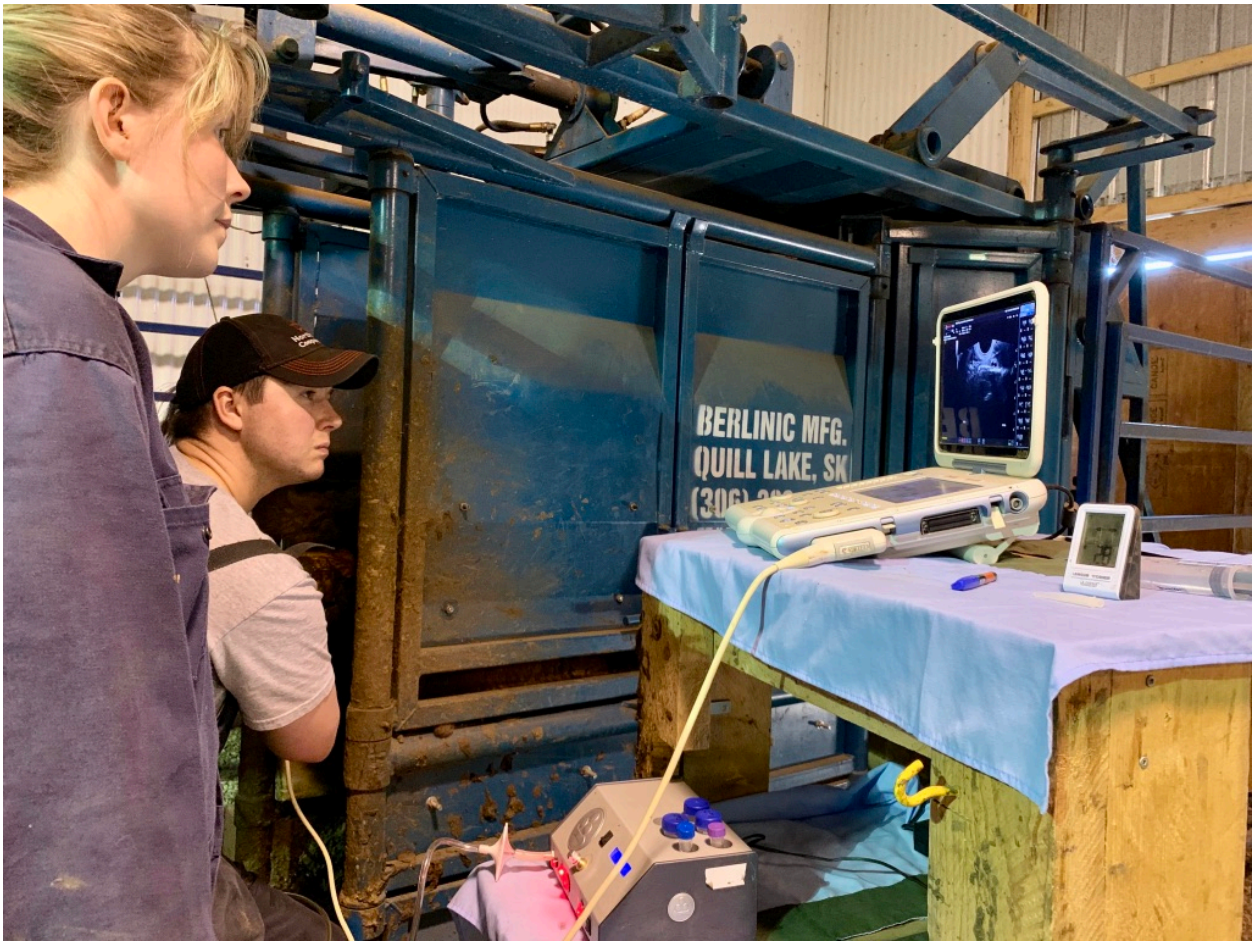


Figure 4.2. Cumulus oocyte complex (COC) collection on bison restrained in a hydraulic chute without sedation during the anovulatory season.

Figure 4.3. Cumulus oocyte complex (COC) collection on a chemically immobilized wood bison during the anovulatory season.

4.3.2. Cumulus oocyte complex collection & classification

Total bison handling time (duration of time the bison was first touched after capture in chute or full sedation until they were released from the chute or drug reversal was given) and COC collection time (duration of time between insertion and removal of the transvaginal probe) was recorded. The COC collection procedure was done in a manner similar to that previously described (Palomino et al., 2014b; Chapter 3). Briefly, the perineum was washed 3 times with surgical disinfectant, and the ovaries were examined and recorded by transvaginal ultrasonography (MyLab Alpha, Esaote North America Inc, Fishers, IN, USA) for later assessment of follicle numbers and size. COC were collected by transvaginal ultrasound-guided aspiration of follicles ≥3 mm in diameter using a short-beveled 18-ga x 2" disposable needle (WTA, Cravinhos, SP, Brazil) connected to a 50 mL conical tube by autoclaved medical grade Polytetrafluoroethylene (PTFE)

micro tubing (internal diameter 0.047 mm; Catalog # BB311-17, Scientific Commodities, Lake Havasu City, AZ, USA). Follicular contents were aspirated using a regulated vacuum pump set at a flow-rate of 12-16 mL/min (BV 003i Digital Vaccum Pump, WTA, Cravinhos, Sao Paulo, Brazil) into collection medium (BO-OPU, Catalog # 51001, IVF bioscience, Falmouth, Cornwall, United Kingdom) maintained at 37ºC. The follicular aspirate was filtered through a 75 µm mesh (IVF Oocyte Filter, Partnar Animal Health, Ilderton, ON, Canada) and stereomicroscopy (M8; Wild Heerbrugg, Heerbrugg, Switzerland and SMZ800, Nikon Instruments Inc., Americas) was used at 10x magnification to recover the COC. The COC were morphologically classified as described previously (Cervantes et al., 2017b, Chapter 3) and combined into a high-quality group (compact good, compact regular, and expanded) and low-quality group (compact poor, denuded and degenerate).

4.3.3. Experiment 2 – Effects of superstimulation treatment and method of administration

A separate group of bison (n=17) underwent transvaginal ultrasound-guided COC collection in a standing position without sedation, at random stages of the follicular wave. Using a 2 x 2 experimental design, bison were assigned randomly to two groups in which ovarian superstimulation was induced using a single dose eCG (n=9) vs multiple doses of FSH plus hCG (n=8; Fig. 4.4). Half of the bison in each superstimulation group were treated by manual intramuscular injection in the chute (n=9) or by field darting (3cc Type "U" aluminum barrel dart with 1.25 inch needle and gelatin collar, Pneu-dart, Williamsport, PA, USA; n=8; Fig. 4.4). Ovarian superstimulation was induced in the eCG group by a single 5,000 IU dose of eCG im (Folligon, Merck animal health, Kirkland, QC, Canada) given at the time of the random start collection (Day -1; Day 0 = expected day of follicular wave emergence; Chapter 3). Superstimulation was induced in the FSH group with 200 mg FSH im (Folltropin-V, Vetoquinol NA Inc., Lavaltrie, Quebec, Canada) on Day 0 and Day 2, and *in vivo* maturation was induced by treatment with 2,000 IU hCG im (Chorulon, Merck Animal Health, Summit, NJ, USA) on Day 4 (Cervantes et al., 2016). The second COC collection was done on Day 4 for the eCG group and on Day 5 for the FSH group (34 hours after hCG; Cervantes et al., 2017b).

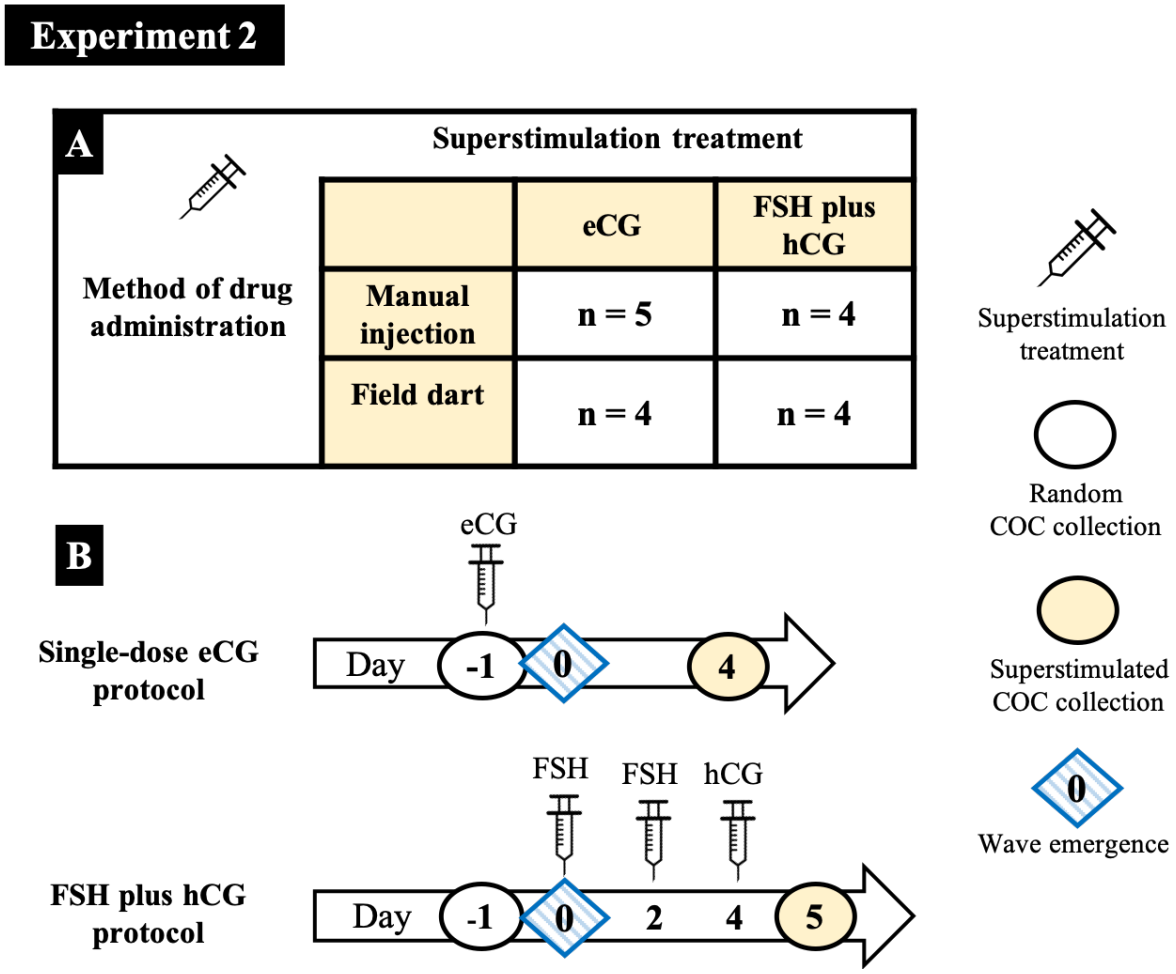


Figure 4.4. A) 2 x 2 experimental design for examining the effects of superstimulation treatment (single dose of eCG vs multiple doses of FSH) and method of administration (field dart vs manual injection) on cumulus oocyte complex (COC) collection efficiency and IVP. B) Ovarian superstimulation treatment protocols, single-dose eCG (5,000 IU equine chorionic gonadotrophin), and FSH plus hCG (two injections of 200 mg follicle stimulating hormone and 2,000 IU human chorionic gonadotrophin), n = number of bison per group (Experiment 2).

4.3.4. In vitro maturation, fertilization and culture

High- and low-quality COC were processed for embryo production, with the exception of degenerate COC which were discarded, as described for Experiment 1. The COC were washed and loaded into 1.5 mL sonification tubes (Active Motif, Carlsbad, CA, USA) with 1 mL (BO-HEPES-IVM; Catalog # 71001, IVF bioscience) into a portable incubator (Lab Mix Portable Incubator, WTA), as previously described (Chapter 3). The COC from the random and eCG

collections were matured *in vitro* for 25-28 hours as described previously (Chapter 3). In the FSH group, COC were collected one day later than in the eCG group to allow *in vivo* maturation (Cervantes et al., 2017b). However, only 20 of 96 COC collected from this group were expanded (matured); therefore, the non-expanded COC (n=76) were matured *in vitro* for 25 to 28 hours while the 20 expanded COC were matured *in vitro* for a maximum of 4 hours.

The COC (n = 5-20 per tube/group) were removed from the maturation tubes and washed once in a prewarmed and equilibrated 500 µl well of bovine IVF medium (BO-IVF, IVF bioscience, United Kingdom). Each group of COC were transferred to a pre-warmed and equilibrated 90 µl drop of BO-IVF covered with oil in a 35 mm petri dish. The dishes were placed in an incubator (Miri® Benchtop Multi-room Incubator, Esco Medical ApS, Denmark) at 38.8°C in 5% O_2, 5% CO_2 and 90% N_2. Two 0.5 mL straws of frozen-thawed wood bison semen were prepared, as described in Chapter 3. Each 90 µl drop of BO-IVF containing COC were fertilized with the prepared semen to a final concentration of 2.0 x 10^6 sperm/mL. The COC and sperm were co-incubated at 38.8°C in 5% O_2, 5% CO_2 and 90% N_2. Eighteen hours after the start of co-incubation with sperm, presumptive zygotes were denuded in wash medium (WASH; Catalog # 51002, IVF bioscience), and washed in 500 µl of the pre-warmed and equilibrated in culture medium (BO-IVC, Catalog #71005, IVF bioscience) at 38.8°C in 5% O_2, 5% CO_2 and 90% N_2. Each group of presumptive zygotes was placed in a pre-warmed and equilibrated 90 µl drop of BO-IVC covered with oil in a 35 mm petri dish and incubated at 38.8°C in 5% O_2, 5% CO_2 and 90% N_2. Presumptive zygotes were evaluated for cleavage (≥ 2-cell) 3 days after *in vitro* fertilization, and embryo evaluation was conducted, as described previously (Chapter 3).

4.3.5. Statistical analyses

Statistical analyses were performed using SAS 9.4 and Enterprise Guide 4.2 (Statistical Analysis System Institute Inc., Cary, NC, USA). Numerical scale data are represented as mean ± SEM and P-values were considered significant if ≤ 0.05.

In Experiment 1, the effect of the animal restraint system (sedation vs chute) on collection time, the number and size of follicles available for aspiration, the number of follicles aspirated, and the number and the quality of COC collected was compared by Student's t-test. The follicle aspiration efficiency (follicles aspirated/follicles available) and COC collection efficiency (COC

collected/follicles aspirated) was compared between groups by chi-square. The effect of synchronization (random start vs synchronized start) on the number and size of follicles available for aspiration, the number of follicles aspirated, and the number and the quality of COC collected was assessed by the Proc Mixed procedure for repeated measures. Follicle aspiration efficiency (follicles aspirated/follicles available) and COC collection efficiency (COC collected/follicles aspirated) were compared between groups by chi-square.

In Experiment 2, the effects of superstimulation treatment (eCG vs FSH) and method of drug administration (manual injection vs field darting) on the number and size of follicles available for aspiration, the number of follicles aspirated, and the number and the quality of COC collected were examined by analysis of variance using a general linear model (GLM) factorial design, and Tukey's *post-hoc* test was used for multiple comparisons if main effects or interactions were statistically different ($P \leq 0.05$). Proportional data including follicle aspiration efficiency, COC collection efficiency, and cleavage and blastocyst rates (based on the total number of COC used in each treatment group) were examined with the GLIMMIX procedure using binomial distribution and link logit function. Chi-square was used to compare the number of embryos produced per bison. The effect of maturation time in the FSH group (4 hours vs 25-28 hours) on cleavage and embryo rates were compared with the GLIMMIX procedure. Superstimulation treatment and method of administration were then combined to create a superstimulation group to be compared to the collection at random stages of the follicular wave. Analysis of variance using a general linear model (GLM) factorial design was used for the numerical endpoints described above, and Tukey's *post-hoc* test was used for multiple comparisons. Proportional data were examined with the GLIMMIX procedure using binomial distribution and link logit function and the number of embryos produced per bison was compared using chi-square.

4.4. Results

4.4.1. Experiment 1 – Effects of restraint and wave synchronization in non-superstimulated bison

Transvaginal ultrasound-guided COC collection was carried out successfully and without complication in all bison in each group (Fig. 4.2 & 4.3). The effects of the bison restraint (sedation

vs chute) on procedure efficiency are summarized in Table 4.1. No differences were detected between restraint systems in total handling time, COC collection time, the size and number follicles available, the number and quality of COC recovered, or follicular aspiration and COC collection efficiencies. In the sedated group, the total time from darting to ambulatory recovery was 50 ± 1.3 minutes. All bison recovered normally after immobilization and no mortality or morbidity was observed as a result of chemical immobilization.

The effects of the synchronization are summarized in Table 4.2. No differences were detected in any follicular or COC numerical endpoints between the random and synchronization groups. However, the random group had a greater COC collection efficiency than the synchronization (102/161 [63%] vs 74/151 [49%], Chi-square P = 0.02; respectively) but there was no difference between follicular aspiration rates (161/192 [84%] vs 151/191 [79%], P = 0.7; respectively).

Table 4.1. The effect of restraint system (hydraulic chute vs sedated) on handling time and cumulus oocyte-complex (COC) collection (mean ± SEM) in wood bison during the anovulatory season (Experiment 1).

	Chute	**Sedated**
Number of bison	8	8
Total handling time[1]	17.5 ± 3.4	23.5 ± 2.0
COC collection procedure time[2]	6.9 ± 1.0	8.9 ± 1.0
Number of follicles		
3-7.5 mm	13.9 ± 2.4	8.4 ± 1.3
≥8 mm	1.5 ± 0.3	1.4 ± 0.2
Total	15.4 ± 2.4	9.9 ± 1.3
Follicles aspirated	11.5 ± 1.88	8.63 ± 1.5
Follicular aspiration efficiency[3]	92/123 (75%)	69/69 (100%)
COC collection efficiency[4]	58/92 (63%)	44/69 (64%)
COC recovered		
High quality	3.4 ± 0.6	4.0 ± 0.9
Low quality	3.6 ± 1.0	1.6 ± 0.3
Total	7.3 ± 1.2	5.5 ± 0.9

[1]Duration of time (minutes) between first touch in the chute or after sedation until release from the chute or administration of drug-reversal
[2]Duration of time (minutes) between insertion and removal of the COC collection probe
[3]Follicles aspirated/follicles ≥3 mm available
[4]COC collected/follicles aspirated

Table 4.2. The effect of ovarian follicular wave synchronization on cumulus oocyte-complex (COC) collection (mean ± SEM) in wood bison during the anovulatory season (Experiment 1).

	Random[1]	Synchronized[2]
Number of bison	16	16
Number of follicles		
3-7.5 mm	12.6 ± 1.5	9.7 ± 1.3
≥8 mm	1.5 ± 0.2	0.9 ± 0.2
Total	12.8 ± 1.6	11.9 ± 1.5
Follicles aspirated	10.1 ± 1.2	9.4 ±1.2
Follicular aspiration efficiency[3]	161/192 (84%)	151/191 (79%)
COC collection efficiency[4]	102/161 (63%)[a]	74/151 (49%)[b]
COC recovered		
High quality	3.7 ± 0.5	3.1 ± 0.7
Low quality	2.6 ± 0.6	1.6 ± 0.4
Total	6.4 ± 0.8	4.6 ± 0.8

[1]COC collection done at random stages of the follicular wave
[2]COC collection done 3 days after random start collection (Day 2 of wave emergence)
[3]Follicles aspirated/follicles ≥3 mm available
[4]COC collected/follicles aspirated
[ab]Within rows, values with no common superscript are different (P ≤ 0.05).

4.4.2. Experiment 2 - Effects of superstimulation treatment and method of administration

The effects of superstimulation treatment (eCG vs FSH) and method of drug administration (manual injection vs field darting) on follicle numbers, cumulus oocyte-complex (COC) collection and *in vitro* embryo production are provided in Table 4.3. The superstimulation treatment and method of drug administration had no difference in the total number of ≥3 mm follicles available (P = 0.4) or the follicles aspirated (P = 0.7). However, the dart administered group had fewer ≥8 mm follicles than the manually injected groups (P = 0.04). The eCG group that was administered by darting had the highest follicular aspiration efficiency (method of administration x superstimulation interaction; P = 0.03; Table 4.3) and the lowest COC collection efficiency (method of administration x superstimulation interaction; P = 0.001; Table 4.3). There were a similar number of COC recovered from all groups (P = 0.5) and all treatment groups resulted in similar embryo production rates (total = 53/189 [28%]) and embryos produced per bison (total = 53/17 [3.1]). There was also no difference between the embryo production rate for oocytes

collected in the FSH group that were expanded and matured for 4 hours vs non-expanded and matured 25 to 28 hours (3/20 [15%] vs 23/76 [30%], P = 0.2; respectively).

Table 4.3. The effects of superstimulation treatment and method of administration on follicle numbers, cumulus oocyte-complex (COC) collection and *in vitro* embryo production (mean ± SEM) in wood bison during the anovulatory season (Experiment 2).

	FSH plus hCG[1]		eCG[2]	
	Manual injection	**Dart**	**Manual injection**	**Dart**
Number of bison	4	4	5	4
Number of follicles				
3-7.5 mm	16.0 ± 6.0	16.3 ± 2.4	12.0 ± 1.5	13.0 ± 2.7
≥8 mm[x]	10.25 ± 1.0	5.5 ± 1.6	10.0 ± 1.3	8.5 ± 1.6
Total	26.3 ± 5.9	21.8 ± 1.6	22.0 ± 2.0	21.5 ± 3.9
Follicles aspirated	23.3 ± 6.2	18.75 ± 3.3	19.0 ± 2.28	21.25 ± 3.99
Follicle asp. eff.[3,xy]	93/105 (89%)[a]	75/87 (86%)[a]	95/110 (86%)[a]	85/86 (99%)[b]
COC collect. eff.[4,xy]	50/93 (54%)[a]	59/75 (79%)[b]	64/95 (67%)[ab]	41/85 (48%)[a]
COC recovered				
High quality	8.3 ± 4.1	10.5 ± 3.0	9.6 ± 2.3	7.0 ± 2.1
Low quality	1.3 ± 0.6	2.3 ± 0.5	0.8 ± 0.8	1.5 ± 0.5
Total	12.5 ± 4.4	14.8 ± 2.8	12.8 ± 2.7	10.3 ± 2.3
Cleavage rate[5]	34/45 (76%)	37/51 (72%)	45/58 (78%)	23/35 (66%)
Embryo rate[6]	13/45 (29%)	13/51 (26%)	15/58 (26%)	12/35 (34%)
Embryos per bison	13/4 (3.3)	13/4 (3.3)	15/5 (3.0)	12/4 (3.0)

[1]200 mg FSH on Days 0 and 2, and 2,000 IU hCG on Day 4 (Day 0 = wave emergence)
[2]5,000 IU eCG on Day -1
[3]Follicles aspirated/follicles ≥3 mm available
[4]COC collected/follicles aspirated
[5]Number of presumptive zygotes cleaved/COC submitted to *in vitro* maturation
[6]Number of morulae and blastocysts produced/COC submitted to *in vitro* maturation
[ab]Within rows, values with no common superscript are different (P ≤ 0.05).
[x]Effect of method of drug administration (P ≤ 0.05)
[xy]Method of administration x superstimulation treatment interaction (P ≤ 0.05)

Data from the superstimulation treatment and method of administration groups were combined into a single superstimulation group to compare with random collection prior to superstimulation (Table 4.4). Although the total number of follicles available did not differ significantly between groups (P = 0.4), both the number of ≥8 mm follicles (P<0.004) and the

follicular aspiration efficiency (P<0.0001) were greater in the superstimulated group than in the non-stimulated random group (Table 4.4). The superstimulated group also had higher rates of cleavage (P = 0.03) and embryo development (P = 0.04), and produced a greater number of embryos than the random non-stimulated group (P = 0.01; Table 4.4).

Table 4.4. Comparison of embryo production after COC collection in non-stimulated bison at random stages of the ovarian follicular wave vs bison given superstimulation treatment after follicular wave synchronization in the anovulatory season (Experiment 2).

	Random, non-stimulated	**Synchronized, superstimulated[1]**
Number of bison	17	17
Number of follicles		
3-7.5 mm	16.4 ± 2.4	14.2 ± 1.6
≥8 mm	3.4 ± 1.5[a]	8.6 ± 0.8[b]
Total	19.8 ± 3.2	22.8 ± 1.7
Follicles aspirated	15.1 ± 9.7	20.5 ± 7.7
Follicular aspiration efficiency[2]	256/336 (76%)[a]	348/388 (90%)[b]
COC collection effiency[3]	151/336 (45%)	214/348 (61%)
COC recovered		
High quality	6.2 ± 1.7	8.9 ± 1.3
Low quality	2.5 ± 2.5	1.4 ± 1.3
Total	8.9 ± 2.0	12.6 ± 1.5
Cleavage rate[4]	70/132 (53%)[a]	139/189 (74%)[b]
Embryo rate[5]	18/132 (14%)[a]	53/189 (28%)[b]
Embryos per bison	18/17 (1.1)[a]	53/17 (3.1)[b]

[1]Superstimulation with either eCG or FSH plus hCG
[2]Follicles aspirated/follicles ≥3 mm available
[3]COC collected/follicles aspirated
[4]Number of presumptive zygotes cleaved/COC submitted to *in vitro* maturation
[5]Number of embryos produced/COC submitted to *in vitro* maturation
[ab]Within rows, values with no common superscript are different (Student's paired t-test P ≤ 0.05)

4.5. Discussion

The ability to collect COC from chemically immobilized bison is an important step for the use of advanced reproductive techniques in wild herds. Results of the present study revealed that the COC collection procedure was neither prolonged nor compromised while the bison was in lateral recumbency after chemical immobilization. The overall handling time for COC collection procedures did not warrant supplementary sedation for any individual. Animal captures in the wild

involve additional time for induction and recovery from sedation in chemically immobilized bison, which was not recorded in this study. However, the COC collection time recorded in the present study was within typical handling times for sedated captures for standard wildlife conservation activities (i.e., 20 minutes.; Slater, 2020). Furthermore, the COC collection procedure may be done concurrent with the activities of others related to the collection of biological data, samples for disease screening and securing tracking collars (Rhyan et al., 2009; Didkowska et al., 2021).

The COC collection efficiencies of the chemically immobilized bison were similar to collections done in a hydraulic chute without sedation. Challenging aspects of COC collection in chemically immobilized bison include down-time between individuals to allow for sedation and reversal, the position of recumbency after sedation, and the requirement of specialized personnel and supplementary oxygen. Sternal recumbency was not ideal as the perineal region was tilted toward the ground making the angle of palpation and transvaginal approach awkward. In the present study, those performing COC collections preferred the bison to be in left lateral recumbency as they palpate transrectally with their left hand. Rolling the bison from one side to the other, and adjustment to avoid obstructions was accomplished relatively easily with 2 or 3 people. A similar model has been established for oocyte collection in the rhinoceros as several sub-species of rhinoceros are critically endangered, including the northern white rhinoceros (*Ceratotherium simum cottoni*). Although rhinoceros can be acclimated to tolerate serial transrectal ultrasonography of the reproductive tract while standing (Pennington et al., 2019), successful oocyte collection has only been described using transrectal ultrasound-guided follicular aspiration while in lateral recumbency after chemical immobilization (Hermes et al., 2009).

The goal of synchronizing ovarian follicular wave emergence is to schedule COC collection at a time when most of the follicles ≥3 mm are in the growing phase (i.e., minimize the number of follicles in static and regressing phases) and to take advantage of a greater proportion of COC with developmental competence (Vassena et al., 2003). Consistent with this expectation, cleavage and embryo development rates were greater in the synchronized group than in the random group in our previous study (Chapter 3). As in the previous study (Chapter 3), the number of follicles available and the number and morphologic grades of COC recovered were similar between the random and synchronized groups in the present study. However, the effect of synchronization on embryo development was not tested in the present study. In cattle, COC collected on Day 2 of the follicular wave had lower embryo development rates than Day 5 (Vassena

et al., 2003). Future studies are required to assess the developmental competence of follicles early in the follicular wave in bison.

In the previous study (Chapter 3), the minimal-handling single-dose eCG superstimulation protocol was effective for embryo production in bison. Prior to this, the most successful superstimulation protocol in bison was a multiple-dose FSH plus hCG protocol (5 handlings; Cervantes et al., 2017b). In addition, the intended *in vivo* maturation effect of hCG (Cervantes et al., 2017b) was modest in the present study. The COC expansion rate in the FSH+hCG group was lower than previously reported in Cervantes (2017b; 20/109 [18.3%] vs 92/148 [62.2%], respectively) and resulted in the unanticipated need for *in vitro* maturation of 76 non-expanded COC in the FSH+hCG group. The ineffectiveness of including *in vivo* maturation in the present study may have resulted from the use of a smaller dose of hCG than that used previously (i.e., 2,000 IU hCG vs 2,500 IU) and/or by diluting the FSH in saline rather than hyaluronan. Interestingly, there was no difference in the rate of embryo development between expanded COC that did not undergo *in vitro* maturation and non-expanded COC that underwent 25 to 28 hours of *in vitro* maturation in the present study. This emphasizes the importance of the expansion of cumulus cells from the oocyte as an indicator for maturation and readiness of the oocyte for *in vitro* fertilization (Merton et al., 2003).

Whether in captive or free-roaming conditions, bison maintain their wild character and demeanor and are more difficult to handle than cattle. The single-dose, 2-time handling protocol provides an alternative to the multiple-dose FSH, 5-time handling protocol, and thereby reduces unwanted stress in wild bison (Caven et al., 2021). The elimination of 3 handlings is an important step in applying these technologies to bison in an effort to minimize damage to themselves, other bison and the handling equipment. In free-roaming herds, the 2-time handling protocol may be feasible for COC collections done on bison sedated from a helicopter, with the assistance of a tracking collar to relocate the animal for the second collection. While dart-administered treatment resulted in fewer follicles ≥8 mm than the manual treatment, there was no difference in the total number follicles available, and no effect on the number of COC recovered or embryos produced. Proper dart placement is important for ensuring drug effectiveness and rapid absorption (Larter et al., 2000), and may be mitigated for ovarian superstimulation by increasing the dose of the superstimulation drug given.

Importantly, the results of the present study document that COC collection at a random stage of the follicular wave is feasible and may be appropriate in the field. However, results also showed that synchronized, superstimulated COC collections resulted in a greater number of large follicles, COC recovered and embryos produced than random collections. Using the embryo production rates reported herein for random and synchronized-superstimulated collections (1.1 and 3.1 embryos per bison, respectively), for example, two captures of the same 5 bison 5 days apart (10 captures) would result in the production of 21 embryos [(1.1 x 5) + (3.1 x 5)]. To produce the same number of embryos using single random collection, 19 bison would need to be collected (19 captures). While the single random collection may be more costly, it would provide greater genetic diversity of germplasm. Embryo production from a single random COC collection may also be expected to be more variable since follicles at different stages contain COC of varying competence; Merton et al., 2003; Vassena et al., 2003). The latter is reflected in a comparison of the results of our previous (Chapter 3) and the present study; embryo production per COC collected was similar in the superstimulation groups but was 6% vs 14% in the random collection groups in respective studies.

Progress has been made on transitioning COC collection protocols for use in bison in free-roaming field conditions. In summary, COC collections can be done as effectively on sedated recumbent bison as those restrained in a standing position in a hydraulic chute, and ovarian superstimulation using a single-dose protocol is as effective as a multiple-dose protocol. Finally, field darting is an effective method of administering superstimulation treatments and ovarian superstimulation improves *in vitro* embryo production. Future studies will involve the implementation of these techniques for COC collection in free-roaming wild bison herds across North America.

4.6. Acknowledgments

The study was supported by a grant from the Natural Sciences and Engineering Research Council of Canada. The authors thank Vetoquinol who covered half of the cost of the Folltropin (FSH). The authors also thank Steve Yang, Victoria Wallace and Chris Jermey for assistance with data collection, and the staff of the Livestock and Forage Centre of Excellence for managing the bison at the Native Hoofstock Centre.

CHAPTER 5:

5. INFLUENCE OF REPRODUCTIVE STATUS ON OOCYTE COLLECTION AND *IN VITRO* EMBRYO PRODUCTION IN BISON

Miranda L Zwiefelhofer[1], Eric M Zwiefelhofer[1,2], Ella G Macquisten[1], Jaswant Singh[1], Gabriela F Mastromonaco[2], Gregg P Adams[1]

[1]Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine
University of Saskatchewan, Saskatoon, Saskatchewan, Canada
[2]Department of Reproductive Sciences, Toronto Zoo, Toronto, Ontario, Canada

Relationship of this study to the dissertation

When selecting female bison in the wild for oocyte collection, the approximate age of each bison can be determined from a distance while the reproductive status (i.e., pregnancy) cannot. This study allowed us to determine if cumulus oocyte complex collection done in pregnant (up to 120 days of gestation) and pre-pubertal yearling bison is feasible.

Authors' contributions

Miranda L Zwiefelhofer: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing-Original Draft, Visualization **Eric M Zwiefelhofer:** Conceptualization, Methodology, Investigation, Writing-Review & Editing **Ella G Macquisten:** Formal analysis, Investigation, Data curation, Visualization **Jaswant Singh:** Conceptualization, Methodology, Writing-Review & Editing **Gabriela F Mastromonaco:** Conceptualization, Writing-Review & Editing **Gregg P Adams:** Conceptualization, Methodology, Resources, Writing-Review & Editing, Supervision, Funding acquisition

***Manuscript in preparation**

5.1. Abstract

The objectives of the study were to determine the effects of reproductive status (non-pregnant, pregnant, and pre-pubertal) and advancing gestation (pregnant bison, 90 vs 120 days) on ovarian follicles, cumulus oocyte complexes (COC) collection and *in vitro* embryo production. A secondary objective was to examine the effect of follicular wave status (random vs synchronized) on *in vitro* embryo production. Transvaginal ultrasound-guided aspiration of follicles ≥3 mm was conducted in non-pregnant, pregnant, and pre-pubertal bison (n=4/group) at a random stage of the follicular wave and again five days later (synchronized) in two replicates (i.e., approximately 90 days and 120 days of gestation). Pre-pubertal bison had a greater number of follicles ≥3 than non-pregnant and pregnant bison (42.0 ± 3.3 vs 23.7 ± 3.1 vs 21.8 ± 2.3; P = 0.0001) and more COC recovered (23.0 ± 2.1 vs 12.9 ± 2.4 vs 5.7 ± 1.0; P = 0.0001. The total number of follicles was not different between random and synchronized collections (29.8 ± 3.4 vs 28.8 ± 2.7, respectively; P = 0.7), but as expected, fewer large follicles were present on Day 4 in the synchronized group (1.8 ± 0.3 vs 2.6 ± 0.4; P = 0.04). Collections on pregnant bison were more difficult with advancing gestation; COC collection efficiencies were lower at 120 vs 90 days (53% vs 79%; P = 0.0001). The embryo development rate tended to be lowest in the pre-pubertal group, intermediate in the pregnant group and highest non-pregnant group (17/332 [5.1%] vs 5/64 [7.8%] vs 19/182 [10.4%], respectively; P = 0.08). The number of embryos produced per bison tended to be lower in the pregnant group than the non-pregnant and pre-pubertal groups (5/16 [0.3] vs 19/16 [1.2] vs 17/16 [1.1]; P = 0.06, data combined between random and synchronized waves). In conclusion, both pregnant bison and pre-pubertal bison are valid options for COC collection in field conditions. Results document successful COC collection and embryo production from pregnant bison, but production was limited by ovary inaccessibility, fewer follicles and lower COC collection efficiency. Yearling pre-pubertal bison provide the most promise as COC donors in the field as they produced a similar number of embryos (approximately 1 freezable embryo per collection) as non-pregnant mature bison, and are available for collection throughout the winter season without the limitations imposed by advanced stages of pregnancy or unknown pregnancy status.

5.2. Introduction

The wood bison (*Bison bison athabascae*), a subspecies native to Canada, is classified by the Canadian Government Species at Risk Act (SARA) as Schedule 1 *threatened* (Environment and Climate Change Canada., 2018). A majority of wood bison conservation herds were derived from 11 founder animals from Elk Island National Park (Environment and Climate Change Canada., 2018). The exception is the greater Wood Buffalo National Park area which is home to the largest and most genetically diverse population of wood bison globally (Joly and Messier, 2005). To safeguard the future of wood bison, we are establishing a germplasm biobank comprised of embryos and semen collected from populations that have been separated in time and space to produce healthy offspring and restore genetic diversity (Adams et al., 2020).

The application of reproductive technologies in wild species is challenging as techniques are less developed in comparison to domestic species such as cattle and are difficult to implement in field conditions (Comizzoli et al., 2000). Transportation to and within remote herds such as Wood Buffalo National Park require helicopters and planes to track, monitor and capture wild species such as bison (Caulkett, 2014; Slater, 2020). The technique of collecting cumulus-oocyte-complexes (COC) has been tested in chemically immobilized, recumbent bison in preparation for collection in free-roaming bison herds (Chapter 4), but additional logistics for COC collection in the field include weather and animal selection. The ideal outdoor temperature for bison capture by aerial drug delivery is between -5°C and -30°C (Slater, 2020). This restricts the capture of bison largely to the winter months. Bison are seasonal breeders (i.e., mature females ovulate a single follicle approximately every 20-21 days from August to March until a pregnancy is established, Adams et al., 2020). As the gestation length of bison is approximately 265 days (Gogan et al., 2005a), and Wood Buffalo National Park reported a pregnancy rate of 72.2% in females ≥2 years of age (Joly and Messier, 2005), a majority of adult females are pregnant during the winter months. Previous research on COC collection in live bison has been done using mature, non-pregnant bison (Cervantes et al., 2017a; b; Palomino et al., 2020; Chapters 2, 3 & 4). Ovaries recovered post-mortem from bison of various reproductive stages, including juveniles and pregnant bison, have been used for COC collection to produce *in vitro* embryos (Benham et al., 2021). While the collection of COC has been reported in pregnant cattle up to approximately 100 days of gestation (Ooe et al., 1997; Eikelmann et al., 2000; Aller et al., 2012), COC collection from live pregnant

bison has not been reported. Similarly, COC collection has been done in pre-pubertal beef calves (Brogliatti and Adams, 1996), although the competence of oocytes to develop into embryos is positively correlated with the onset of puberty (Revel et al., 1995; Palma et al., 2001; Landry et al., 2016). Since the majority of mature female bison will be pregnant during the winter months, yearling bison are a group that warrants further investigation as COC donors for *in vitro* embryo production (IVP) and can be visually distinguished from other groups based on physical characteristics such as body size, horn size and shape, and pelage (Olson, 2012).

In support of an overall goal to enable COC collection from bison in a wild setting for the purposes of IVP and contribution to a bison genome biobank, the primary objectives were to determine the effects of reproductive status (non-pregnant, pregnant, and pre-pubertal) and advancing gestation (pregnant bison, 90 vs 120 days) on the size distribution of ovarian follicles, COC collection and IVP. A secondary objective was to determine the effect of follicular wave status (random vs synchronized) on IVP.

5.3. Materials and methods

5.3.1. Animals

The study was conducted in two replicates during the anovulatory season (May and June, 2021; respectively) using female wood and plains bison (n=8 and 4, respectively) between the ages of 1 and 10 years. During each replicate, the bison were maintained in corrals with free access to water and grass hay. Before and after each replicate, they were maintained on pasture. Animal procedures were approved by the University of Saskatchewan's Animal Care Committee and performed in accordance with the guidelines of the Canadian Council on Animal Care.

During the month of May, three female bison groups were used (n=4 per group); 1) mature, non-pregnant wood bison (age = 5.3 ± 0.3 years, weight = 520.3 ± 11.1 kg; mean ± SEM), 2) mature pregnant wood bison (age = 6.5 ± 1.2 years, weight = 523.1 ± 10.3 kg), and 3) pre-pubertal, non-pregnant plains bison (age = 12 months, weight = 193.3 ± 6.9 kg).

For each replicate, COC collection was done on the day the bison were brought in from pasture (i.e., random stage of their follicular wave), and again 5 days later (synchronized follicular wave; Fig. 5.1). The same 12 bison were used in the same three experimental groups (n=4 per group) for replicates 1 and 2; 1) mature, non-pregnant, 2) mature pregnant (Replicate 1 at

approximately 90 days of gestation; Replicate 2 at approximately 120 days of gestation), and 3) pre-pubertal (Replicate 1 at approximately 12 months of age; Replicate 2 at approximately 13 months of age). Bison were brought in from the pasture 22 days after their last collection (Replicate 1) and had COC collected again at a random stage of follicular wave and 5 days later for a synchronized collection.

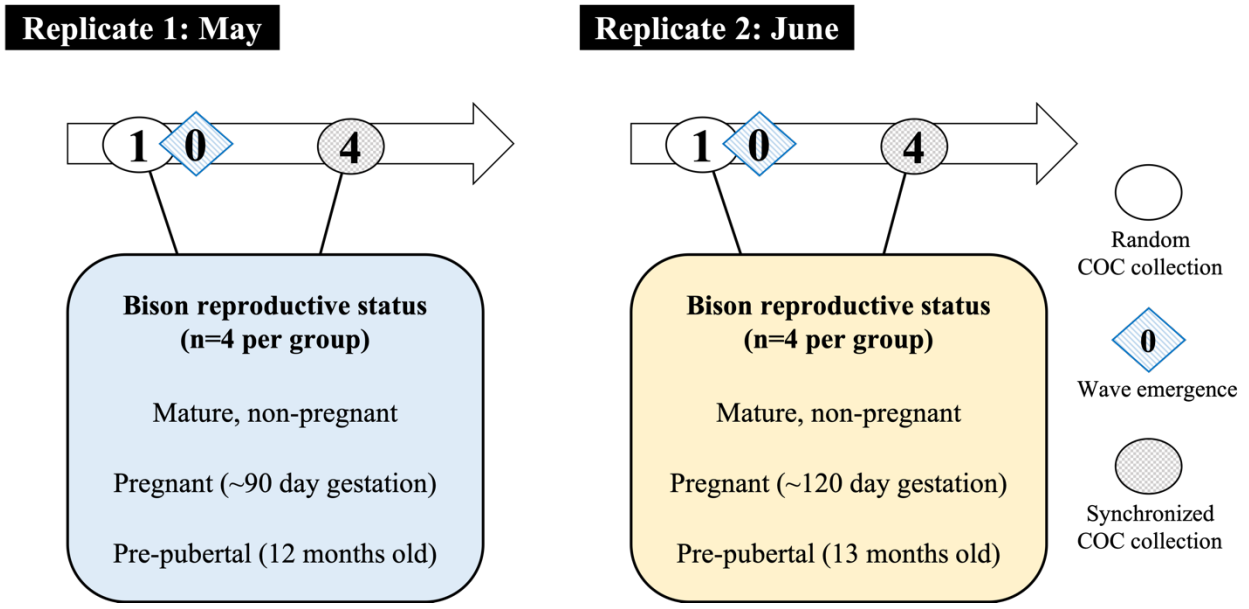


Figure 5.1. Experimental time-line for cumulus oocyte complex (COC) collection in non-pregnant, pregnant, and pre-pubertal bison, n = the number of bison used per group, random COC collection = collection at random stage of follicular wave.

5.3.2. Cumulus oocyte complex collection, classification, and in vitro maturation

Bison were restrained in a hydraulic chute; the perineum was washed and caudal epidural anesthesia was induced, as previously described (Palomino et al., 2014, Chapters 3 & 4) in preparation for transvaginal ultrasound-guided COC collection. The same technician and collection team were used throughout the experiment. A 5-8 MHz convex-array transvaginal probe (MyLab Alpha, Esaote North America Inc, Fishers, IN, USA) fitted to an extended handle was used to examine and record cine-loops of the ovarian structures. Cine-loops were examined on a later date to determine the number of follicles in small (3-4 mm), medium (4.5-7.5 mm) and large (>8 mm) categories. A vacuum pump (BV 003i Digital Vacuum Pump, WTA) was set at a 16-18 mL/min aspiration flow-rate. Aspiration of follicles ≥3 mm was done with a disposable 18-gauge

short-bevel needle (WTA, Cravinhos, SP, Brazil) connected to autoclaved medical grade polytetrafluoroethylene (PTFE) micro tubing (internal diameter 0.047 mm; Catalog # BB311-17, Scientific Commodities, Lake Havasu City, AZ, USA) and a 50 mL Falcon tube containing collection medium (Dulbecco's phosphate buffered saline; DPBS with Ca^{2+} and Mg^{+} [Catalog # D8662, Sigma-Aldrich, Oakville, ON, CAN] with 1% v/v pluronic F-68 non-ionic surfactant [100X; Catalog # 24040032, Gibco] and 2% v/v heparin sodium injection [1,000 USP/mL]) at 37°C. A 75 μm mesh oocyte filter (Partnar Animal Health, Ilderton, ON, Canada) was used to filter the follicular aspirate. Stereomicroscopy at 10x magnification (SMZ800, Nikon Instruments Inc., Americas) was used to locate and morphologically assess the COC. Degenerate COC were discarded while viable COC (compact, expanded and denuded COC [Cervantes et al., 2017a, Chapter 3]) were washed in oocyte and embryo wash medium (WASH; Catalog #51002, IVF bioscience, Falmouth, Cornwall, United Kingdom) three times and once in bovine hepes-buffered oocyte maturation medium (BO-HEPES-IVM; Catalog # 71001, IVF bioscience) prior to being placed in a 1.5 mL sonification tubes (Active Motif, Carlsbad, CA, USA) filled with BO-HEPES-IVM. Each tube contained COC from an individual bison with no more than 20 COC per tube. Sealed tubes were put in a portable incubator (Lab Mix Portable Incubator, WTA) maintained at 38.8°C (in air; i.e., without CO_2 incubation) for 24 to 28 hours.

5.3.3. In vitro fertilization and embryo culture

The COC, with individual bison ID maintained, were washed twice in 500 μl wells of bovine fertilization medium (BO-IVF, Catalog # 71004, IVF bioscience) prior to transfer into a prewarmed and equilibrated 35 mm petri dish with a 90 μl drop of BO-IVF covered with oil. The COC plates were put in 5% O_2, 5% CO_2 and 90% N_2 incubator (Miri Benchtop Multi-room Incubator, Esco Medical ApS, Egaa, Denmark) at 38.8°C. Two semen straws of a single wood bison bull, previously frozen and extended in Triladyl (REF: 13500/0250 Minitube, Ingersoll, Ontario, Canada) were thawed and processed through a 1 mL / 1 mL PureSperm 80 / 40 gradient (Catalog # PS40-100 & PS80-100; Nidacon, Gothenburg, Sweden) at room temperature. The semen pellet was then washed once in bovine semen preparation medium (BO-SemenPrep, Catalog # 71003, IVF bioscience) at 37.0°C. The sperm solution was added to the 90 μl drops with COC for a final concentration of 2.0×10^6 sperm/mL. The COC and sperm were then co-incubated

at 38.8°C in 5% O_2, 5% CO_2 and 90% N_2 for 18 hours. Oocytes were denuded of cumulus cells and the remaining sperm in BO-WASH prior to being washed in bovine embryo culture medium (BO-IVC, Catalog # 71005, IVF bioscience) and placed in a pre-warmed and equilibrated 90 µl drop of BO-IVC covered with oil in a 35 mm petri dish and incubated at 38.8°C in 5% O_2, 5% CO_2 and 90% N_2. Cleavage (≥ 2-cell) was evaluated at 3 days after *in vitro* fertilization and the embryos were evaluated for morula and blastocyst development 7 to 9 days after fertilization (Chapters 3 & 4).

5.3.4. Statistical analyses

Statistical analyses were performed using SAS 9.4 and Enterprise Guide 4.2 (Statistical Analysis System Institute Inc., Cary, NC, USA). Numerical scale data are represented as mean ± SEM and P-values are considered significant if ≤ 0.05.

The effects of reproductive status (non-pregnant, pregnant and pre-pubertal) and ovarian follicular wave status (random vs synchronized start) on the number and size of follicles available for aspiration, the number of follicles aspirated and the number and the quality of COC collected were examined by analysis of variance using a Proc Mixed procedure for repeated measures. If main effects or interactions were significant ($P \leq 0.05$), Tukey's *post-hoc* test was used for multiple comparisons. Proportional data (follicle aspiration efficiency [follicles aspirated/follicles ≥3 mm], COC collection efficiency [COC collected/follicles aspirated] as well as cleavage and blastocyst rates [based on the total number of COC used in each treatment group]), were examined with the GLIMMIX procedure using binomial distribution and link logit function. The number of embryos produced per bison was compared using Fisher's exact test. The effect of gestation stage (90 vs 120 days) on the number and size of follicles available for aspiration, the number of follicles aspirated and the number and the quality of COC collected were examined by paired t-test. Proportional data was compared by Chi-square.

5.4. Results

5.4.1. Effects of reproductive status and ovarian follicular wave status

Initial analysis revealed a replicate effect for only two endpoints: COC collection efficiency (COC collected/follicles aspirated) in the pregnant group was greater during the first replicate (~90 days gestation) vs the second replicate (120 days gestation), and follicle aspiration efficiency (follicles aspirated/follicles ≥3 mm) increased from the first to the second replicate. For all other endpoints, data from Replicates 1 and 2 were combined to determine the effects of reproductive status (non-pregnant, pregnant and pre-pubertal) and ovarian follicular wave status (random vs synchronized start) on ovarian follicle distribution, COC collection and embryo development (Table 5.1). The total number of follicles was greater in the pre-pubertal group than in the non-pregnant and pregnant groups (42.0 ± 3.3 vs 23.7 ± 3.1 vs 21.8 ± 2.3, respectively; P = 0.0001; Fig. 5.2) as a result of more follicles in the small- and medium-size categories (P < 0.001). Fewer large follicles were detected in the pregnant group vs other groups (P = 0.001). Consequently, more follicles were aspirated in the pre-pubertal group than in the non-pregnant and pregnant groups (33.6 ± 3.1 vs 17.7 ± 2.9 vs 8.6 ± 1.2; P = 0.0001).

The total number of follicles was not different between random and synchronized collections (29.8 ± 3.4 vs 28.8 ± 2.7, respectively; P = 0.7), but as expected, fewer large follicles were present on Day 4 in the synchronized group (1.8 ± 0.3 vs 2.6 ± 0.4; P = 0.04). Follicle aspiration efficiency was greater after synchronized vs random collection in non-pregnant and pre-pubertal groups, but not in the pregnant group (reproductive status by wave status interaction, P = 0.001). The COC collection efficiency was highest after random collection in the non-pregnant group, but was otherwise similar among groups (reproductive status by wave status interaction, P = 0.03).

The number of viable COC recovered differed among each of the reproductive status groups (P = 0.0001); it was greatest in the pre-pubertal group, intermediate in the non-pregnant group and lowest in the pregnant group (20.4 ± 2.1 vs 10.7 ± 2.1 vs 4.3 ± 0.9). Fewer degenerate COC were recovered after wave synchronization than at random stages of the wave (1.2 ± 0.3 vs 2.6 ± 0.6; P = 0.03). A reproductive status x wave status interaction (P = 0.02) on cleavage rate was attributed to an increase in the synchronized vs random group for pre-pubertal bison only; no difference was detected in non-pregnant and pregnant groups. No main effect or interaction was detected in embryo development rate; however, it tended to be lowest in the pre-pubertal group, intermediate in the pregnant group and highest in the non-pregnant group (17/332 [5.1%] vs 5/64 [7.8%] vs 19/182 [10.4%], respectively; P = 0.08). The overall number of embryos produced per

bison tended to be lower in the pregnant group than the non-pregnant and pre-pubertal groups (5/16 [0.3] vs 19/16 [1.2] vs 17/16 [1.1], respectively; P = 0.06, data combined between random and synchronized waves).

Table 5.1. The effect of reproductive status on follicle numbers, cumulus oocyte-complex (COC) collection and *in vitro* embryo production (mean ± SEM) in bison. COC collection was done on a group of bison at a random stage of the follicular wave (Day -1; Wave emergence = Day 0) and five days later (synchronized; Day 4).

	Non-pregnant		Pregnant		Pre-pubertal	
Follicular wave	**Random[1]**	**Synch[2]**	**Random**	**Synch**	**Random**	**Synch**
Number of bison	8	8	8	8	8	8
Number of follicles						
3-4 mm[x]	14.5 ± 3.9	12.3 ± 2.6	12.6 ± 3.0	18.0 ± 2.3	29.6 ± 3.9	23.1 ± 4.2
4.5-7.5 mm[x]	6.6 ± 1.5	8.3 ± 2.0	4.3 ± 0.8	7.1 ± 1.3	14.0 ± 2.4	12.1 ± 1.7
≥8 mm[x,y]	3.4 ± 0.5	2.4 ± 0.4	1.3 ± 0.3	0.9 ± 0.3	3.1 ± 0.8	2.0 ± 0.4
Total[x]	24.5 ± 4.5	22.9 ± 4.7	18.1 ± 3.2	26.0 ± 2.7	46.8 ± 4.2	37.3 ± 4.8
Follicles aspirated[x]	15.5 ± 3.3	19.9 ± 5.0	6.0 ± 1.2	11.6 ± 1.5	32.4 ± 4.9	34.8 ± 4.1
Follicle asp. eff.[3,xy]	124/196[a] (63.3%)	159/183[b] (86.9%)	48/145[c] (33.1%)	81/182[c] (44.5%)	259/374[a] (69.3%)	278/298[b] (93.3%)
COC collect. eff.[4,xy]	107/124[a] (86.3%)	99/159[b] (62.3%)	34/48[b] (70.8%)	51/81[b] (63.0%)	192/259[ab] (74.1%)	176/278[b] (63.3%)
COC recovered						
Viable[x]	10.1 ± 3.2	11.3 ± 3.1	2.3 ± 0.6	6.6 ± 1.2	20.6 ± 3.3	20.3 ± 3.0
Degenerate[x,y]	3.1 ± 1.0	1.1 ± 0.4	0.8 ± 0.4	0.7 ± 0.7	3.9 ± 1.1	1.8 ± 0.5
Total[x]	13.4 ± 3.8	12.4 ± 3.1	4.3 ± 1.3	7.3 ± 1.3	24.0 ± 2.8	22.0 ± 3.3
Cleavage rate[4,xy]	67/92[a] (72.8%)	62/90[a] (68.9%)	13/18[ab] (72.2%)	27/46[ab] (58.7%)	88/170[b] (51.7%)	113/162[a] (69.8%)
Embryo rate[5]	11/92 (12.0%)	8/90 (9.0%)	2/18 (11.1%)	3/46 (6.5%)	12/170 (7.0%)	5/162 (3.1%)
Embryos per bison	11/8 (1.4)	8/8 (1.0)	2/8 (0.3)	3/8 (0.4)	12/8 (1.5)	5/8 (0.6)

[1]COC collection occurred at a random stage of the follicular wave
[2]COC collection occurred three days later after the initial random collection
[3]Follicles aspirated/follicles ≥3 mm
[4]Number of presumptive zygotes cleaved/COC submitted to *in vitro* maturation
[5]Number of morulae and blastocysts produced/COC submitted to *in vitro* maturation
[abc]Within rows, values with no common superscript are different ($P \leq 0.05$).
[x]Effect of reproductive status ($P \leq 0.05$)
[y]Effect of follicular synchronization ($P \leq 0.05$)
[xy]Reproductive status x follicular synchronization interaction ($P \leq 0.05$)

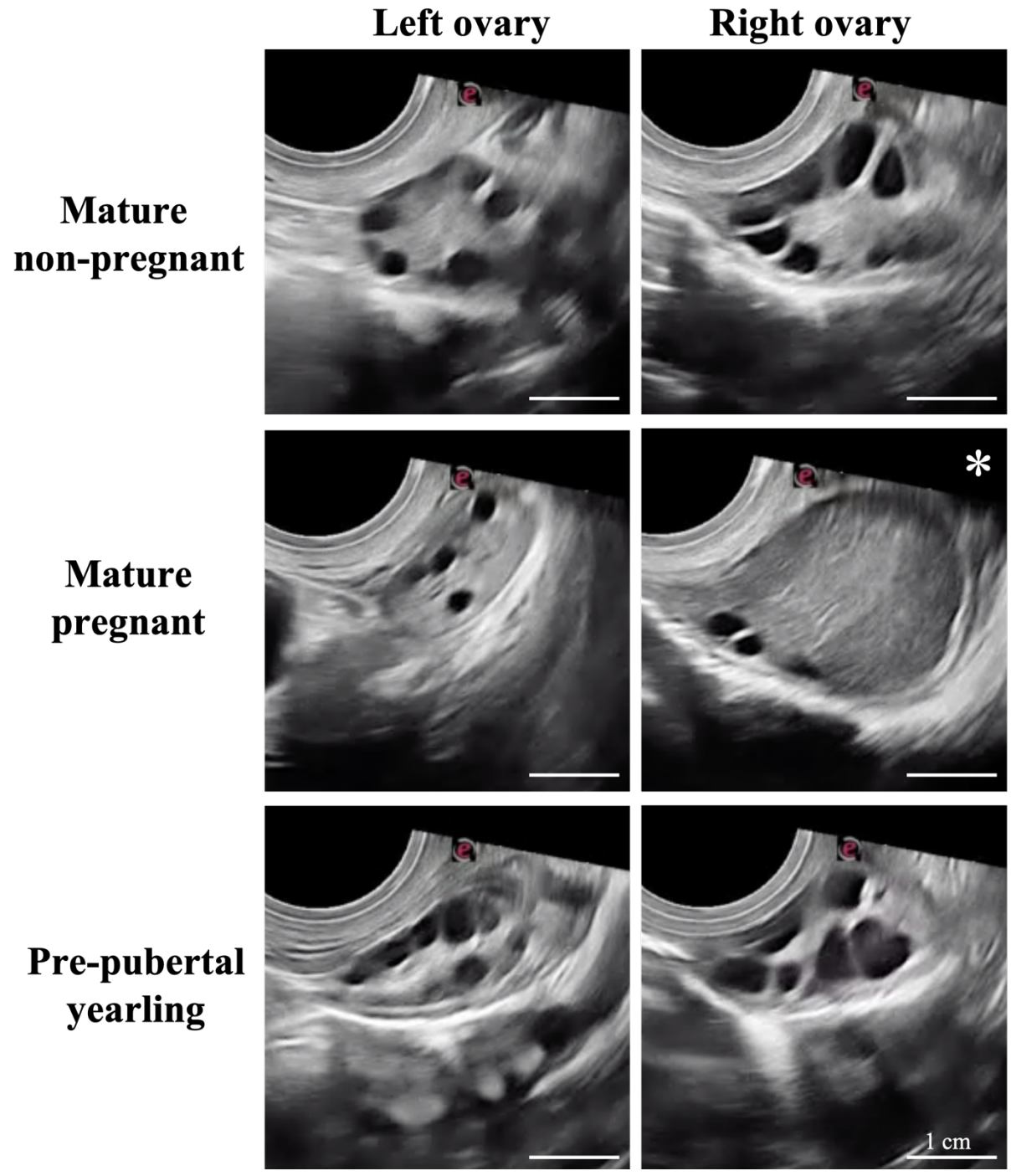


Figure 5.2 Transvaginal ultrasound images of ovarian structures at a synchronized stage (4 days after wave emergence) during the anovulatory season (May) for mature non-pregnant, mature pregnant (90 days of gestation), and pre-pubertal yearling bison. (*the ovary with the CL, ipsilateral to the gravid uterine horn).

5.4.2. Effects of advancing gestation (90 vs 120 days)

The effects of advancing gestation (i.e., Replicate 1 vs Replicate 2) are summarized in Table 5.2). As a result of a slight decrease in the number of follicles in each size category, the total number of follicles detected tended to be lower at 120 days gestation than at 90 days ($P = 0.09$). A single corpus luteum was detected in the ovary ipsilateral to the gravid uterine horn in all pregnancies, and fewer follicles were detected in the ipsilateral vs contralateral ovary (mean = 7.3 ± 1.6 vs 14.7 ± 8.4, respectively; $P < 0.05$). Ovary accessibility during COC collection (number of ovaries successfully collected from / number of ovaries) was greater for the contralateral ovary than the ipsilateral ovary in pregnant bison (14/16 [87.5%] vs 9/16 [56.3%], respectively; $P = 0.05$). Compared to 90 days of gestation, COC collection at 120 days was associated with lower follicle aspiration efficiency ($P < 0.001$) and a lower collection efficiency ($P = 0.0001$).

Table 5.2. The effect of advancing gestational age (90 vs 120 days) on follicle numbers (mean±SEM) and cumulus oocyte-complex (COC) collection in the anovulatory season in wood bison.

Gestational age	**90 days**	**120 days**
Number of bison	8	8
Ovary accessibility relative to gravid horn (and CL)[1]		
Contralateral ovary	7/8 (87.5%)	7/8 (87.5%)
Ipsilateral ovary	5/8 (62.5%)	4/8 (50.0%)
Number of follicles		
3-4 mm	17.4 ± 3.1	12.6 ± 2.2
4.5-7.5 mm	6.8 ± 1.0	4.3 ± 1.1
≥8 mm	1.3 ± 0.4	0.9 ± 0.1
Total	25.4 ± 3.1	17.7 ± 2.9
Follicles aspirated	7.9 ± 1.6	9.4 ± 1.8
Follicle aspiration efficiency[2]	63/203[a] (31.0%)	66/124[b] (26.7%)
COC collection efficiency[3]	50/63[a] (79.4%)	35/66[b] (53.0%)
COC recovered		
Viable	4.8 ± 1.6	3.7 ± 0.5
Degenerate	1.0 ± 0.7	0.4 ± 0.3
Total	6.3 ± 1.5	5.0 ± 1.3

[1]Number of ovaries successfully collected from/number of ovaries
[2]Follicles aspirated/follicles ≥3 mm
[3]COC collected/follicles aspirated
[ab]Within rows, values with no common superscript are different ($P \leq 0.05$)

5.5. Discussion

The present study was designed to develop feasible COC collection protocols in free-roaming bison herds for use in a genome biobank for bison. The objectives of the study were to determine the effects of reproductive status (non-pregnant, pregnant, and pre-pubertal), advancing gestation (pregnant bison, 90 vs 120 days) and follicular wave status (random vs synchronized) on COC collection and IVP. As the capture of wild bison is primarily conducted in the winter months, when most mature female bison are pregnant, we were interested in the feasibility of COC collection in pregnant bison. Oocyte collection has been reported in cattle up to approximately 100 days of gestation (Ooe et al., 1997; Eikelmann et al., 2000; Aller et al., 2012). In cattle, attempts at COC collection were increasingly difficult due to the anatomical position of the ovaries as gestation progressed (Eikelmann et al., 2000). In the present study in pregnant bison, the operator was unable to palpate one or both of the ovaries in 7 of 16 attempts; i.e., transvaginal ultrasound-guided follicle aspiration was not possible for all pregnant bison. Both follicle aspiration efficiency and COC collection efficiency were lower at 120 days vs 90 days of gestation in bison resulting in fewer COC recovered and fewer embryos produced per bison overall. However, embryos were produced from pregnant bison without disrupting gestation; hence, pregnant bison may be a realistic option for collections in the wild, particularly during the earlier part of the breeding season from September to December. The added stress of capture and anesthesia during collections in free-roaming bison warrants further investigation.

Ultrasound-guided COC collections have been reported on cattle as young as 6 weeks of age (Brogliatti and Adams, 1996). Puberty in beef cattle, defined as the onset of ovulatory cyclicity, occurs at around 13 months of age (reviewed in Adams, 1999). However, unlike domestic cattle, the onset of ovulatory cyclicity in bison is affected by both age and season. Although a small proportion of bison may become sexually receptive at one year of age (i.e., the first breeding season of their life; Halloran, 1968), the first ovulation was detected in approximately 50% of bison at 28 months of age (i.e., the second breeding season of their life) and in 100% of bison at 40 months of age (the third breeding season of their life; reviewed in Adams et al., 2020). In the present study, the pre-pubertal (yearling) bison had a greater number of follicles available for aspiration, resulting in a greater number of COC recovered than all other groups. However, the embryo development rate per COC tended to be lower in the pre-pubertal group than

the non-pregnant group (5% vs 10%) resulting overall in a similar number of embryos per bison in the two groups (1.1 vs 1.2 embryos per bison). Similar embryo production rates were reported for plains bison after collection of COC from slaughterhouse ovaries from juveniles (1.5 to 2.5 years old) and mature plains bison (8% vs 8%; Benham et al., 2021). High follicle counts in pre-pubertal bison is consistent with studies in cattle where 4-month old heifers had higher antral follicle counts than 7-month old heifers (Evans et al., 1994; Krause, 2019). As well, high numbers of COC have been recovered from pre-pubertal cattle, but with lower COC competence, resulting in similar overall embryo production to that of adult cows (Landry et al., 2016). Others have reported no difference in developmental competence of COC from prepubertal heifers but a higher incidence of early embryonic loss post-transfer, possibly due to a delay in the kinetics of nuclear maturation (Khatir et al., 1998). Ovarian superstimulation in pre-pubertal cattle increased the developmental competence of COC and subsequent embryo development (Brogliatti et al., 1997; Baldassarre et al., 2018; Krause et al., 2020). Ovarian superstimulation protocols designed specifically for bison (Chapter 3 & 4) may be applied to increase the effectiveness of COC collection in yearling bison to offset the effects of sexual immaturity. The selection of yearling bison appears to be a promising reproductive subgroup for COC collection in free-roaming bison herds. They are easily identified, unlikely to be pregnant and have produced approximately 1 embryo per collection.

A single COC collection at a random stage of the follicular wave is the most plausible scenario for use in wild conditions, and the present study allowed another opportunity to assess the effect of random vs synchronized COC collections (see Chapters 2, 3 & 4). Surprisingly, results showed no detectable differences between random vs synchronized collections in follicular and COC endpoints or embryo production, contrary to results reported in Chapter 3 where lower embryo development rates were observed in the random vs synchronized collections. One limitation of the present study, however, was that the second COC collection (Replicate 2) may not have represented a true random stage of the follicular wave, as intended. The synchronizing effect of Replicate 1 may have extended to Replicate 2, done 22 days later, particularly since the inter-wave interval during the anovulatory season was reported as 7 days (McCorkell et al., 2013). Hence, the collection in Replicate 2 of the present study was inadvertently done around the time one might expect a third follicular wave to emerge (3 waves x 7 days = 21 days). Results of previous studies (Chapter 3 & 4) demonstrated the positive effects of ovarian synchronization and

superstimulation on embryo production in bison. However, these additional procedures may not always be feasible in field conditions. Results of the present study suggest that COC collection at random and unstimulated stages of ovarian follicular development are viable options for use in wild free-roaming herds where bison can be handled only once.

Embryo development in the present study was low (overall = 7% [ranged 3-12%]), but similar to previous reports. As a technical laboratory control for the present study, hybrid embryos (cattle oocyte x bison sperm) were used during two IVF runs, resulting in an acceptable 27% embryo production rate. Previous studies utilizing bison post-mortem samples (i.e., random stage of follicular wave) reported 5 to 16% blastocyst rates (Thundathil et al., 2007; Aurini et al., 2009; Barfield and Seidel, 2011; Krishnakumar et al., 2015; Benham et al., 2021). We previously reported embryo production rates for non-superstimulated random collections to be 6% and 14% and synchronized collections to be 20% in wood bison (Chapters 3 and 4). The use of ovarian superstimulation and the selection of morphologically graded compact-good COC for bison IVP has resulted in embryo production rates as high as 54% (Cervantes et al., 2017a). Although COC may be divided into separate groups and processed (high vs low quality), all COC recovered from bison in the wild will likely be used because of their genetic value and resource cost, and therefore lower embryo production rates would be expected. Variability between individual animals may be a factor in the inconsistency of embryo production rates. In the present study, bison identities were maintained through the experiments in preparation for future biobanking, where genetic identity is very important. All 3 of the embryos produced in the pregnant group were from a single animal. In the pre-pubertal group, individual bison produced 4, 5, 2 and 0 embryos. In the non-pregnant group, individual bison produced 4, 3, 1 and 0 embryos. In several observations, including the present study, a single chamber in the incubator (6 chambers total) will contain a culture plate that produces 0 freezable embryos while another culture plate produces 5 freezable embryos right next to it while in the same culture conditions. Another observed factor was that many embryos were low-quality (Grade 3 or lower; IETS standards) non-compact morulae at the embryo evaluation stage and therefore not counted as freezable embryos. Further studies are warranted to investigate embryo development from the time of first cleavage to the embryo evaluation stages.

In conclusion, bison in various reproductive states (non-pregnant, pregnant, pre-pubertal) are valid options for COC collection in field conditions for IVP. Oocytes were collected and embryos were produced from bison at both 90 and 120 days of gestation, but inaccessibility of the

ovaries at the more advanced stage reduced COC collection efficiency. Yearling pre-pubertal bison provide the most promise as COC donors in the field as they produced a similar number of embryos (approximately 1 freezable embryo per collection) as non-pregnant mature bison and are available for collection throughout the winter season without the limitations imposed by advanced stages of pregnancy or unknown pregnancy status. The present study has shown new and feasible options for COC collections for IVP in free-roaming bison herds that can contribute to bison genome biobanks.

5.6. Acknowledgments

The authors would like to acknowledge the Natural Sciences and Engineering Research Council of Canada and Boehringer Ingelheim Veterinary Scholarship for research funding. The authors would also like to thank the staff of the Livestock and Forage Centre of Excellence for managing the bison at the Native Hoofstock Centre.

CHAPTER 6:

6. FACTORS AFFECTING SURVIVABILITY OF *IN VITRO* PRODUCED WOOD BISON (*BISON BISON ATHABASCAE*) EMBRYOS AFTER CRYOPRESERVATION

Miranda L Zwiefelhofer[1], Gabriela F Mastromonaco[2], Eric M Zwiefelhofer[1,2], Jaswant Singh[1], Gregg P Adams[1]

[1]Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine
University of Saskatchewan, Saskatoon, Saskatchewan, Canada
[2]Department of Reproductive Sciences, Toronto Zoo, Toronto, Ontario, Canada

Relationship of this study to the dissertation

There is a need to create a species-specific bison embryo banking protocol to select embryos of a specific developmental stage and morphologic grade on specific freeze days for optimal cryopreservation. This study aided in the identification of specific factors that can help embryo survival after cryopreservation and warming resulting in pregnancies and subsequent calves born. The study also allowed us to compare the effect of two embryo cryopreservation methods on pregnancy rate.

Authors' contributions

Miranda L Zwiefelhofer: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing-Original Draft **Gabriela F Mastromonaco:** Conceptualization, Methodology, Investigation, Writing-Review **Eric Zwiefelhofer:** Conceptualization, Methodology, Investigation, Writing-Review **Jaswant Singh:** Conceptualization, Methodology, Investigation, Resources, Writing-Review **Gregg P Adams:** Conceptualization, Methodology, Resources, Writing-Review & Editing, Supervision, Funding acquisition

***Manuscript in preparation**

6.1. Abstract

As part of an overall effort to establish a functional germplasm biobank for bison conservation, three experiments were designed to determine factors affecting the post-thaw viability of pre-implantation *in vitro* produced (IVP) embryos in wood bison. Exp. 1 determined the effect of *in vitro* embryo characteristics on post-thaw survivability parameters of vitrified embryos (n=67 bison embryos, 15 hybrid [cattle x bison] embryos) by TUNEL and DAPI staining. Exp. 2 compared the pregnancy rates after the transfer of IVP embryos vitrified at different stages of development and days post-fertilization (n=28 embryo transfers), while Exp. 3 determined the effect of vitrification vs programmed freezing in glycerol to fresh embryo transfers (n=26 embryo transfers). In Exp. 1, the morulae cryopreserved on Day 7 or 8 tended to have a greater proportion of nuclear morphology score 1 (i.e., fewer abnormal nuclei post-thaw) than Day 7 or 8 blastocysts (10/12 [83.3%] vs 6/12 [50.0%]; P = 0.09). The blastocyst embryos cryopreserved on Day 7 or 8 (high quality embryos) tended to have higher cell counts than Day 9 or 10 blastocysts (P = 0.08). The high quality (Grade 1 and 2 combined) blastocysts had higher cell counts than low quality blastocysts (cryopreserved on Day 9 or 10 groups only; P=0.03). Embryos produced from hybrids had a higher proportion of embryos on Day 7 or 8 than embryos produced from bison (Day 7 or 8 embryos/total embryos produced, 15/15 [100.0%] vs 25/67 [37.3%]; P<0.0001). In Exp. 2, five pregnancies were confirmed at the 30 day pregnancy diagnosis and two were lost by 60 days. The morula group had a greater 60-day pregnancy rate than the blastocyst group (3/7 [42.9%] vs 0/21 [0%]; P=0.02). The day the embryo was cryopreserved after fertilization (Day 7 or 8 vs Day 9 or 10) had no effect on pregnancy rate at 30 or 60 days (P=0.6). All pregnancies made it full term and two live calves were born. In Exp. 3, pregnancies were detected at 30-days after the transfer of fresh (2/9 [22.2%]) and vitrified (2/8 [25.0%]) embryos but not from slow frozen in glycerol embryos (0/9 [0%], P=0.2) with the live birth of three calves. In conclusion, IVP embryos of higher quality had less cryo-damage and higher cell counts. Morulae survived cryopreservation with less damage than blastocysts, and subsequent transfer resulted in a higher pregnancy rate at 30-days and lower pregnancy loss between 30 to 60 days than blastocysts. Transfer of vitrified embryos resulted in a similar pregnancy rate to the transfer of fresh embryos. Future efforts will focus on bison-specific IVP culture conditions to improve embryo development and pregnancy rates.

6.2. Introduction

Assisted reproductive techniques such as gamete recovery, artificial insemination, and embryo production and transfer have been explored as biosecure options for the retrieval of genetics from isolated bison herds and for the creation of a bison genome biobank (Robison et al., 1998; Thundathil et al., 2007). Wood bison (*Bison bison athabascae*) are classified as Schedule 1 *threatened* by the Canadian Government Species at Risk Act (SARA; Environment and Climate Change Canada, 2018). The use of cryo-stored genome biobanks facilitates gene flow between *in situ* (i.e., free-ranging herds) and *ex situ* (i.e., captive herds and cryo-stored germplasm) herds (Comizzoli et al., 2000) without translocating stress-susceptible wild animals, which is dangerous and a biosecurity risk (Wildt, 2000). Protocols have been developed to collect cumulus oocyte complexes (COC) by transvaginal ultrasound-guided collection and by postmortem retrieval of reproductive material to produce *in vitro*-derived (IVP) embryos (Palomino et al., 2020; Benham et al., 2021). However, the post-thaw viability of IVP bison embryos has not been critically investigated to date.

Studies using cattle have shown that *in vitro* culture conditions have produced embryos that differed physically and chemically from *in vivo* embryos due to an inadequate culture system that does not mimic the natural *in vivo* environment (Leibo et al., 1995; Hasler, 2000). *In vitro*-derived cattle embryos are of lower morphological grade (Rizos et al., 2002) and result in lower pregnancy rates following transfer than *in vivo*-derived embryos (Hasler et al., 1995). Selection of embryos with specific characteristics during culture, such as stage, age and morphologic grade, improved pregnancy rates after transfer in cattle (Hasler, 2000). The additional stress of cryopreservation on embryos also resulted in lower pregnancy rates after transfer (Hasler et al., 1995). Previous studies have shown that IVP cattle embryos are sensitive to the process of chilling and cryopreservation (Pollard and Leibo, 1994). The cryopreservation process damaged the embryo through physical, chemical and osmotic damages to the cells (Overstrom, 1996; Baguisi et al., 1999). Moreover, the developmental stage, species, and culture conditions impacted cryotolerance (Pollard and Leibo, 1994).

In vitro analysis of embryos post-thaw using staining and microscopy techniques allows assessment of embryo quality and viability without the necessity of transferring embryos into recipients. Studies in domestic cattle have shown earlier and increased levels of apoptosis in IVP

embryos (Gjorret, 2004) and cryopreserved embryos (Park et al., 2006; Sudano et al., 2014). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays have been used to identify apoptotic DNA fragmentation, an indicator of programmed cell death, in a variety of species including mice, pigs, cattle, gaur and bison x cattle hybrid embryos (Brison and Schultz, 1997; Hao et al., 2003; Gjorret, 2004; Mastromonaco et al., 2007; Seaby et al., 2012). While *in vitro* analytical tools provide valuable information to determine the developmental potential of embryos, live-birth after embryo transfer is an essential endpoint to assess the effect of factors related to the *in vitro* production and cryopreservation processes.

The overall objective of this study was to begin to establish criteria upon which to base interpretations and decisions regarding the viability of IVP bison embryos to be used in a germplasm biobank. The specific objective of Experiment 1 was to determine the effect of embryo factors (embryo stage, morphologic grade, freeze-day and post-thaw culture) on post-thaw survivability assessed by apoptosis, nuclear and cell morphology, and total cell count of IVP bison embryos. The data permitted test of 4 hypotheses; 1) Morulae have fewer abnormal and apoptotic nuclei than blastocysts when cryopreserved on Day 7 or 8, 2) Blastocysts cryopreserved on Day 7 or 8 have fewer abnormal and apoptotic nuclei than blastocysts cryopreserved on Day 9 or 10, 3) Morphologic grading is positively related to quantitative characteristics of viability (abnormal and apoptotic nuclei, total cell count) and 4) Hybrid embryos have fewer abnormal and apoptotic nuclei than bison embryos. The objectives of subsequent studies were to determine the effect of embryo factors (Exp. 2) and cryopreservation method (Exp. 3) on pregnancy and calving rates following transfer of IVP wood bison embryos.

6.3. Materials and methods

Embryo recipients were wood bison (n=61) ranging in age from 3 to 16 years old. Bison were maintained in corrals during recipient synchronization until embryo transfer and given free access to water and grass hay. After embryo transfer, bison were released to pasture and brought back into the facility for pregnancy diagnosis before being released to pasture again. Animal use was approved by the University of Saskatchewan's Animal Research Ethics Board (Protocol No. 20090058) and in accordance with the guidelines of the Canadian Council on Animal Care.

6.3.1. Experiment 1 - Embryo factors related to post-thaw viability

The IVP wood bison embryos used for Experiment 1 were produced and vitrified in previous studies (n=72, Chapter 3) using a commercial bovine media kit (IVF bioscience, Falmouth, Cornwall, United Kingdom). In brief, cumulus oocyte complexes (COC) were collected from female wood bison donors using transvaginal ultrasound-guided follicular aspiration (5-8 MHz convex-array probe; MyLab Alpha, Esaote, Fishers, IN, USA), matured *in vitro* for 25 to 28 hours, co-incubated with frozen-thawed wood bison semen overnight, denuded and cultured. The developmental stage and morphologic grade of the embryos were monitored daily from 7 to 10 days after fertilization using 40X stereomicroscopy (SMZ800, Nikon Instruments Inc., Americas). Hybrid embryos (wood bison semen x cattle oocyte), produced in Chapter 3, were used in the present study as a technical control (n=21). The COC for the technical control group were aspirated from slaughterhouse cattle ovaries immediately after the bison collections chute-side, and were matured *in vitro* for 20-24 hours. The hybrid embryos were graded according to the bison embryo scale described below to be consistent with the bison embryos.

Identification of the development stage of bison embryos was based on standards used for cattle (Bó and Mapletoft, 2013); morula, early blastocyst, blastocyst and expanded blastocyst (Fig. 6.1). However, bison-specific characteristics made staging and grading more challenging. In general, bison oocytes and embryos have exhibited darker cellular matter and higher incidences of vacuolization than cattle, making them more difficult to assess (Thundathil et al., 2007; Barfield, 2019). The staging and grading of bison and hybrid embryos are illustrated in Fig. 6.1. The distribution of embryos analyzed, excluding those lost or damaged during thawing, fixing and staining is shown in Table 6.1 (n=67 bison embryos, n=15 hybrid embryos).

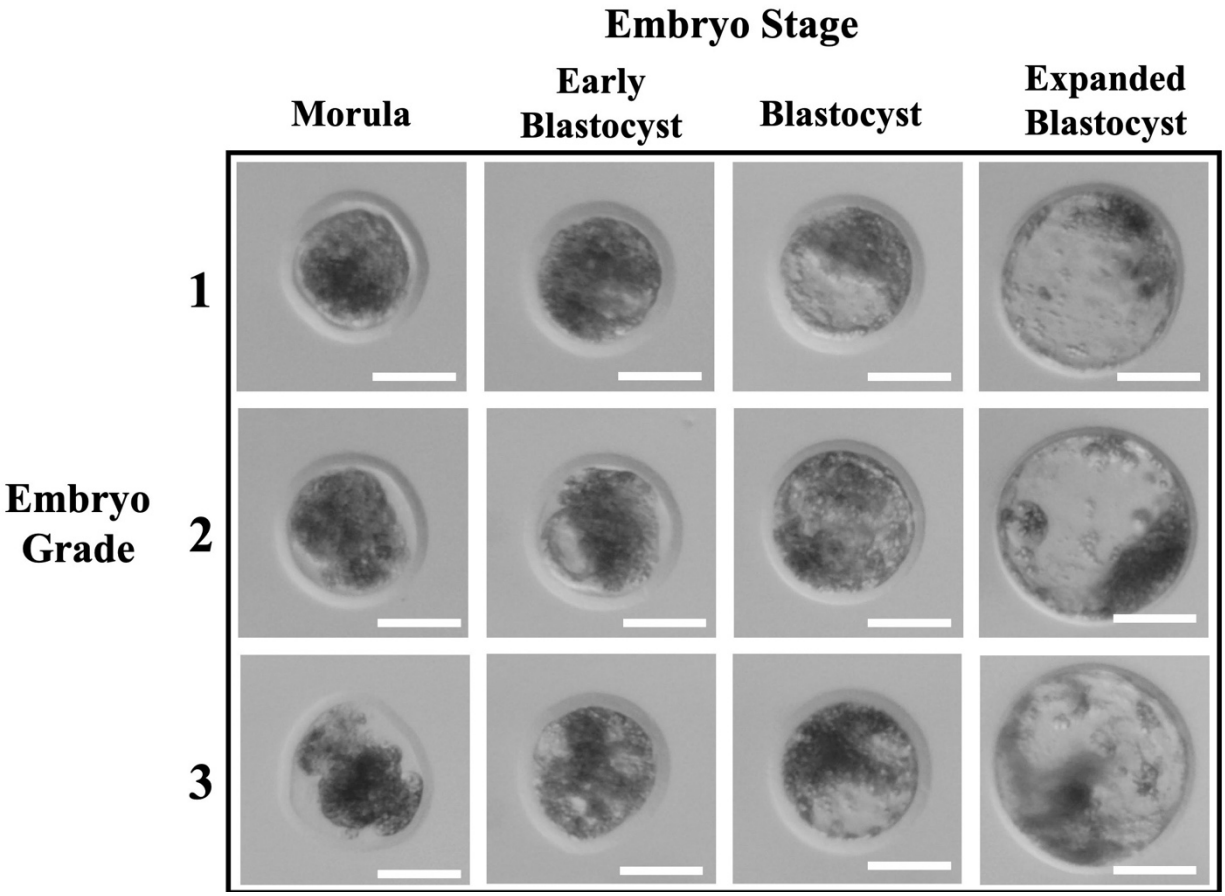


Figure 6.1. Classification (embryo stage and morphologic grade) of *in vitro*-produced bison embryos (scale bar = 50 µm). **Embryo stage: morula**, **early blastocyst**, **blastocyst,** and **expanded blastocyst**. **Embryo Grade 1:** At least 75% of the of the cellular material is intact (i.e., not extruded into the perivitelline space). Vacuoles, if present, are minimal in size and represent ≤25% of cellular material. The cellular material has a consistent texture and overall color. No dark cells, no cracks or adhesions to the zona pellucida, and the zona pellucida is either spherical, oval or pear-shaped. **Embryo Grade 2:** At least 50% of the of the cellular material is intact (i.e., not extruded into the perivitelline space). Vacuoles, if present, vary from small to large in size and represent up to 50% of cellular material. No cracks or adhesions to the zona pellucida, and the zona pellucida is either spherical, oval or pear-shaped. **Embryo Grade 3:** At least 25% of the cellular material is intact (i.e., not extruded into the perivitelline space). Vacuoles, if present, vary from small to large in size and represent up to 75% of cellular material. The zona pellucida may have cracks or adhesions and is either spherical, oval or pear-shaped.

Table 6.1. Distribution of post-thaw embryo factors of wood bison and hybrid (wood bison semen x cattle oocyte) embryos analyzed in Experiment 1 for post-thaw survivability characteristics.

Wood bison (n=67)*				
Freeze day[1]	**Day 7 or 8**		**Day 9 or 10**	
Embryo stage	**Morula**	**Blastocyst[2]**	**Morula**	**Blastocyst**
High quality[3]	12	3 + 3 + 6 = 12	0	1 + 7 + 9 = 17
Low quality[4]	0	1 + 0 + 0 = 1	0	5 + 18 + 2 = 25
Hybrid (cattle x bison; n=15)*				
Freeze day	**Day 7 or 8**		**Day 9 or 10**	
Embryo stage	**Morula**	**Blastocyst**	**Morula**	**Blastocyst**
High quality	5	4 + 3 + 3 = 10	0	0
Low quality	0	0	0	0

*n = number of embryos analyzed, excluding those lost or damaged during thawing, fixing and staining
[1]Number of days after *in vitro* fertilization that the embryo was cryopreserved
[2]Early blastocyst + blastocyst + expanded blastocyst = combined blastocyst group
[3]High quality = Grade 1 & 2
[4]Low quality = Grade 3

Embryos were cryopreserved using a commercial vitrification kit (VitriCool, Catalog #63001, IVF bioscience) according to the manufacturer's protocol, in 50 µl drops at room temperature. A single embryo was moved using a stripper micropipetter with a 290 µl EZ-tip (CooperSurgical, Trumbull, CT, USA) into the *pre-incubation* medium for 2 minutes, *cooling 1* medium for 2 minutes, and *cooling 2* medium for <30 seconds. The embryo was then transferred with 1-2 µl of the medium onto a Cryotop device (Kitazato, Tokyo, Japan) and plunged directly into liquid nitrogen, capped, loaded into canes and stored in liquid nitrogen for future use. The number of days after fertilization that each embryo was cryopreserved was referred to as the freeze day (Days 7-10). Embryo stage, grade and freeze day assessment occurred at the same time immediately before cryopreservation.

Embryos were warmed individually using a commercial warming kit (VitriWarm, Catalog # 63002, IVF bioscience). Each vitrification device containing a single embryo was uncapped

under liquid nitrogen and immediately placed in *warming 1* medium for 3 minutes, *warming 2* medium for 2 minutes, *warming 3* medium for 2 minutes and *warming 4* medium for 30 seconds at 37°C. Individual embryos were thawed and cultured separately to maintain embryo identity. Each embryo was washed once in bovine embryo culture medium (BO-IVC, Catalog #71005, IVF bioscience) and placed into a 90 µl drop of BO-IVC covered with oil in a 35 mm petri dish which was equilibrated and incubated at 38.8°C in 5% O_2, 5% CO_2 and 90% N_2 (Miri Benchtop Multi-room Incubator, Esco Medical ApS, Egaa, Denmark). Half of the bison and hybrid embryos were photographed at 0- and 1- hour of culture and fixed at 1 hour. The remaining half were photographed at 0-, 1-, 24- and 36- hours and fixed at 36 hours post-thaw. If developmental progress was observed between 0 and 36 hours, expansion progress was recorded as YES if the embryo advanced in development (blastocyst creation, blastocyst expansion, expansion + collapsing and hatching) or NO if no advancement in development occurred.

Individual embryos were stained and fixed in multi-well dishes to maintain identity. Embryos were washed three times in 0.2% polyvinyl alcohol (PVA) in PBS (wt/vol), and then fixed using 4% paraformaldehyde (PFA) in PBS at room temperature for 1 hour and maintained in 1% PFA at 4°C until staining (Gjorret, 2004). Embryos were permeabilized in 0.5% Triton X-100 (BIO-RAD, Hercules, CA, USA) in PBS at room temperature for 1 hour. Embryos were then washed three times in PBS-PVA. The TUNEL reaction was done using a commercial kit (*In situ cell death detection kit, Fluorescein;* Roche Diagnostics, Mannheim, Germany) as previously described (Brison and Schultz, 1997) with minor changes. Each embryo was incubated in 10 µl of the TUNEL mixture (1 µl terminal deoxynucleotidyl transferase [TDT] & 9 µl FITC labelled nucleotide mixture) for 1 hour at 37°C in the dark. The embryos were then washed twice in 0.5% Triton X-100 in PBS and twice in PBS PVA at room temperature. Embryos were washed in increasing concentrations of vectashield antifade mounting medium with DAPI (4′,6-diamidino-2-phenylindole, Vector Labs., Burlingame, CA, USA; Catalog # H-2000) and individually mounted in the center of reinforcement tags (Avery, Pickering, ON, Canada). A coverslip was then placed on the slide and sealed with nail polish. To serve as technical positive controls for validating the staining procedure, cattle embryos (cattle sperm x cattle oocyte) were incubated in 10 µl of 25% DNase 1 (GE Healthcare Life Sciences, Piscataway, NJ, USA) in PBS (v/v) for 1 hour at 37°C after permeabilization to induce DNA fragmentation. Cattle embryos incubated without TDT served as negative controls.

Embryos were maintained at 4°C in the dark after staining. Within one day of staining, an epifluorescence laser microscope (Zeiss Axioskop 5 Carl Zeiss Ltd., Toronto, ON, Canada) with a camera (Nikon 1 V1, Nikon Canada Inc., Mississauga, ON, Canada) was used to immediately record the full scan of the embryo as the TUNEL stain was short-lived. A subset of embryos was selected to undergo further imaging using the Zeiss LSM 710 confocal microscope (Carl Zeiss Microscopy, White plains, NY, USA). Embryo videos were then assessed by an evaluator blinded to the original stage/grade groups using a clear grid system attached to the computer screen. The number of nuclei was used for total cell count (i.e., number of DAPI positive structures). Each embryo was given a TUNEL score using the proportion of nuclei with TUNEL labeling (Score 1: <50% apoptotic nuclei; Score 2: ≥50% apoptotic nuclei) and a nuclear morphology score using the proportion of nuclei abnormal morphology (fragmented or condensed; Score 1: <50% abnormal nuclei; Score 2: ≥50% abnormal nuclei, Gjorret, 2004).

6.3.2. Experiment 2: Embryo factors related to pregnancy rates after embryo transfer

The wood bison embryos used were produced and vitrified in previous studies (Chapters 3 & 4), as described in Experiment 1. The embryos used were those that were cryopreserved using three different vitrification devices (Cryotop, Kitazato, Tokyo, Japan; Cryolock, Irvine Scientific, Santa Ana, CA, USA; Vitrifit, Origio, Måløv, Denmark). The distribution of embryos is shown in Table 6.2 (n=28 embryos). Embryos were warmed individually in a field lab adjacent to the bison handling facility using the protocol described in Experiment 1. The embryo was then washed once in transfer medium (Catalog # 61006, IVF bioscience) and loaded individually into a 0.25 mL straw with embryo transfer and transport medium. The embryo straw was loaded into an embryo transfer catheter (YTgun; YamaneTech, Nagano, Japan). The perineum of recipient bison was washed using an iodine-based detergent and rinsed with saline. A single embryo was transferred into the uterine horn ipsilateral to the corpus luteum (CL) in recipients with a CL ≥15 mm.

Embryo recipients were mature female wood bison (n=32) ranging between the ages of 3 to 15 years. Embryo transfers were done during October (ovulatory season) in 2019. The wood bison recipients were synchronized for ovulation in two replicates on successive days. To confirm ovarian cyclicity before the initiation of the study, transrectal ultrasonography (7.5 MHz linear array probe, MyLab Alpha, Esaote, Fishers, IN, USA) was done to detect the presence of a corpus

luteum (CL). Each bison was fitted with an electronic estrus detection sensor (Heatwatch, Cow Chips, Manalapan, NJ, USA) to aid in estrus detection. Ultrasound-guided follicular ablation was done on Day -1 to induce emergence of a new follicular wave (Day = 0; Toosi et al., 2013b) and was immediately followed by the insertion of an progesterone-releasing intravaginal device (Eazi-Breed CIDR, Zoetis, Kirkland, QC, Canada; Fig. 6.2). On Day 5, the progesterone device was removed, and bison were given 500 μg cloprostenol im (PGF; Bioestrovet, Vetoquinol, Lavaltrie, QC, Canada). On Day 8, 2,500 IU human chorionic gonadotrophin (hCG; Chorulon, Merck, Kirkland, QC, Canada) was given im. Transrectal ultrasonography was done at 12-hour intervals from Day 7 to 9 (for 48 hours) to assess ovulatory follicle growth and ovulation. Embryo transfer was done 7 days after ovulation. Bison that did not ovulate were not used as embryo recipients. Heatwatch software was used to determine if expression of estrus (≥2 mounts within an hour) was related to pregnancy rate after embryo transfer.

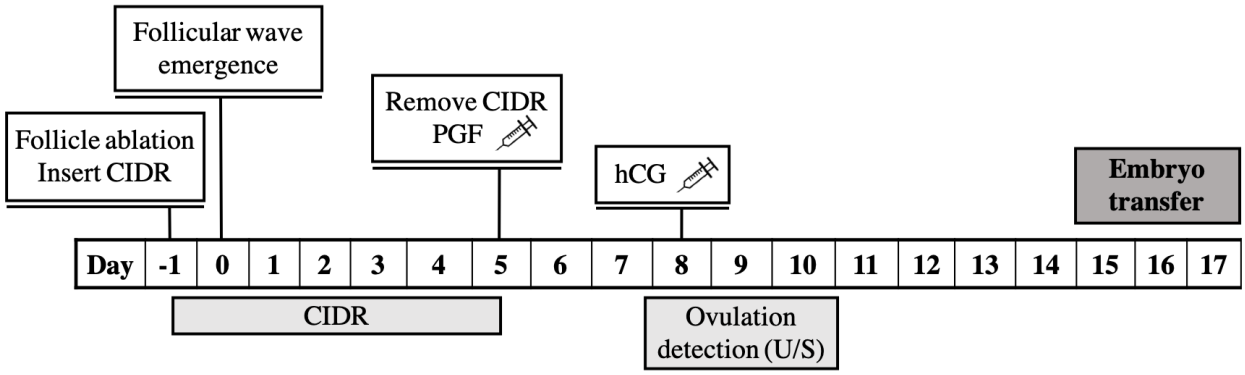


Figure 6.2. Synchronization protocol for the transfer of *in vitro*-produced wood bison embryos wood bison recipients (Experiments 2 & 3). Embryos were transferred to recipients 6 or 7 days after the recipient ovulated. CIDR (controlled internal drug release) = Zoetis Eazi-Breed CIDR, PGF = 500 μg cloprostenol im, hCG = 2,500 IU human chorionic gonadotrophin.

Transrectal ultrasonography was done at 30- and 60-days post-ovulation for pregnancy diagnosis, confirmed by visualization of the embryo proper. At 30 days, embryos were assessed for the heartbeat and length. At 60 days, the umbilicus and transabdominal diameter were measured. Gestation length was calculated as the age of the embryo transferred (i.e., Day 8 embryo) plus the number of days *in utero* (i.e., from embryo transfer to calving).

6.3.3. Experiment 3 - Effect of embryo cryopreservation method on pregnancy rates after embryo transfer

Only Grade 1 morulae, 7 or 8 days after fertilization, were used in Experiment 3 (n=26). The experiment had 3 treatment groups (vitrified, slow-freeze and fresh). The vitrified and slow-freeze embryos were produced previously as described in Experiment 1. The vitrified embryos were cryopreserved as described in Experiment 1 using the Cryotop devices. The slow-freeze embryos were placed in 10% glycerol for 8 to 10 minutes at room temperature (20°C) and individually loaded into 0.25 mL straws. The straws were immediately transferred into the freezing chamber (Freeze control, CryoLogic, Blackburn, Victoria, Australia) at -7°C, held for 5 minutes and seeded. The straws were held at -7°C for an additional 10 minutes and cooled at 0.5°C/min to -38°C. They were then submerged in liquid nitrogen and stored. Fresh embryos were produced, as described in Experiment 1, the week before transfer using COC collected from a new group of wood bison (n=12). On the morning of the transfer, embryos in culture (7 days after fertilization) were washed in transfer medium and placed into 1.5 mL tubes (sonification tubes, Active Motif, Carlsbad, CA, USA) with 1 mL of the transfer medium. The embryos were kept in a portable incubator (Lab Mix Portable Incubator, WTA) maintained at 38.8°C in ambient air (i.e., without CO_2) until transfer.

Recipient wood bison (n=29) were synchronized for ovulation as described in Experiment 2, during the month of October in 2020 (Fig. 6.2). Estrus patches (Estrotect, Rockway Inc., Spring Valley, WI, USA) were placed just cranial to the tailhead on Day 5 and scored on a 4-point scale based on color change (0 = unchanged, 1 = 50% color change, 2 = > 50% color change, 3 = missing; Colazo et al., 2018) to determine estrus (Fig. 6.5). Transrectal ultrasonography was done at 24-hour intervals beginning at the time of hCG treatment until ovulation was detected. Embryos were transferred 8 days after hCG was given (i.e., 6 or 7 days after the recipient ovulated, Fig. 6.2).

Embryos were processed individually in a field lab adjacent to the bison handling facility on the day of transfer. The vitrified embryos were thawed using the protocol described in Experiment 1. The embryos slow-frozen with glycerol were thawed in air for 10 seconds before being submerged into a 35°C water bath for 1 minute. The embryos were then washed in the 3-step dilution of sucrose (3-step Thaw Media; ABT 360, Pullman, WA, USA) for 5 minutes each. One at a time, the embryos (vitrified, slow-freeze or fresh) were washed once in the transfer

medium and loaded individually into 0.25 mL straws with transfer medium. The embryo straw was then placed into a standard 0.25 mL embryo transfer gun with a metal-tipped embryo transfer sheath (3 mm, Eastgen, Guelph, ON, Canada). Embryo transfer, pregnancy diagnosis and calving evaluation were done as described in Experiment 2.

Table 6.2. The distribution of IVP embryos transferred into recipient wood bison in Experiments 2 and 3.

Experiment 2 (2019)[1]				
Embryo stage	**Morula**	**Early blastocyst**	**Blastocyst**	**Expanded blastocyst**
Freeze day[2]				
Day 7	5	1	1	1
Day 8	2	3	4	2
Day 9	0	3	3	3

Experiment 3 (2020)		
	Day 7 - Morula	**Day 8 - Morula**
Cryopreservation method		
Fresh	9	0
Vitrified	4	4
Slow-freeze	6	3

[1]Embryos in Experiment 2 were vitrified
[2]Number of days after *in vitro* fertilization the embryo was cryopreserved

6.3.4. Statistical analyses

Statistical analyses were performed using SAS (Enterprise Guide 4.2; Statistical Analysis System Institute Inc., Cary, NC, USA). Numerical scale data are represented as mean ± SEM and P-values are considered significant if ≤0.05. Each embryo or embryo recipient was considered as an experimental unit.

In Experiment 1, Proc Freq procedure for Fisher exact test was used to compare the effects of embryo stage, grade, freeze day and post-thaw culture time on the post-thaw survivability parameters (TUNEL score, nuclear morphology score and expansion rate). A Proc Mixed procedure was used to determine the effects of embryo stage, grade, freeze day and culture time for total cell counts. To compare the effect of species (bison and hybrid) and embryo stage the GLIMMIX procedure with binary distribution and link logit function was used.

In Experiment 2 and 3, the Proc Freq procedure for Fisher exact test was used to evaluate the effect of embryo factors and cryopreservation method on pregnancy rate. The Proc Mixed procedure was used to compare the effects of recipient age, ovulatory follicle size, corpus luteum size after embryo transfer and estrus on pregnancy data.

6.4. Results

6.4.1. Experiment 1 - Embryo factors related to post-thaw viability

A total of 72 IVP bison embryos were thawed, and five were lost or damaged during thawing, fixation and staining (n=67 bison embryos analyzed). A total of 21 hybrid embryos were thawed, and six were lost or damaged during thawing, fixation and staining (n=15 hybrid embryos analyzed). The effects of embryo factors on post-thaw survivability are summarized in Table 6.3. No effect of time in post-thaw culture (n=35 for 1 h vs n=32 for 36 h) was detected for any endpoint, therefore data were combined. Similarly, no effect of blastocyst stage (early blastocyst, blastocyst, and expanded blastocyst) was detected for any endpoint, therefore data were combined into a single blastocyst group. Morulae cryopreserved on Day 7 or 8 tended to have a greater proportion of nuclear morphology score 1 than Day 7 or 8 blastocysts (10/12 [83.3%] vs 6/12 [50.0%]; P = 0.09). Morphologically high quality blastocysts (Grades 1 and 2 combined) cryopreserved on Day 7 or 8 tended to have higher cell counts than those cryopreserved on Day 9 or 10 (115.0 ± 11.7 vs 90.5 ± 8.1; P = 0.08). The high quality (Grade 1 and 2 combined) blastocyst embryos had higher cell counts than low quality blastocysts (cryopreserved on Day 9 or 10 groups only; 100.7 ± 6.9 vs 57.6 ± 7.2; P = 0.03).

Hybrid embryos (bison oocytes and cattle semen) had a higher proportion of embryos produced on Day 7 or 8 than bison embryos (Day 7 or 8 embryos/total embryos produced, 15/15 [100.0%] vs 25/67 [37.3%]; P < 0.0001, Fisher's exact test; Table 6.4). For both bison and hybrid embryos, blastocysts had higher cell counts than morulae (P = 0.0007).

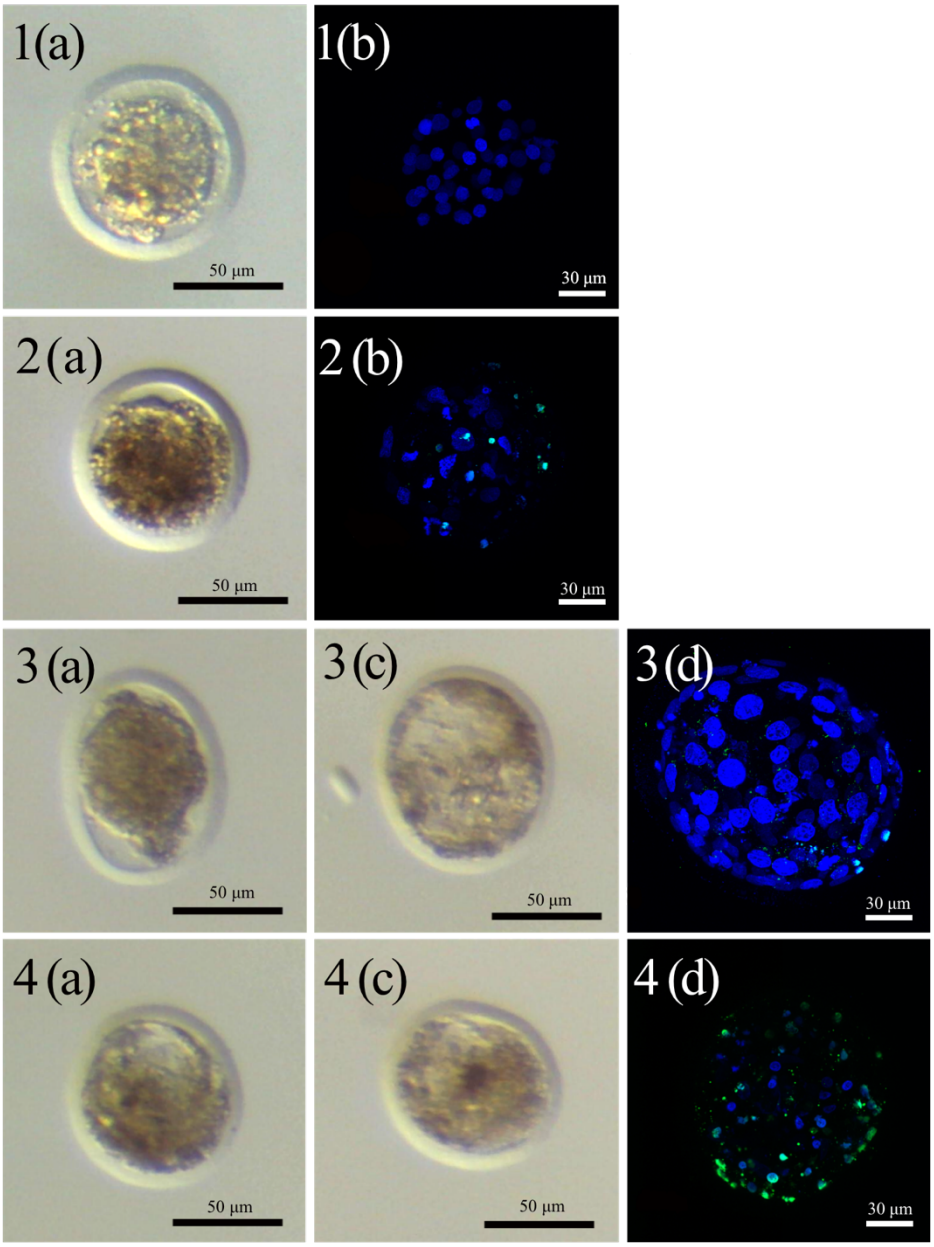


Figure 6.3. Stereomicrographs (bright field) and confocal images (dark field) of vitrified-warmed wood bison embryos after 1 hour (a and b) or 36 hours (c and d) in culture with 4′,6-diamidino-2-phenylindole (DAPI; blue stain) and terminal deoxinucleotil transferase mediated dUTP nick end labeling (TUNEL; green stain) staining. 1) Morula, Grade 1, Day 8, 1 hour in post-thaw culture, TUNEL score 1, nuclear morphology score 1, 2) Blastocyst, Grade 3, Day 9, 1 hour in post-thaw culture, TUNEL score 1, nuclear morphology score 2, 3) Morula, Grade 2, Day 8, 36 hours in post-thaw culture [expanded to blastocyst, 3c], TUNEL score 1, nuclear morphology score 1, and 4) blastocyst, Grade 2, Day 9, 36 hours in post-thaw culture TUNEL score 2, nuclear morphology score 2.

Table 6.3. The effect of *in vitro* wood bison embryo factors on post-thaw survivability at 1 and 36 hours after culture (mean ± SEM; Experiment 1).

Embryo freeze day[1]	**Day 7 or 8**	
Embryo stage[2]	**Morula**	**Blastocyst**
TUNEL score of 1[3]	8/9 (88.9%)	7/9 (77.8%)
Nuclear morphology score of 1[4]	10/12 (83.3%)	6/12 (50.0%)
Expansion rate[5]	1/5 (20.0%)	3/7 (42.9%)
Total embryo cell count[6]	87.3 ± 9.7	107.4 ± 12.9
Embryo stage	**Blastocyst**[7]	
Embryo freeze day	**7 or 8**	**Day 9 or 10**
TUNEL score of 1	6/8 (75.0%)	8/15 (53.3%)
Nuclear morphology score of 1	6/11 (54.6%)	12/17 (70.6%)
Expansion rate	3/6 (50.0%)	4/7 (57.1%)
Total embryo cell count	115.0 ± 11.7	90.5 ± 8.1
Embryo type	**Day 9 or 10 Blastocysts**	
Embryo grade[8]	**High**	**Low**
TUNEL score of 1	14/23 (60.9%)	6/17 (35.3%)
Nuclear morphology score of 1	12/26 (46.2%)	18/28 (64.3%)
Expansion rate	6/13 (46.2%)	3/13 (23.1%)
Total embryo cell count	100.7 ± 6.9[a]	57.6 ± 7.2[b]

[1]Number of days after *in vitro* fertilization
[2]Morula vs blastocyst (early blastocysts, blastocysts, and expanded blastocysts combined)
[3]Number of embryos with <50% of apoptotic nuclei / number of stained embryos
[4]Number of embryos with <50% morphologically abnormal nuclei / number of stained embryos
[5]Number of embryos with progressive development between 0 and 36 hours / number of embryos cultured for 36 hours
[6]Number of DAPI positive structures
[7]Blastocysts of high quality (Grades 1 and 2)
[8]High- (Grades 1 and 2) vs low- quality (Grade 3)
[ab]Within rows, values with no common superscript are different (Fisher's exact test, $P \leq 0.05$).

Table 6.4. Effect of species (wood bison vs hybrid) and embryo stage on post-thaw preimplantation embryo viability (Experiment 1, all Day 7 or 8, high quality [Grade 1 & 2] embryos).

Embryo species[1]	**Wood bison**		**Hybrid**	
Embryo stage[2]	**Morula**	**Blastocyst**	**Morula**	**Blastocyst**
TUNEL score of 1[3]	8/9 (88.9%)	6/8 (75.0%)	4/4 (100.0%)	9/9 (100.0%)
Nuclear morphology score of 1[4]	10/12 (83.3%)	6/11 (54.5%)	4/5 (80.0%)	10/10 (100.0%)
Expansion rate[5]	5/6 (83.3%)	3/6 (50.0%)	3/3 (100.0%)	2/6 (33.3%)
Total embryo cell count[6]	87.3 ± 9.7[a]	115.0 ± 11.3[b]	70.4 ± 12.4[a]	141.3 ± 15.0[b]

[1]Wood bison (wood bison oocyte x wood bison semen) vs hybrid (cattle oocyte x wood bison semen)
[2]Morula vs blastocyst (early blastocysts, blastocysts, and expanded blastocysts combined)
[3]Number of embryos with <50% of apoptotic nuclei / number of stained embryos
[4]Number of embryos with <50% morphologically abnormal nuclei / number of stained embryos
[5]Number of embryos with progressive development between 0 and 36 hours / number of embryos cultured for 36 hours
[6]Number of DAPI positive structures
[ab]Within rows, values with no common superscript are different (Fisher's exact test, $P \leq 0.05$).

6.4.2. Experiment 2 - Embryo factors related to pregnancy rates after embryo transfer

The effects of embryo stage, freeze day, and vitrification device on pregnancy rate (per embryo transferred) at 30 and 60 days post-ovulation is provided in Table 6.5. Five wood bison were diagnosed pregnant at 30 days with ultrasonographic visualization of an embryo proper with a heartbeat. Four additional bison had intra-uterine fluid consistent with the appearance of embryo loss; an embryo proper was not detected and they were classified as non-pregnant. The vitrification device impacted the pregnancy rate with the Cryotop device resulting in all of the pregnancies in comparison to the Cryolock and Vitrifit devices, which failed to produce pregnancies (5/10 [50%] vs 0/11 [0%] and 0/7 [0%], respectively; $P = 0.003$, Fisher's exact test). Pregnancy rates for three different types of blastocyst (early blastocyst, blastocyst and expanded blastocyst) were similar ($P = 1.0$, Fisher's exact test) and therefore data were combined. Pregnancy rates were also similar for Day 7 and 8 embryos ($P = 0.6$, Fisher's exact test) and therefore data were combined into an Day 7 or 8 embryo group. The pregnancy rate at 30 days tended to be higher in the morulae group than in the blastocyst group (stages combined; $P \leq 0.1$, Table 6.5). At the 60-day pregnancy diagnosis, two of the five confirmed pregnancies were lost. The morula group had a greater 60-day pregnancy

rate than the blastocyst group (3/7 [42.9%] vs 0/21 [0%]; P = 0.02). No effect of embryo freeze day was detected on pregnancy rate at 30 day or 60 days (P = 0.3).

The length of the embryo proper at 30 days of gestation for bison that maintained pregnancies vs those that experienced pregnancy loss was 9.5 ± 1.1 vs 7.8 ± 0.3 mm, respectively; P = 0.8). At 60 days of gestation, the transabdominal diameter was 19.9 ± 0.6 mm and the umbilical diameter was 5.4 ± 0.3 mm. The three pregnancies confirmed at 60 days were carried to term with gestation lengths of 264, 266 & 267 days, and were born in July 2020 (Fig. 6.4). One of the recipients experienced dystocia; a 46 kg calf with a crown-rump length of 102 cm died during obstetrical intervention. The male to female ratio of resulting pregnancies was 2:1, which was accurately classified at the 60- day pregnancy check.

Table 6.5. Effect of embryo stage and freeze day on pregnancy rates after embryo transfer at 30 & 60 days post-ovulation for wood bison (Experiment 2).

Embryo Variables		Pregnancy rate after embryo transfer	
		30 days	60 days
Embryo stage[1]	**Morula**	3/7 (42.9%)	3/7 (42.9%)[a]
	Blastocyst	2/21 (9.5%)	0/21 (0%)[b]
Freeze day[2]	**Day 7 or 8**	4/19 (21.1%)	3/19 (15.8%)
	Day 9	1/9 (11.1%)	0/9 (0%)

[1]Morula vs blastocyst (early blastocyst, blastocyst, expanded blastocyst)
[2]Number of days after *in vitro* fertilization the embryo was cryopreserved
[ab]Within columns for each variable, values with no common superscript are different (Fisher's exact test, P ≤ 0.05).

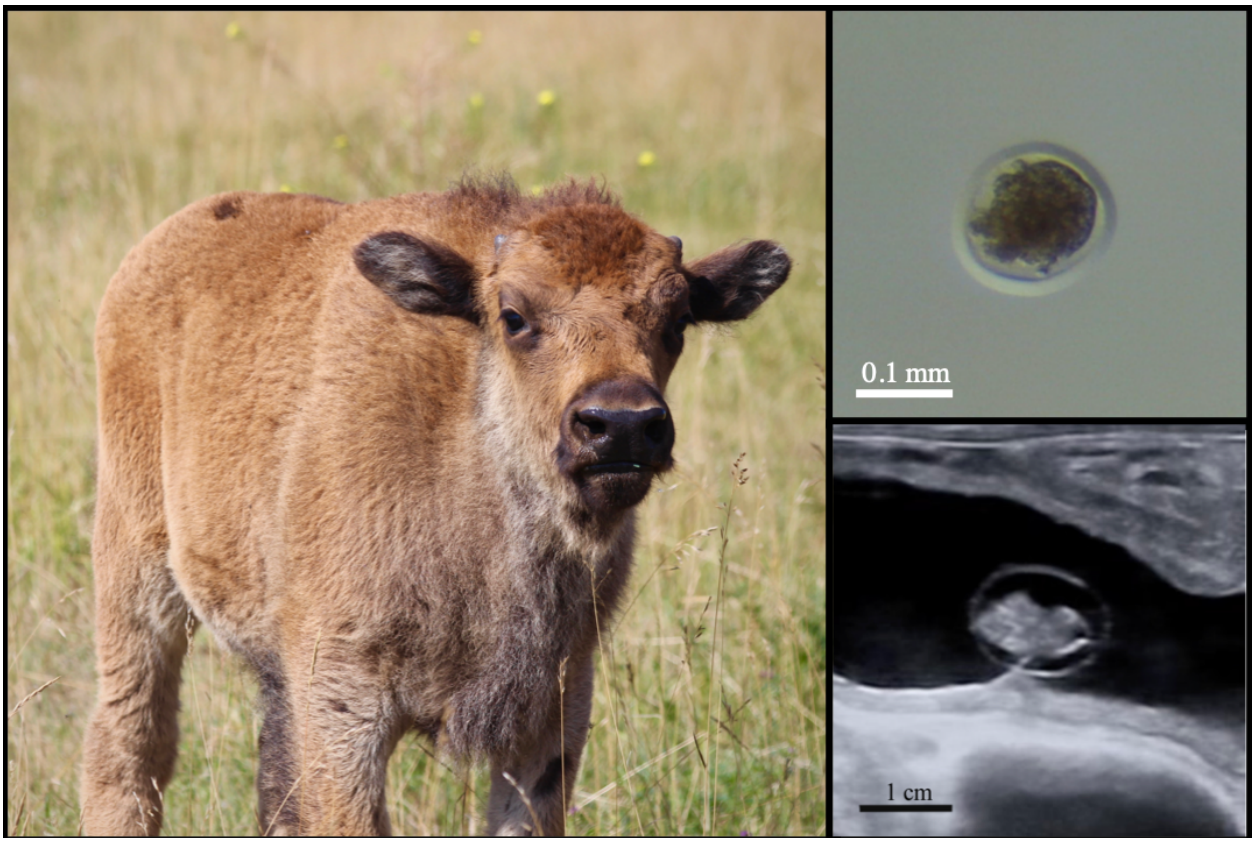


Figure 6.4. One month old wood bison calf (left) born after the transfer of an *in vitro* derived (*in vitro* matured) wood bison embryo (top right image; Grade 1 morula immediately before vitrification on cryotop device, 8 days after fertilization). Bottom right image = fetus with fetal membranes at 30 days post-ovulation. (Experiment 2)

6.4.3. Experiment 3 – Effect of embryo cryopreservation method on pregnancy rates after embryo transfer

The effect of cryopreservation method on pregnancy rates for Experiment 3 is provided in Table 6.6. At the 30-day pregnancy diagnosis, four recipient bison were confirmed pregnant with an embryo proper. Pregnancies resulted after the transfer of fresh or vitrified embryos, but not from the embryos cryopreserved in glycerol by slow- freezing procedure (30 day pregnancy check; 2/9 [22.2%] vs 2/8 [25.0%] vs 0/9 [0%], respectively; P = 0.5). The male to female ratio of the four pregnancies at 60 days was 2:2. A pregnancy loss was detected in the fresh group at 180 days during transrectal ultrasonography of the herd. Three calves were carried to term with gestation lengths of 266, 266, and 269 days and were born in July 2021. The mean ± SE conceptus measurements by transrectal ultrasonography at 60 days were: transabdominal diameter = 17.9 ±

1.3 mm and umbilicus diameter = 6.7 ± 0.4 mm. When conceptus measurements were combined between Experiments 2 and 3 the 30- day (n=9 fetuses) crown-rump length measurement was 9.7 ± 0.7, the 60- day (n=7 fetuses) transabdominal diameter was 18.7 ± 0.8, and the 60- day umbilicus diameter was 6.1 ± 0.3.

Table 6.6. Embryo endpoints for pregnancy rates after embryo transfer for wood bison (Experiment 3).

Predictor Variables	Pregnancy rates after embryo transfer			
	30 days[1]	60 days	180 days	Full term
Fresh	2/9 (22.2%)*	2/9 (22.2%)	1/9 (11.1%)	1/9 (11.1%)
Vitrified	2/8 (25.0%)*	2/8 (25.0%)	2/8 (25.0%)	2/8 (25.0%)
Slow-freeze	0/9 (0%)	-	-	-

[1]Age of pregnancy after ovulation
*embryonic loss was observed at 30-day pregnancy check

6.4.4. Recipient effect

Table 6.7 is provided to show the recipient characteristics of Experiments 2 & 3. Recipient bison that did not ovulate were removed from analysis (n=26 and 28 embryos transferred, respectively). The age of the recipients did not differ between non-pregnant and pregnant bison (7.7 ± 0.6 vs 7.5 ± 1.3 years, respectively; P = 0.6, Experiment 2 & 3 combined). The ovulatory follicle size and the size of the CL at the time of embryo transfer were similar between groups. A total of 24/54 (44.4%) bison displayed estrus, but the display of estrus was similar between pregnant and non-pregnant bison (P = 0.9) and experimental years (P = 0.9). The interval to ovulation following hCG was shorter in Experiment 2 (ovulation detection done every 12 h) compared to Experiment 3 (ovulation detection done every 24 h; 21.9 ± 2.1 vs 38.8 ± 2.7, respectively, P = 0.004).

Table 6.7. Wood bison recipient endpoints for pregnancy per embryo transfer at 30 days (Data represented as mean ± SEM or proportional data; Experiment 2 & 3).

	Pregnancy per embryo transferred (30 days)			
	Experiment 2[1]		Experiment 3[2]	
	Non-pregnant	Pregnant	Non-pregnant	Pregnant
Bison (n)	23	5	22	4
Recipient age (years)	7.2 ± 0.9	8.6 ± 1.7	8.2 ± 0.9	5.7 ± 1.7
Ovulatory follicle size (mm)	14.2 ± 0.4	14.0 ± 0.7	13.1 ± 0.4	13.8 ± 0.9
Corpus luteum size at ET[3] (mm)	20.0 ± 0.6	18.6 ± 0.5	21.0 ± 0.7	19.5 ± 1.0
Ovulation time after hCG[4] (h)	20.9 ± 2.4[a]	26.4 ± 4.5[a]	39.3 ± 3.0[b]	36.0 ± 6.9[b]
Displayed estrus[5]	15/23 (65.2%)	1/5 (20%)	8/22 (36.4%)	0/4 (0%)

No differences detected among groups
[1]4 bison excluded that did not ovulate (n=28)
[2]3 bison excluded that did not ovulate (n=26)
[3]ET=embryo transfer
[4]hCG=human chorionic gonadotrophin
[5]Proportion of bison that displayed bison/total bison per group
[ab]Within rows, values with no common superscript are different (Fisher's exact test, $P \leq 0.05$).

Figure 6.5. Estrus display of wood bison (Experiment 3) evaluated using estrus patches. A) white arrow = wood bison exhibiting standing estrus, B) Estrus patches scored: 0 = unchanged, 1 = 50% color change, 2 = > 50% color change, 3 = missing, C) wood bison that displayed estrus

6.5. Discussion

The present study was designed to determine factors affecting the post-thaw viability of pre-implantation IVP embryos characterized by microscopic morphology and establishment of pregnancy after embryo transfer in wood bison. As IVP embryos are developing in culture, it is important to decide which stage of development and day the embryo should be cryopreserved to result in the greatest number of calves. Overall embryo morphology, commonly referred to as quality, is an important factor for producing pregnancies in all species (Lindner and Wright, 1983; Irani et al., 2017). Embryos that are morphologically graded lower (i.e., blastomere number, size and symmetry, membrane definition, cytoplasmic clarity, cell compaction, and fragmentation) have physical DNA damage and an increased incidence of apoptosis (Jurisicova et al., 1996). Even parthenogenetic cattle blastocysts, which do not differ morphologically, had an increased incidence of apoptosis and lower total cell counts, demonstrating TUNEL as a more reliable viability indicator than visual assessment (Neuber et al., 2002). In cattle, pregnancy rates improved after the transfer of high quality embryos (Grade 1 embryos; Hasler, 2001). These observations were consistent with the results of Experiment 1, where high quality blastocysts (Grade 1 & 2 combined) had higher cell counts than low quality blastocysts. As a result of Experiment 1, only Grade 1 embryos were selected for the embryo transfer trials (Experiments 2 & 3); the effect of grade was not investigated.

A previous study showed that increased time *in vitro* resulted in increased levels of damage caused by apoptosis (Hao et al., 2003). In general, compact morulae and blastocysts had been shown to have similar survival rates after cryopreservation in cattle and perform better than earlier embryos and hatched blastocysts (Hasler et al., 1995; Mapletoft and Hasler, 2018). However, De Rosa et al. (2007) reported that expanded blastocysts had greater post-thaw expansion than morula, early blastocysts and blastocysts in vitrified buffalo (*Bubalus bubalis*) and cattle IVP embryos. In the present study, morulae tended to have less abnormal nuclei (Experiment 1). Morulae also had more pregnancies diagnosed and calves born after transfer than blastocyst-stage embryos (Experiment 2). Although pregnancies were established at 30 days post-ovulation from morulae, early blastocysts, and blastocysts, the transfer of morulae resulted in all of the full-term pregnancies in comparison to the blastocysts, which produced none. Cattle embryos that developed faster and were therefore cryopreserved earlier (7 days after fertilization), produced greater

pregnancy rates than those cryopreserved on Day 8 (42% vs 20%, respectively; Hasler et al., 1995). In the present study, bison blastocysts cryopreserved on Day 7 or 8 tended to have higher cell counts than blastocysts cryopreserved on Day 9 or 10 (Experiment 1). Although selecting embryos on Day 7 or 8 did not result in more pregnancies at 30 days (Experiment 2), every pregnancy that made it full-term was from a Day 7 or 8 embryo (Experiment 2 & 3).

To date, only two bison calves have been born from cryopreserved IVP embryos before the present study. One calf (wood bison) was produced from an IVP embryo derived from mature COC collected by transrectal ultrasound-guided COC collection, cryopreserved at the University of Saskatchewan and shipped to the Toronto Zoo (1/4 [25%] pregnancy rate; unpublished Mastromonaco, James, 2018). The second calf (plains bison) was produced from a cryopreserved IVP embryo derived from postmortem reproductive material (1/12 [8.3%] pregnancy rate; Benham et al., 2021). The present study is the first to report the production of multiple calves derived from cryopreserved IVP embryos in a single trial. The calving rate (calves that made it full term/embryos transferred) of all vitrified embryos in the present study (5/37 [13.5%]) was similar to the previously reported IVP embryo transfer trials. The present trial differed from previous attempts as it successfully identified embryo factors that increased pregnancy rates and the number of calves on the ground (i.e., vitrified day 7 & 8 morulae combined; 5/16 [31.3%]).

As minimal data concerning bison embryo transfer are available, commercial bovine embryo transfer data has been used as a reference point. The selection of Grade 1 fresh IVP embryos resulted in higher pregnancy rates than Grade 1 cryopreserved IVP embryos (56% vs 42%, respectively; Hasler, 1998). Moreover, the transfer of cryopreserved IVP bison embryos is less effective than the bovine model (31% vs 42%). As the transfer of vitrified embryos in Experiment 3 resulted in a pregnancy rate similar to that after the transfer of fresh embryos (2/8 [25.0%] vs 2/9 [22.2%]), the use of vitrification for bison embryos was supported. Cryopreservation tolerance has been shown to vary between inter- and intra-species, with porcine being less cryotolerant than bovine (Niemanna and Rath, 2001) and some cattle breeds (i.e., Jersey) exhibiting lower cryotolerance than others (Steel and Hasler, 2004). It has been hypothesized that the primary limiting factor in bison IVP embryo development is the oocyte, as pure wood bison embryos had lower developmental rates, lower cell numbers, higher incidence of apoptosis and elevated ATP levels per blastomere than hybrid (cattle COC x wood bison semen) embryos (Seaby et al., 2012). The present study showed hybrid embryos had a higher proportion of embryos

produced on Day 7 or 8 than bison embryos (100.0% vs 38%). Previous studies have reported that bison embryos had features (developmental timing, darkness of cellular matter and zona pellucida shape) different from cattle (Thundathil et al., 2007; Barfield, 2019). Therefore, the bison oocyte may be stressed in the cattle *in vitro* culture system. We observed dark ooplasms and unusual zona pellucida shapes at the time of COC collection, which may suggest that bison oocytes are innately different from cattle oocytes before any *in vitro* intervention. To improve the overall quality of IVP bison embryos and subsequent cryotolerance, *in vitro* culture conditions may need to be modified.

It is important to note that bison are a wild species and even chute-acclimated animals are stressed during handling (Toosi et al., 2013a). However, the effect of stress on pregnancy rates has not been investigated to date in bison. In the present study, 7 out of the 54 synchronized recipient bison failed to ovulate (13%; Experiments 2 & 3 combined) and were therefore excluded as embryo recipients, further reducing our numbers. At the 30-day pregnancy check, six recipient bison (n = 3 & 3, Exp. 2 & 3, respectively) exhibited apparent embryonic loss (small intrauterine fluid collection without an embryo proper. Two additional pregnancies were lost between 30 and 60 days in Experiment 2. With the inclusion of all early losses, the total pregnancy loss by 60 days of gestation was 8/15 [53%]. In cattle, embryos of lower morphologic grade have been reported to have higher incidence of early embryonic mortality than high grade embryos (Farin and Farin, 1995) suggesting that future efforts should focus on embryo quality.

In summary, the *in vitro* (staining) and *in vivo* (embryo transfer) viability assessments of pre-implantation wood bison embryo factors resulted in similar outcomes. Morphologic grading was positively related to quantitative viability characteristics, with higher quality embryos having less cryo-damage and higher cell counts than low quality embryos. Morulae survived cryopreservation with less damage than blastocysts, and subsequent transfer resulted in a higher pregnancy rate at 30-days and lower pregnancy loss between 30 to 60 days than blastocysts. The vitrification method of cryopreserving embryos resulted in a pregnancy rate similar to the transfer of fresh embryos. The study produced nine pregnancies at 30 days post-ovulation, of which 6 made it to term and the birth of five calves. The insight provided in the present study will contribute to selecting embryos for future bison genome biobanks.

6.6. Acknowledgments

The authors would like to thank the Natural Sciences and Engineering Research Council (NSERC) for funding support. We acknowledge the Livestock and Centre of Excellence for the care and maintenance of our bison herd including Kris Ringwall, Brad Blackmore, Brian Klassen and Trent Presnell. Thank you to Steve Yang and Sergio Enrique Pezo Alencastre for assistance with bison handling. Also special thanks to Murray Woodbury for the establishment of the bison research group and for ensuring the care and well-being of the bison.

6.7. Supplementary figures

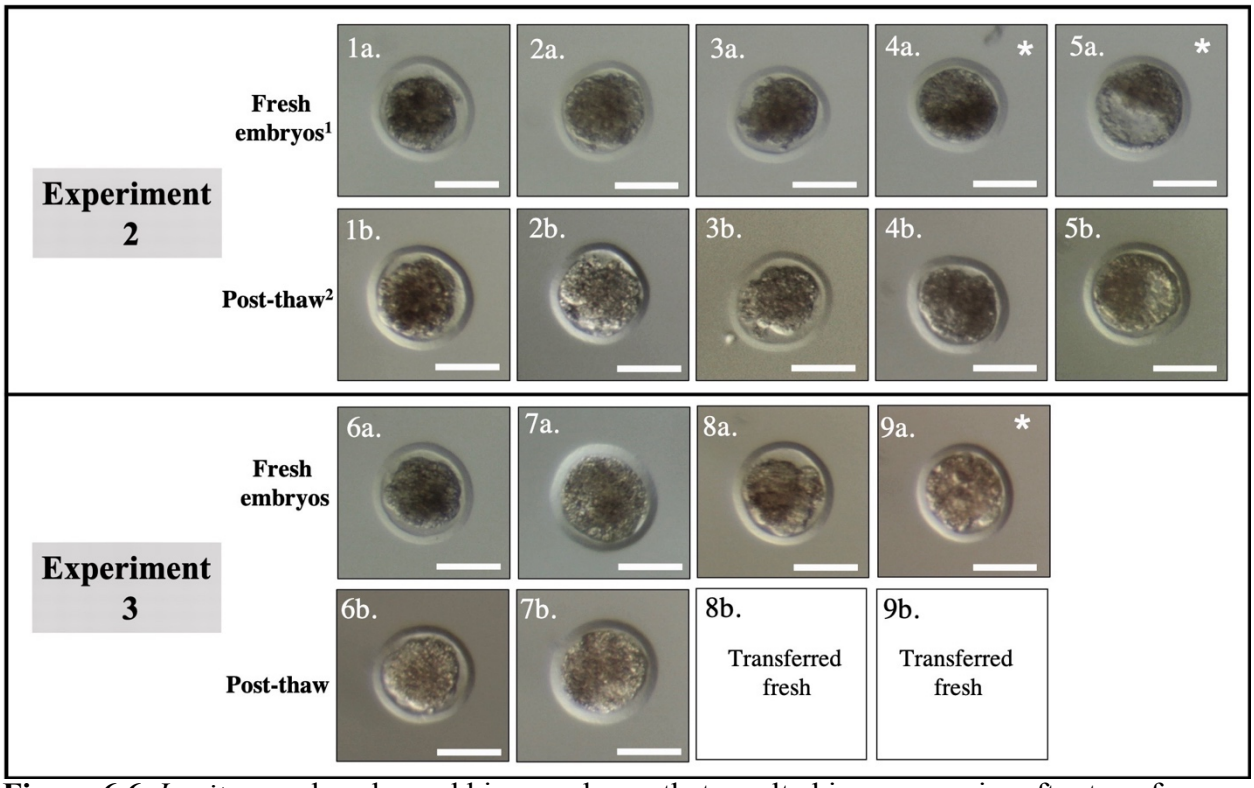


Figure 6.6. *In vitro* produced wood bison embryos that resulted in pregnancies after transfer.
[1]Embryos were imaged immediately before vitrification or if they were transferred fresh
[2]Embryos were imaged post-thaw and before transfer
* = pregnancy was lost during gestation.
Individual embryo characteristics:
1) Vitrified, Morula, Grade 1, Day 7 (full term)
2) Vitrified, Morula, Grade 1, Day 7 (full term)
3) Vitrified, Morula, Grade 1, Day 8 (full term)
4) Vitrified, Early blastocyst, Grade 1, Day 8 (pregnancy lost before 60 days gestation)
5) Vitrified, Blastocyst, Grade 1, Day 9 (pregnancy lost before 60 days gestation)
6) Vitrified, Morula, Grade 1, Day 7 (full term)
7) Vitrified, Morula, Grade 1, Day 8 (full term)
8) Transferred fresh, Morula, Grade 1, Day 7 (full term)
9) Transferred fresh, Morula, Grade 1, Day 7 (pregnancy lost before 180 days gestation)

Figure 6.7. One day old male wood bison calf (IVF-1) born after the transfer of an *in vitro* embryo (Vitrified, Morula, Grade 1, Day 8; Gestation length = 266 [age of the embryo transferred plus the number of days in utero]; *Embryo #3 in figure 6.6*).

Figure 6.8. One day old male wood bison calf (IVF-2) born after the transfer of an *in vitro* embryo (Vitrified, Morula, Grade 1, Day 7; Gestation length = 267 [age of the embryo transferred plus the number of days in utero]; *Embryo #1 in figure 6.6*).

Figure 6.9. Female wood bison calf, produced after transfer of an *in vitro* embryo (Vitrified, Morula, Grade 1, Day 7; Gestation length = 264 [age of the embryo transferred plus the number of days in utero]; *Embryo #2 in figure 6.6*), was born dead after dystocia.

Figure 6.10. One day old female wood bison calf (IVF-4) born after the transfer of an *in vitro* embryo (Vitrified, Morula, Grade 1, Day 8; Gestation length = 266 [age of the embryo transferred plus the number of days in utero]; *Embryo #7 in figure 6.6*).

Figure 6.11. Two day old male wood bison calf (IVF-3) born after the transfer of an *in vitro* embryo (Transferred fresh, Morula, Grade 1, Day 7; Gestation length = 266 [age of the embryo transferred plus the number of days in utero]; *Embryo #8 in figure 6.6*).

Figure 6.12. Three day old male wood bison calf (IVF-5) born after the transfer of an *in vitro* embryo (Vitrified, Morula, Grade 1, Day 7; Gestation length = 269 [age of the embryo transferred plus the number of days in utero]; *Embryo #6 in figure 6.6*).

CHAPTER 7. GENERAL DISCUSSION

7.1. Simplified ovarian superstimulation

The use of reproductive technologies and specifically embryo production in wild species has enormous possibilities. Prior to the work done in this thesis, great strides were taken by implementing ovarian superstimulation and COC collection protocols that resulted in *in vitro* produced bison embryos. These studies utilized protocols that were created for domestic cattle and required extensive handling of the bison. Bison acclimated to repetitive handling through chute systems have been superstimulated for COC collection, but stress still remains a limitation when working with a wild species such as bison. As bison are more easily stressed then cattle, bison are more likely to cause injury to themselves, other bison or personnel. Past studies have emphasized that stressed bison can impede superstimulation (Dorn, 1995).

To limit stress, several minimal-handling superstimulation protocols were investigated in this thesis. The sustained-release follicle stimulating hormone (FSH) and the low dose equine chorionic gonadotrophin (eCG; 2500 IU) examined in Chapters 2 & 3, respectively, did not result in a suitable superstimulatory response. The high dose of 5000 IU eCG in Chapter 3 resulted in the greatest number of follicles available, COC recovered and embryos produced per bison. This protocol incorporated a single dose of eCG at the time of a follicular ablation followed by COC collection 5 days later. When testing the new single dose eCG protocol against the most productive multiple handling protocol to-date in Chapter 4, the eCG had a similar number of embryos produced per bison (3.0 vs 3.3, respectively). Interestingly, the dosage is much higher than the recommended 1500 to 3000 IU of eCG in domestic cattle (Bó and Mapletoft, 2014). It could be speculated that bison metabolize hormones (specifically eCG) at a faster rate than beef cattle as they are a different species. This would be consistent with the massive dose of eCG required as stress (i.e., cortisol) inhibits the effectiveness of superstimulation treatment. In a study done with superstimulated beef heifers, the addition of stress (i.e., stock trailer movement) tended to effect (reduce) the total CL at the time of embryo collection (Fernandez-Novo et al., 2020). The single dose protocol allows for less handlings and had the same efficacy all while using significantly cheaper products. The 5000 IU of eCG is priced around $50.00 CAD while the 400 mg of FSH

required for one collection is $200 CAD. One downfall of eCG, also known as pregnant mare serum gonadotrophin, is that it is currently restricted for use in the United States. However, it is approved in Canada and would be applicable in Wood Buffalo National Park.

7.2. Random and synchronized COC collections

There is a fine line between finding a protocol that is feasible in the field which requires the least number of handlings and results in a sufficient number of embryos produced per bison. In an effort to create the most simplified protocol to be implemented in the wild, collections at random stages of the follicular wave were investigated. In theory, random collections would be the most ideal and field-friendly method to collect COC from bison as all that would be required is animal selection, capture, COC collection and release. The efficacy of a collection at a random timepoint has been variable throughout the results of Chapters 2, 3, 4 & 5 ranging from 0.0 to 1.5 embryos per bison. For example, where ≥1 embryos were produced per bison, bison had many follicles available and were likely near follicular wave emergence. In instances where ≤0.5 embryos per bison were produced, it is speculated that the follicles were undergoing atresia which resulted in decreased COC competency and lower embryo production. Although the random collection has a lower embryo production rate than the superstimulation protocols, it may be the most feasible and realistic protocol for use in the wild.

The random collections were also used to synchronize the follicular wave allowing for a synchronized collection. The incorporation of a synchronized, non-superstimulated COC collection protocol allowed for the collection of similarly sized (and aged), developmentally competent COC. It required an additional handling (i.e., the random COC collection) to initiate the follicular synchrony. Throughout the thesis, the results varied from 0.6 to 1.25 embryos produced per bison. Although we attempted multiple timings, the synchronized collection produced the best results 5 days after the random collection (Day -1 and Day 4; Day 0 = wave emergence). It may also be a beneficial protocol as you can repeat the protocol with a collection every 5 days. It may be worthwhile to investigate the use of treatment of estradiol-progesterone to induce follicular wave emergence rather than follicular ablation (i.e., random COC collection). It may be feasible to give the treatment by field darting similar to the administration of superstimulation drugs in Chapter 4 prior to collection. Another uninvestigated option would give

an injection of hCG and an injection of eCG at a random stage of the follicular wave and collect the COC 5 days later. This would induce ovulation and initiate a new follicular wave (hCG) and subsequently induce superstimulation (eCG) in a single handling. If repetitive access to the bison is achievable and superstimulation is not an option, synchronized collections are a viable option for COC collection.

7.3. COC collections on sedated bison

One of the primary goals of creating a bison genome biobank is to be able to collect germplasm from isolated wood and plains bison herds that contain unique and valuable genetics in an effort to protect from future bottlenecks and safe-guard the genetics. Bison in these locations will be truly wild. In some locations it may be possible to bring in a portable chute and corral system to handle the bison. The implementation of reproductive interventions using repetitive handlings in captive bison is feasible but would be difficult to implement in free-roaming bison herds located in the wild. Conservation herds that supplement feed, have corrals and/or handling systems may be able to implement these complex strategies. Moreover, prior ovarian superstimulation and COC collection protocols that require a handling system are not feasible for bison located in remote locations. Studies in this thesis investigated how a genome biobank can be created using genetics from wild bison herds that are not able to be rounded up and handled in a chute system. Transportation to and within remote herds such as Wood Buffalo National Park or the interior areas of Alaska require helicopters and planes to track, monitor and capture wild species such as bison (Caulkett, 2014; Slater, 2020). Therefore, it would be feasible to sedate a female bison for either the sole or partial purpose of collecting COC. In Chapter 4, COC collections were performed on sedated female bison which was the first time it has been reported. This is a huge step for being able to access live bison genetics in remote areas without removing them from their natural habitat. The COC collections on sedated bison had the same follicular aspiration and COC recovery rates as collections performed in the hydraulic chute system. Oocyte collections on sedated animals are done in zoos and conducted surgically through laparoscopy rather than transvaginally (non-surgical). In many instances, this is because many endangered animals' valuable genetics are isolated in the zoos as they have already either partially or completely disappeared from the wild. The recovery of bison genetics from wild and isolated populations has

been recommended before they are wiped out or hybridize. The use of sedated collections can either be used for COC collections at random stages of the follicular wave or even the synchronized or superstimulated collections with the use of a GPS or radio frequency collar. Chapter 4 also explored the efficacy of field darts for the administration of superstimulation drugs which their effectiveness did not differ from the manual injection of the drugs. However, if a helicopter would be required every time to relocate the bison, even without capturing the bison, it would be costly. In some locations alternative off-road vehicles may be plausible for use to locate, relocate and/or dart bison.

7.4. COC collections on pre-pubertal bison

An additional factor that is present in COC collections in wild herds is animal selection. Bison can be categorized into different sex and age classes by visual assessment (Olson, 2012). The capture of bison is preferred to occur in the winter months (Slater, 2020), however it may be possible during cooler times throughout the year. As pregnancy status is unknown in mature females, immature females are an alternative donor. Previous attempts of COC collections in live bison have been limited to non-pregnant mature bison. Chapter 4 investigated COC collection and embryo production in bison of varying reproductive statuses including pregnant, non-pregnant mature bison and pre-pubertal yearling bison. Interestingly, all of the pre-pubertal calves (n = 4) that were collected were in the HIGH antral follicle group (Figure 7.1). The pre-pubertal bison produced embryos at the same rate as the non-pregnant mature females without superstimulation. Therefore, the selection of yearling pre-pubertal bison is a worthwhile option for COC collection in wild herds. However, nutritional intake of bison in the wild may have an impact on the time of maturity and subsequent COC competence. More research and development is required on creating adaptations to the COC collection system that can keep equipment and samples warm during the winter.

7.5. Antral follicle counts

One of the biggest issues observed in COC collection and embryo production in bison throughout this thesis is the variability of follicle numbers. After combining the total follicle data

for every bison that has had ≥2 COC collections done throughout the studies conducted in this thesis (n = 248 total COC collections), the mean ± SE of total ≥3 mm follicles available was 19.5 ± 0.8 with a median of 17. Surprisingly, the synchronized, superstimulated and synchronized, non-superstimulated COC collections had a similar number of total follicles compared to COC collections at random stages of the follicular wave (18.4 ± 1.0 vs 21.3 ± 1.4; P = 0.09). Figure 7.1 displays the variation observed across individual bison for follicle numbers ranging from 2 to 82 total follicles (n = 42 bison). Interquartile ranges of combined data divided the bison into LOW (below the 25th percentile), MODERATE (between the 25th and 75th percentile) and HIGH (above the 25th percentile) antral follicle counts at ≤12, 13 to 23, and ≥24 mean follicles available, respectively (Figure 7.1). Although data varied among individuals, it was repeatable within an individual which is consistent with antral follicle counts in cattle (Morotti et al., 2017). In the LOW group (mean = ≤12 follicles), 7 out of 7 bison had <15 follicles available for collection for every single collection (Figure 7.1). Bison #27 has been collected 12 times and has never had >10 follicles available for aspiration. Overall, 2 bison had data points consistently in the LOW range, 10 bison were in the LOW/MODERATE range, 6 bison in the MODERATE range, 7 in the MODERATE/HIGH range, 3 in the HIGH range and 7 which varied between LOW, MODERATE and HIGH groups.

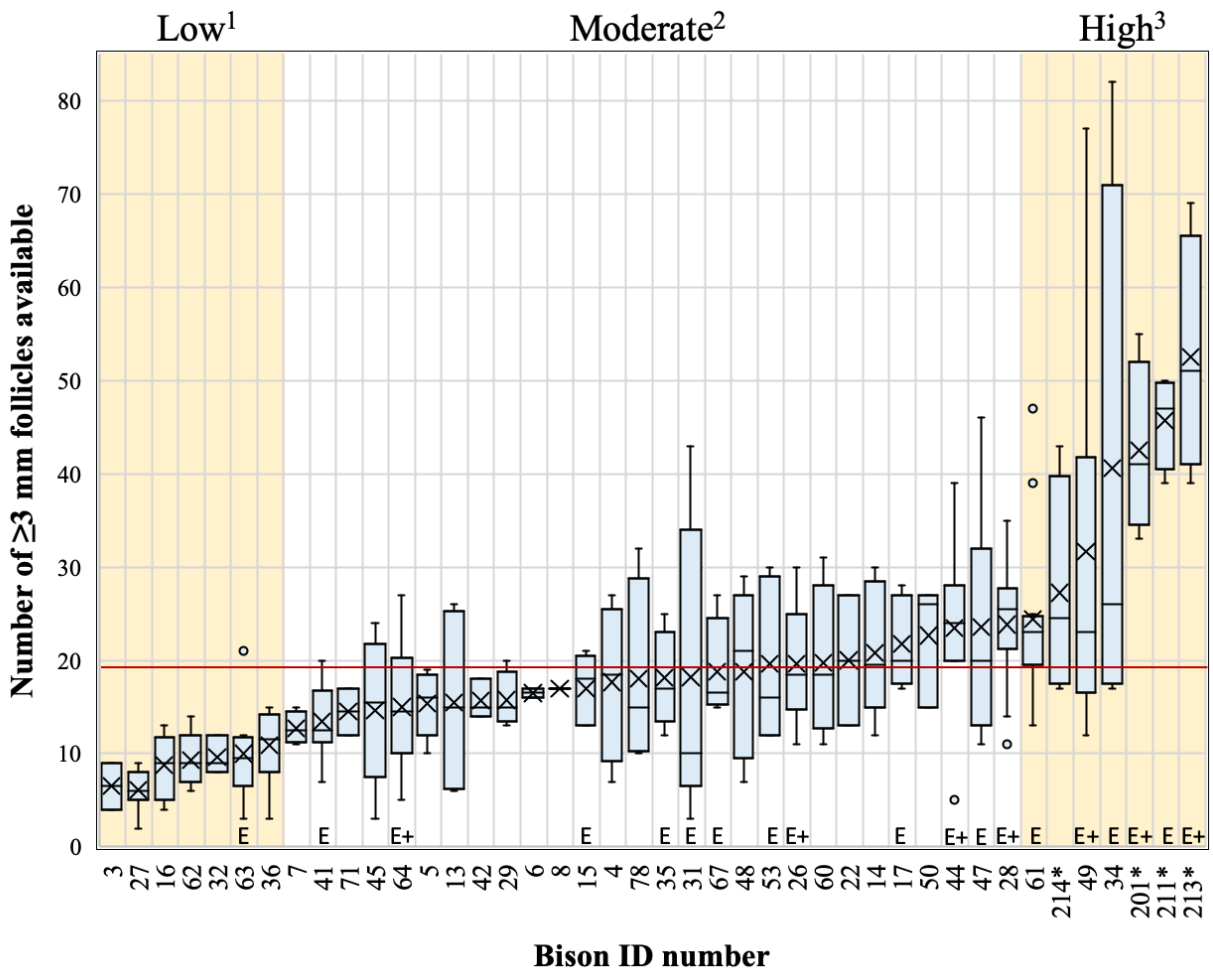


Figure 7.1. Boxplot of the number of follicles available (≥3 mm) for aspiration during cumulus-oocyte-complex collection for individual wood bison that had multiple collections done (2017-2021). X = mean for individual bison, Red line = overall mean ± SEM (19.5 ± 0.8).
[1]Low; ≤12 mean follicle count
[2]Medium; 13 to 23 mean follicle count
[3]High; ≥24 mean follicle count
*pre-pubertal yearling bison
[E]Bison has been documented as produced ≥1 embryo
[E+]High embryo producing bison; has produced ≥4 embryos in a single collection

7.6. Embryo production

The overall embryo production rate after combining each embryo production run included in the thesis (random, synchronized, and superstimulated collections) was 14.6% (209 embryos / 1428 COC matured). This is consistent to the 15% reported for embryo production in bison with

abattoir ovaries (Benham et al., 2021). However, bison production was not equal among all bison as bison with higher available follicles counts produced a greater number of embryos. Bison which produced ≥4 embryos in a single collection were located in the MODERATE (n = 4) and HIGH (n = 3) groups but not in the LOW group (Figure 7.1). Bison #49 has consistently produced a high number of *in vitro* embryos. In Chapter 4, she produced 10 embryos in her random collection and 10 embryos after superstimulation. Several bison have not produced a single embryo. The bison with higher follicle counts had a higher chance of producing an embryo. One of 7 (14%) bison with low follicle counts, 12 of 28 (43%) bison with moderate follicle counts and 6 of 7 (86%) bison with high follicle counts produced a single embryo (Chi-square comparison; P = 0.03). Results of Chapter 6 suggest that cryopreservation of embryos appear to be best done at the morula stage through the use of vitrification with cryotop devices. Embryos at the morula stage resulted in less apoptosis damage, higher pregnancy rates, less embryonic loss and more calves born. The embryos in general appear to be of a lower grade than cattle embryos and develop slower in culture. The viability of embryos after thaw and transfer is also variable. Low pregnancy rates and embryonic loss is still common. Future studies may be warranted to adapt the *in vitro* culture system specifically for bison and to directly compare *in vitro* and *in vivo* embryo stage and quality and closed vitrification systems for higher security during storage and transport.

7.7. Recommendations for COC collection and in vitro embryo production in the field

The body of work represented in this thesis explored the efficacy and feasibility of COC collection and *in vitro* embryo production for use in a bison genome biobank. A combination of techniques explored appears promising for use in free-roaming bison herds. In the field, yearling bison can be selected and sedated for COC collection without having to worry about if mature bison are pregnant or if they have a calf still with them. The collection can be conducted immediately (at random a random follicular wave), however, embryo rates will be variable. If a second handling is possible with the use of GPS or a radio collar, a synchronized COC collection is advised. The collection can be done without superstimulation or with the single dose of eCG at the time of the first collection. An additional study would be recommended to test the eCG protocol on sedated pre-pubertal bison and work out any technical issues in the system prior to transition to the field. Temporary laboratories will need to be constructed relatively near the herd (accessible

by helicopter; maximum of 30 minutes away). This lab will require power (electrical or by generator) for temperature control (i.e., heat, 20-30°C), lighting and to run equipment (i.e., microscopes, additional heating elements). The laboratories can be established in buildings already available or in simple tents or sheds. After oocytes are recovered, washed and placed in media in a portable incubator, they must be transported within 24 hours to a complete IVF laboratory. Transportation options include private or commercial flights and driving. Detailed planning is required for embryo production from bison in wild herds.

7.8. Future of reproductive technologies in bison

Reproductive technologies are very promising tools to be implemented in the conservation of wood and plains bison. The management of bison in the wild and conservation herds is best managed through a combination of methods. Natural breeding is still required in wild populations and in captive situations where the bison cannot not get pregnant from AI or embryo production. Semen collection can be done in wild and captive settings and the dissemination of genetics through artificial insemination is an efficient method to bring in new male genetics into captive herds. Although *in vivo* embryo production could be feasible in captive herds, it is not realistic in the field. Meanwhile, the collection of COC for *in vitro* embryo production in the field is feasible. This method allows entirely new genetics (male and female) to be introduced into a captive herd. The use of AI and embryo transfer to introduce genetics into a wild herd is a constraint as handling for synchronization is too complex for remote areas. Moreover, germplasm collected from wild herds can be used to strengthen the genetic diversity of the captive herds. If wild herds require supplementary bison/genetics, it would still require the release of live animals.

In conclusion, the minimal-handling protocols for COC collection and *in vitro* embryo production are feasible for bison. The specific COC collection protocol must be tailored for each individual herd varying based on herd location, accessibility and the time of year. Results suggest that COC collections from yearling bison a random stage of the follicular wave would be the simplest collection and use of the single dose eCG ovarian superstimulation would be the most efficient. The methods investigated in the present thesis have resulted in more options for COC collection and embryo production for the efficient creation of a germplasm biobank. The thesis

was also able to determine a repeatable bison specific embryo freezing protocol that resulted in the birth of multiple calves in two consecutive years.

CHAPTER 8: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

8.1. General conclusions

Chapter 2

a) A greater proportion of active mitochondria was located in the central region of the ooplasm and greater mitochondrial clustering was present in low quality oocytes
b) The minimal-handling ovarian superstimulation treatments implemented in the present study to reduce the number of bison handlings did not increase the number of COC collected or improve embryo production rates

Chapter 3

a) A single dose of 5,000 IU eCG increased the number and size of follicles available for COC collection, more than doubled the number of COC collected for IVP and resulted in the production of more embryos than other groups
b) Collections done after follicle wave synchronization resulted in greater embryo production than collections done at random stages of the follicular wave
c) Repeated COC collections after successive wave synchronization resulted in similar follicular counts and embryo production rates within individuals
d) The greatest number of follicles aspirated, COC collected, and embryos produced was in the anovulatory season

Chapter 4

a) COC collections can be done as effectively on sedated recumbent bison as those restrained in a standing position in a hydraulic chute
b) Ovarian superstimulation using a single-dose eCG protocol was as effective as a multiple-dose FSH+hCG protocol
c) Field darting was an effective method of administering superstimulation treatments
d) Ovarian superstimulation improved *in vitro* embryo production

Chapter 5

a) Oocytes were collected and embryos were produced from bison at both 90- and 120- days of gestation, but the position of the ovaries at the more advanced stage reduced COC collection efficiency
b) Yearling pre-pubertal bison provided the most promise as COC donors in the field as they produced a similar number of embryos (approximately 1 freezable embryo per collection) as non-pregnant mature bison and are available for collection throughout the winter season without the limitations imposed by advanced stages of pregnancy or unknown pregnancy status

Chapter 6

a) IVP embryos of higher quality had less cryo-damage and higher cell counts
b) Morulae survived cryopreservation with less damage than blastocysts, and subsequent transfer resulted in a higher pregnancy rate at 30-days and lower pregnancy loss between 30 to 60 days than blastocysts
c) The vitrification method of cryopreserving embryos resulted in a pregnancy rate similar to the transfer of fresh embryo

8.2. Future studies

Future studies could explore the following:

a) Investigate COC collections (i.e., at random stages and after a single dose of eCG) on sedated yearling bison

b) Compare *in vivo* vs *in vitro* embryo kinetics (developmental stage/time) in bison

c) Compare lipid content in *in vivo* vs *in vitro* bison embryos

d) Investigate closed vitrification systems for higher security during storage and transport

e) Create an *in vitro* culture system specifically for bison

f) Compare follicular counts and embryo production efficiency for bison in free-roaming, non-supplemented bison vs captive, supplemented bison

g) Develop portable COC collection equipment for off-grid use in cold weather conditions

h) Investigate the effect of superstimulation on wild and free-roaming bison

i) Conduct a risk assessment to determine interventions required to collect COC from bison of unknown disease status

j) Investigate whether washing techniques on embryos produced from Brucella positive bison can result in disease-free bison calves and embryo recipients

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APPENDIX A:

Bison IVF embryo production protocol - 2022
Miranda L Zwiefelhofer

Day prior to collection (morning)

- Prepare holding, OPU and rinsing media (recipes in Appendix D)
- Place the media into the fridge until needed

Day prior to collection (afternoon/night)

- Turn on travel incubators
    - (WTA oocyte transporter [LabMix] – no CO_2, only temperature [38.5°C])
    - MicroQ – 37°C
- Place appropriate number of OPU and rinsing media bottles in incubator at farm (37°C)
    - Estimate 200 mL of rinsing media per animal
- Turn heat on in barn and field lab

Morning of collection (In IVF lab – 5:30am)

- Pipet 1 mL of BO-HEPES-IVM into x number of 1.5 sonification tubes and replace their caps
- Number each of the tubes
- Place all the IVM tubes into the LabMix incubator
- Pipet 0.5 mL of BO-HEPES-IVM into 0.6 mL graduated tubes
- Pipet 1.5 mL of BO-WASH into of 1.5 mL graduated tubes
- Place both the 0.6 mL and 1.5 mL graduated tubes into an empty pipete tip container inside the MicroQ

Morning of collection (Field lab – 6:30am)

- Arrive at farm and plug in all warming plates
- Set up and warm filters, petri plates
- Make grid for searching on plates
- Fill OPU media in 3 - 50 mL tubes and 1 – 15 mL conical tubes and put in vacuum pump heater by chute
- Fill 8 - 35 mL syringes with warmed rinsing media, put back in paper coverings (just cracked open when syringes were removed) and place back in incubator
    - Will have to refill throughout day during “breaks”

Morning of collection (At chute – 6:30am)

- Wipe dust off tables and place washed drapes on the tables (tape down if windy)
- Plug in ultrasound and vacuum pump
- Check hydraulic system is working properly
- Set up aspiration lines with needle and OPU rods

- Adjust flow rate to 12-16 mL of fluid per minute

Start of collection (Chute - 8:00 am)

- Communication is key - make sure all workers are on the same page****
- After bison is securely restrained in the chute the bison is given an epidural of ~6 mL of lidocaine
- Perineum is scrubbed with alternating betadine and 70% alcohol scrub followed by a final rinse of 0.9% saline solution
- Transvaginal ultrasound probe is inserted into the vaginal fornix and needle is placed into the needle guide
- Hand is inserted into the rectum and manure is evacuated
- Ovaries are palpated and manipulated caudally until visualized on the ultrasound
- Follicles ≥3 mm aspirated using a vacuum pressure of 12-16 mL of fluid per minute
- Once follicles on both ovaries are collected the probe/needle is removed
- Tube containing aspirates is brought to the laboratory

Oocyte Retrieval, Washing and Maturation (Lab Day -1)

- Important to move quickly – If the lab gets behind, ask aspiration team to take a break
- Maximum 20 minutes total for - receiving tube, filtering, searching, washing and placing in tubes
- Once the aspirate is received from the aspiration team, place the 50 mL tube into the warmer (37°C) until filtering can be done
- Record the bison number, time the tube was received, and the number of follicles aspirated
- The aspirate will be transferred into an IVF oocyte filter with a 75 μm mesh to be filtered
- Approximately four 35 mL syringes of BO-OPU and a 18G x 1.5" needle will be used to aid in filtering the aspirate
- The filtered aspirate will be transferred to a labeled 100 mm x 15 mm Petri dish to locate the oocytes using a stereomicroscope
- Transfer oocytes to a labeled 35 mm x 10 mm dish containing 2 mL holding media on a hot plate (37°C) using a pipette based on their grading (GOOD – compact good, compact regular and BAD – compact poor, expanded, denuded, and degenerate)
- COC grading is as follows (record in data sheet)
    - compact-good (>3 layers of unexpanded cumulus cells and homogeneous ooplasm)
    - compact-regular (1 to 3 layers of unexpanded cumulus cells and homogeneous ooplasm)
    - compact-poor (>1 layers of unexpanded cumulus cells and heterogeneous ooplasm)
    - expanded (cumulus cells expanded or partially dissociated)

    - denuded (oocyte without cumulus cells)
    - degenerate (pyknotic granulosa cells or vacuolated ooplasm)
- If plates have lots of additional cells "dirty plate", wash plate in holding medium until plate is clear
- The oocytes will then be washed thrice in a four well dish containing 0.5 mL of BO-WASH in three of the wells then once in 0.5 mL of BO-HEPES-IVM
- After washing, the oocytes are transferred to 1.5 sonification tubes containing 1 mL BO-HEPES-IVM from the small, red (LabMix) incubator and placed back into the incubator
- Record the tube number, bison number, treatment, time tube was placed into the incubator, and grading
- Lab mix incubator with COC are transported back to the laboratory for **24-28 total hours of maturation** in the large incubator
    - Or you can keep them in the small travel incubator if closely monitoring and it is plugged in

Night of collection (Lab Day -1)

- Prepare IVF dishes and four-well plates
- Acquire x number of 35 mm x 10 mm dishes the day prior to IVF
- Label the dishes IVF, collection number, plate number, and treatment
- Pipet 10 µL of BO-IVF into the center of the dish as a droplet
- Pipet 5 mL of oil to the dish careful not to disturb the droplet
- Pipet another 80 µL of BO-IVF into the droplet slowly careful not to let the droplet bubble out
- Transfer the dishes to the incubator to allow for equilibration overnight at 38.8°C and 5% CO_2 in humidified atmospheric air (21% O_2) or at 38.8°C in 5% O2, 5% C CO_2 and 90% N_2
- Pull out x number of four-well dishes
- Pipet 0.5 mL of BO-IVF into two of the wells
- Transfer the four-well plates to the incubator to equilibrate overnight at 38.8°C and 5% CO_2 in humidified atmospheric air (21% O2) or at 38.8°C in 5% O_2, 5% CO_2 and 90% N_2

(Lab Day 0)

Wash oocytes prior to IVF

- Calculate maturation time ranges for first and last COC processed
- Make sure to stay within 24 to 28 hours (follicular aspirate brought to field lab → time of semen placed with oocytes)
- Count backwards to know when to start washing COC and when to thaw semen
- Record lab data in sheet
- Remove the sonification tubes (one at a time) from the incubator
- Remove the oocytes from the 1.5 mL sonification tubes and pipet into one well of a four-well dish

- Transfer the oocytes to an equilibrated four-well dish containing two wells of 0.5 mL BO-IVF to wash the oocytes twice
- Remove the equilibrated IVF dish from the incubator
- Transfer the oocytes to the 80 µL droplet in their labelled dish which will be returned to the incubator until semen is added to the droplet
- Record the number of oocytes transferred into the dish

Preparing semen for IVF

- Make 2 PureSperm 80 / 40 gradients (Catalog # PS40-100 & PS80-100; Nidacon, Gothenburg, Sweden) with 0.5 mL layers in 1.5 mL tubes and keep at room temperature
- Warm (37°C) 2 mL of Semen-prep (IVF-bioscience)
- Fill a 15 mL tube with 1 mL distilled water and place into the fridge for cooling
- Remove two semen straws from the liquid nitrogen
- Ensure all the liquid nitrogen is removed from the straw by flicking the straw quickly then placing it in a water bath at 37°C for 30-60 seconds
- Place on a KimWipe to dry off straws ensuring all water is removed and the straws remain warm
- Bring thawed straws back to IVF lab
- Cut the sealed end of each straw and empty into a pre-warmed 1.5 mL tube (1 straw per tube)
- Transfer contents of each semen tube to the top of each gradient using a 1 mL pipete (Drip down the side of the tube slowly)
- Place the tubes into the centrifuge to spin at 300 x g for 20 minutes
- Remove the supernatant using a 1 mL pipete leaving the semen pellet
- Add ~1 mL of Semen-prep to each semen pellet
- Place the tubes into the centrifuge to spin at 300 x g for 5 minutes
- Remove the supernatant using a 1 mL and 200 µl pipete and only leave the semen pellet
    - Semen of high quality is processed easier
    - Low quality or sexed semen is more difficult to work with
    - Semen pellet concentration and volume will differ (flexibility and feel are required during processing)
- Combine semen pellets (20 µl pipet)

Sperm motility

- Pipet ~10 µL of the combined semen pellet onto a labeled slide and place a cover slip over the semen to determine motility
    - If semen is of poor quality you can preserve sample by using a smaller volume so the semen does not get wasted in quality tests
    - Small and light coverslips work best

Sperm concentration

- Remove the 1 mL tube of water from the fridge
- Pipet 10 µL of the mixed semen into the blue top tube
    - If poor quality of semen – preserve sample (smaller proportion and volume)
- Pipet 10 µL of diluted semen onto each side of the hematocytometer and place onto the phase-contrast microscope
- Count the sperm on each of the two grids (25 squares per side) and calculate the average per side (average value = X)
- Calculate the volume of semen required so that 2.0 x 10^6 sperm are present and record the value
    - Math = 200 / X = volume of prepared semen
    - Example: 200 / 50 average count = 4 µL sperm
    - Example: 200 / 20 average count = 10 µL sperm
    - If concentration very low – can use (100 / X) or may need to process new semen straws
    - Keep in mind the maturation time frame**
- Pipet the calculated volume of semen into each of the 35 mm x 10 mm IVF dishes
- Return the dishes to the incubator where they will remain approximately 16 - 20 hours

Preparing Culture dishes (Day 0)

- A day prior to culturing, acquire x number of 35 mm x 10 mm dishes
- Pipet 10 µL of BO-IVC into the center of the dish as a droplet
- Pipet 5 mL of oil to the dish careful not to disturb the droplet
- Pipet 80 µL of BO-IVC into the droplet slowly careful not to let the droplet bubble out
- Place the dishes in the incubator to equilibrate at 38.8°C in 5% O2, 5% CO_2 and 90% N_2
- Pipet 1.5 mL of BO-WASH into 1.5 mL graduated tubes to be placed in the CO_2 incubator for prewarming
- Also, pipet 0.5 mL of BO-IVC into two wells of a four-well dish and place in the incubator to be equilibrated at 38.8°C in 5% O2, 5% CO_2 and 90% N_2

(Day 1)

Denuding and preparing zygotes for culture

- On the morning of day of denuding and culturing, fill and warm BO-WASH in 1.5 mL graduated tubes
- Once warm, pipet 0.5 mL of BO-WASH from the prewarmed 1.5 mL graduated tube into three of the four wells of a four-well dish
- Remove the IVF dish from the incubator and transfer the zygotes to the first well using a 200 µl pipete
- Using a pipet with a 170 µm tip, transfer the zygotes from the first well to the second making sure to not move any debris
    - Try to avoid the floating oil during transfer as they will clog the tips

- In the second well, use a pipet with a 135 μm tip to pick up and pipet out the zygotes several times to remove any remaining cumulus cells (approximately repeat the process eight times in total)
    - 130 μL tips can be too small for bison oocytes
- Transfer the zygotes to the third well and repeat the above process
- Remove the second 1.5 mL graduated tube of BO-WASH from the incubator and pipet 0.5 mL into the fourth well
- Remove the four-well dish containing two wells of BO-IVC to pipet the remaining 1 mL of BO-WASH into the other two wells
- Repeat the denuding/washing process above through the remaining three wells until no cumulus cells are left on oocytes or floating in dish.
    - Contamination issues will occur otherwise
- The zygotes should be completely denuded when the zygotes are transferred to IVC
- Transfer the zygotes into the first well containing BO-IVC and then transfer to the second well of BO-IVC
- Remove the equilibrated culture dish from the incubator and carefully transfer the zygotes from the last well into the droplet
- Return the culture dish to the incubator to culture for approximately 56 hours
- Record the number of oocytes transferred to the culture dishes

(Day 3)

Confirming cleavage

- Day prior make a new set of IVC plates (I labeled as IVCC)
- After approximately 56 hours in the culture dish, remove the IVC dish from the incubator to determine whether cleavage has occurred
- Record all zygotes that have cleaved, move cleaved zygotes to new IVCC dish
- Place IVCC dish in the incubator

(Day 6.5-9)

Embryo evaluation

- Start checking embryos at Day 6.5 and every 12 hours
- Record the stage and grade of embryos at each check (grading and development details and images in Chapter 6)
- Image embryos for records

Confirming embryo formation and vitrification process

- Ensure the three bottles of the BO-VITRICOOL kit are at room temperature (Pre-incubation, Cooling 1, and Cooling 2) and warm the holding medium on the hot plate
- Acquire a small Styrofoam box and fill ½ - ¾ full of liquid nitrogen from stored canister
- Remove the culture dish from the incubator

- Check each of the dishes to confirm embryo status then return to the incubator quickly
- Record the stage and grade of embryos
- Acquire the appropriate number of cryotops and covers needed for vitrification
- Record the embryo number, date, cryotop color, type, stage name, stage number, grade, treatment, collection number, and day since fertilization (day 0 = fertilization)
- Acquire labels for each of the cryotops and label with embryo number, date, species, stage number, grade, day since fertilization
    - I use brady labels for cryotops (BMP71 BMP61 M611 TLS 2200 Self-Laminating Vinyl Wire and Cable Labels)
- Remove the culture dish again and transfer the embryos to be vitrified into a four-well plate
- Return the remaining zygotes to the incubator
- Wash embryos in 12 step IETS trypsin wash with commercial holding media and trypsin
- Transfer the embryos through each of the wells for washing – new tip for each new well
- Pipet 50 µL droplets of the pre-incubator solution at the top of a 100 mm Petri dish or 500 µL in a 4 well plate
- Pipet 30 µL droplets from the containers of Cooling 1 and Cooling 2 onto the same Petri dish below which is divided and labelled 1 and 2 on either side based on the number of embryos to be vitrified
- Transfer an embryo from the ten-well dish to pre-incubator solution droplets for 2 minutes using the **290 µm tip stripper pipettor**
- Transfer the embryo from the pre-incubator solution to a droplet of cooling 1 for another 2 minutes
- Transfer the embryo to the droplet of cooling 2 and pipet some of the solution to allow for contact with the embryo
- Carefully pipet up the embryo and some of the solution using the 290 µm tip so that the embryo is within ¼ end of the tip
- Pipet droplets of solution along the cryotop tip containing the embryo within 30 seconds of removal from the cooling 1 solution then plunge into the liquid nitrogen
- Repeat the process with the remaining embryos
- Plunge the covers of the cryotops into the liquid nitrogen
- Place the cover of the cryotops over the tips under liquid nitrogen using the clamps
- **Do not let the clear tips of the cryotops leave the liquid nitrogen**
- Gently tap against the side of the box to ensure the covers and securely attached
- Acquire x number of canes, holders and tags
- Place a tag onto the holder and label with the cane number, date, and day since fertilization
- Record the cane number, and cane top color into the chart
- Place two canes into a holder and cut the edge of the cane closest to the tag so that air bubbles can escape when under the liquid nitrogen
- Plunge the holders and canes into the liquid nitrogen

- Using clamps, carefully move up to four cryotops into the bottom cane with the covers over the tip entering first
- Push the top cane over the labels of the cryotops ensuring that the canes cover the entirety of the cryotops
- Quickly transfer embryos to liquid nitrogen tank
- Record the tank name and position number of the holder and canes

APPENDIX B:

Below is a QR code and web link for a movie demonstrating oocyte collection and *in vitro* embryo production procedures in bison. The visualization of overall flow and techniques may be beneficial for future researchers.

https://youtu.be/bQ1NsrShzwM

Note: The video was created in 2019 by Miranda Zwiefelhofer. Some embryo production details may differ from the current 2022 SOP provided in Appendix A.

APPENDIX C:

Bison OPU/IVF supply list - 2022
Miranda L Zwiefelhofer

Animal side

- MyLab Alpha ultrasound
- Transrectal probe
- Transvaginal probe
- WTA vacuum pump
- Silicone vacuum line with filter
- Ultrasound handles
- Ablation rods (made by Jaswant's 3D printer)
- 2 drapes
- WTA 18G follicular aspiration needles (Catalog # 13853; wings shaved off with pencil sharpener)
- Pencil sharpener
- Aspiration lines (autoclaved medical grade Polytetrafluoroethylene (PTFE) micro tubing (internal diameter 0.047 mm; Catalog # BB311-17, Scientific Commodities, Lake Havasu City, AZ, USA).
- Blunt 18g needles-for flushing system
- Lidocaine
- 18g needles
- 6 mL and 60 mL regular syringes
- 1000mL Saline bags
- Handheld WTA pocket heater
- 50mL tubes
- OPU media for collection
- Record book
- Pencils
- Sharpies
- Gloves (small and large)
- Palpation sleeves
- Alcohol
- Betadine solution (Blue)
- Betadine detergent (Red)
- Infusion pipettes
- 2-20 L pails (for placing probe in and washing)

Field Lab

- Counter coverings
- 2 stereomicroscopes
- large heating block
- small heating block
- filter holder
- 75 micron filter
- Small incubator
- Heated OPU media bottles
- 50 ml tubes
- 15 ml tubes
- 18 g needles
- 100 mm petri dishes
- 30 mm petri dishes
- 4 well dishes
- Micro Q incubator
- WTA portable incubator
- IVM-hepes medium in 0.6 mL tubes
- IVM-hepes medium for wash in 0.6 mL tubes
- BO-wash in 1.5 mL tubes
- Pipettors (1000 and 100)
- Tips (1000 and 200)
- 30 mL syringes
- data book
- scissors

IVF lab

- Steromicroscope
- Heating block
- Microscope heating block
- Large CO_2 incubator
- Miri incubator
- Pipettors (1000, 100 and 10)
- Tips (1000, 200 and 10)
- Stripper tips (135, 170)
- 5 mL pasteur pipettes
- BO-IVF medium
- BO-IVC medium
- BO-Wash medium
- Filtered oil
- 30 mm petri dishes
- 4 well dishes
- Microscope camera
- Gloves
- Data book

APPENDIX D:

Homemade oocyte handling media protocols

OPU collection medium

- 500 mL DPBS + Ca + Mg
- 2 mL Heparin (1000 USP Units/mL)
- 1.5 mL Sterile pluronic surfactant (1% v/v pluronic F-68 non-ionic surfactant [100X; Catalog # 24040032, Gibco])

*Combine in DPBS bottle and seal with parafilm under biosafety cabinet

Filter rinsing medium

- 500 mL DPBS + Ca + Mg
- 2 mL Heparin (1000 USP Units/mL)

*Combine in DPBS bottle and seal with parafilm under biosafety cabinet

Holding medium

- 95 mL DPBS + Ca + Mg
- 5 mL Calf Serum

*Filtered with .22 micron filter
*Keep in refrigerator until use

APPENDIX E:

Bison embryo transfer supply list - 2022
Miranda L Zwiefelhofer

Large Equipment
Microscope
Microscope heater plate w/ cords
Heated slide plate with cover (37°C)
2nd heater plate
Gun warmer with charger
Dry shipper w/ embryos
Styrofoam box for liquid nitrogen
Liquid nitrogen (in dry shipper or tank)
Portable heater
Ultrasound with transrectal probe

Embryo Handling
Pipettors (200 µL and 1000 µL)
Tips (200 µL and 1000 µL)
Large petri dishes (1 rack)
Transfer media in 1.5 mL tubes
4 well dishes (IVF ones are best) – 2x4-pack
Stripper pipette
290 µL stripper tips
0.25 mL straws
1 mL syringe plus adapter
Hemostats
ET guns x 2
Blue sheaths
Chemises
Ring stoppers

Media
Vitri-warm kit (IVF-bioscience)
Transfer medium

At chute
Lidocaine
6 mL syringes and 18 ga needles
Saline
Betadine/iodine scrub
60 mL syringes
Paper towels
Alcohol gauze
Lubricant

Other
Scissors
Gloves (small and large)
Palpation gloves
Kim wipes
Pencils, sharpies, highlighters
Table covering