IN VITRO EMBRYO PRODUCTION IN WOOD BISON *(Bison bison athabascae)*

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By

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“Most of the important things in the world have been accomplished by people who have kept on trying when there seemed to be no hope at all.” Dale Carnegie

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ABSTRACT

The studies presented in this thesis were designed to develop efficient protocols for collecting competent oocytes and to produce disease-free wood bison embryos by *in vitro* fertilization and culture. In Chapter 3, the *in vivo* and *in vitro* maturational characteristics of cumulus-oocyte complexes (COC) collected from live wood bison during the anovulatory and ovulatory seasons were compared. The stages of nuclear maturation: germinal vesicle (GV), germinal vesicle break down (GVBD), metaphase I (MI) and metaphase II (MII) were determined in wood bison oocytes using anti-Lamin AC/DAPI staining. Additionally, the optimal interval of time after human chorionic gonadotrophin (hCG) treatment required for *in vivo* oocyte maturation in wood bison was determined. Nuclear maturation occurred more rapidly during *in vitro* versus *in vivo* maturation, but was associated with less cumulus cell expansion than with *in vivo* maturation. *In vitro* oocyte maturation was maximal after 24 h of *in vitro* maturation. *In vivo* oocyte maturation was more complete at 30 than 24 h after hCG treatment. Season had no effect on the maturational capacity of wood bison oocytes. Competence of the *in vitro* (Chapter 4) or *in vivo* (Chapter 5) matured oocytes to develop to the blastocyst stage in culture was evaluated in subsequent studies. In Chapter 4, the hypothesis that the morphological characteristics of wood bison cumulus-oocyte complexes (COC) affect the ability of the immature oocyte to develop *in vitro* following *in vitro* fertilization was tested. The effect of extending from 48 h to 72 h the FSH starvation period after superstimulation (FSH diluted in 0.5% hyaluronan) on number and size of the follicles at the time of collection by transvaginal ultrasound-guided follicular aspiration, on COC morphological characteristic, and on blastocyst development rate, was also investigated. Compact COC classified as good (>3 layers of cumulus cells) resulted in the highest blastocyst rate following *in vitro*

maturation, fertilization and culture. There was no effect of extending the FSH starvation period by 24 h on the number of follicles ≥ 5 mm at the time of collection, the morphology of the COC or blastocyst rate on Day 7 or 8 after fertilization. The morphological characteristics of immature wood bison COC affect oocyte *in vitro* developmental potential to the blastocyst stage. In Chapter 5, the effect of an additional 4 h of *in vitro* maturation of *in vivo* matured oocytes collected 30 h after hCG treatment on subsequent embryo development was evaluated. In addition, the effects of extending the interval between hCG treatment and COC collection from 30 to 34 h on *in vitro* embryo production was evaluated. Results confirmed that an additional short period of *in vitro* maturation, or an extended period of *in vivo* maturation increased *in vitro* embryo production rates in wood bison. In the final chapter (Chapter 6), the effectiveness of the IETS washing procedures with or without antibiotics for removing *Brucella abortus* from *in vitro*-produced embryos infected *in vitro* with the pathogen was determined. *Brucella abortus* was removed from 100% of *in vitro*-exposed embryos following 10 washes of 100 fold dilution, with or without antibiotics. Results validated the embryo washing procedures for producing *Brucella*-free *in vitro*-produced wood bison embryos.

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DEDICATION

I would like to dedicate this thesis to my husband Manuel, who has been by my side throughout this whole journey, for making available his support in a number of ways and for his incredible understanding during late nights of typing; and to my precious son, Ricardo, whose smile can always brighten my day and keep me going day after day.

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LIST OF ABBREVIATIONS

AI	Artificial insemination
ANOVA	Analysis of variance
BO	Brackett-Oliphant
BSA	Bovine serum albumin
cc	Cubic centimeter
CFU	Colonia forming units
CITES	Convention on the International Trade of Endangered Species of Fauna and Flora
COC	Cumulus-oocyte complexes
CO_2	Carbon dioxide
CL	Corpus luteum
COSEWIC	Committee on the Status of Endangered Wildlife in Canada
CR1aa	Charles Rosenkran's aminoacid
DAPI	Diaminophenylindole
DPBS	Dulbecco's phosphate-buffered saline
eCG	equine chorionic gonadotropin
EINP	Elk Island National Park
ET	Embryo transfer
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
h	hour
hCG	Human chorionic gonadotropin
IETS	International Embryo Transfer Society
IUCN	International Union for Conservation of Nature and Natural Resources
i.m.	Intramuscular
IOI	Interovulatory interval
IU	International units
IVC	*In vitro* culture
IVM	*In vitro* maturation

IVP	*In vitro* embryo production
IVF	*In vitro* fertilization
IWI	Interwave interval
L	Liter
LH	Luteinizing hormone
mg	Milligram
MHz	Megahertz
MI	Metaphase I
MII	Metaphase II
mL	Milliliter
mm	Millimeters
NIH-FSH-P1	Porcine FSH
N_2	Nitrogen
O_2	Oxygen
P4	Progesterone
pFSH	Porcine FSH
pLH	Porcine LH
PGF	Prostaglandin F2α
SARA	Species At Risk Act
SAS	Statistical analysis system
SEM	Standard error of the mean
TALP	Tyrode's albumin lactate pyruvate
TCM-199	Tissue culture medium-199
µg	Microgram
µm	Micrometer
USA	United States of America
vol	volume
vs.	Versus
WBNP	Wood Buffalo National Park
ZP	Zona Pellucida

CHAPTER 1. GENERAL INTRODUCTION

The following literature review provides general information about the conservation status of the wood bison, reproductive physiology of female bison, the biological aspects of oogenesis and folliculogenesis, oocyte maturation and superstimulation in cattle, and an overview of the reproductive technologies that have been applied in bison. Of particular interest, the technique of producing embryos *in vitro* is reviewed.

1.1. Conservation status of the wood bison

Wood bison (*Bison bison athabascae*) is the largest terrestrial mammal in North America (Reynolds et al., 2003) and one of the most emblematic species in Canada (Harper et al., 2000). However, its population has suffered several challenges over the past two centuries (Gates et al., 2001). The historical range of the wood bison comprised the western part of northern North America (van Zyll de Jong, 1986). This area included the provinces of Saskatchewan, Alberta, British Columbia, the Northwest Territories, Yukon, and Alaska (van Zyll de Jong, 1986; Stephenson et al., 2001). During the late 1800s, approximately 168,000 wood bison were roaming freely throughout this area (Soper, 1941). Unfortunately, this number decreased dramatically until approximately 250 individuals remained at the beginning of the 1900s (Soper, 1941). Overhunting associated with the fur trade was considered the main factor for the near extermination of the wood bison during this period (Soper, 1941; Harper et al., 2000; Gates et al., 2001). By 1914, the population of wood bison had increased to approximately 500 individuals associated with conservation efforts (e.g. hunting ban) of the Canadian government (Banfield and Novakowski, 1960). Later, in 1922, Wood Buffalo National Park was created to protect and maintain the growing wood bison population (~1,500 individuals) in a natural habitat (Environmental Assessment Panel, 1990; Gates et al., 2001).

Wood Buffalo National Park (WBNP) is situated in northern Alberta and southern Northwest Territories (Soper, 1941). The park not only contains the largest population of wood bison in Canada, but also most genetically diverse herd of wood bison in the region (McFarlane et al., 2006). However, for the past eight decades, the population of wood bison has been threatened by cattle diseases that came from an external source (Shury et al., 2015). After the creation of WBNP, the wood bison population increased steadily to reach approximately 12,000 individuals by the end of the 1940s (Environmental Assessment Panel, 1990). Nevertheless, the population of wood bison declined to around 2,300 individuals by the late 1990s (Mitchell and Gates, 2002). Studies have supported the hypothesis that the presence of bovine tuberculosis and brucellosis may have played a major role in the decrease of the wood bison population from the 1970s to the 1990s (Tessaro, 1986; Tessaro et al., 1990). These cattle diseases were introduced into the park during the translocation of 6,673 plains bison from the Wainwright Buffalo Park in Alberta to the WBNP between 1925 and 1928 (Soper, 1941; Environmental Assessment Panel, 1990). Tuberculosis and brucellosis affect the population dynamics of wood bison in the park and are thought to be involved in the population decline of this species (Joly and Messier, 2005). Eradication of cattle diseases in the park will not only help with the reclamation of the wood bison population in the region, but will also prevent the spread of these diseases to healthy wood bison and livestock in and around this area.

Currently, the International Union for Conservation of Nature and Natural Resources (IUCN) considers the wood bison as a near threatened species (Gates and Aune, 2008) whereas the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) lists the wood bison as a species of special concern (COSEWIC, 2013). Likewise, the Convention on the International Trade of Endangered Species of Fauna and Flora (CITES) places the wood bison into Appendix II of the CITES regulations (CITES, 2016). According to CITES, international trade of wood bison

is not prohibited but regulated. In any case, it is clear that there is still some concern on the fate of the wood bison population in Canada. Serious actions have been taken to deal with cattle diseases and eliminate these diseases in this species.

1.2. Reproductive physiology of the female bison

Mammals can be classified as seasonal or non-seasonal breeding species. Seasonal breeders (e.g., bison, sheep, goat, horse) exhibit regular estrous cyclicity and active breeding during a specific time of the year (Vasantha, 2016) that is followed by an anestrus period. They restrict their reproductive efforts to ensure their offspring are born and weaned during a period with the most favourable temperature conditions and plentiful energy resources (Bronson, 1989). Non-seasonal breeders (e.g. pig, domestic cattle) undergo estrous cyclicity and breeding throughout the year irrespective of the season or time of the year (Vasantha, 2016). Changes in day length (i.e., photoperiod) regulates cyclicity in seasonal breeders, and melatonin plays a major role in controlling such events (Tamarkin et al., 1985).

Female wood bison are considered seasonal breeders; the ovulatory season (i.e., breeding season) in western Canada occurs between August and February, and the anovulatory season (i.e., non-breeding season) occurs between March and July (McCorkell et al., 2013; Palomino et al., 2013; Palomino et al., 2014b). Ovarian function in bison females differs between these seasons; as described in following sections.

1.2.1 Ovulatory Season

In wood bison, follicular and luteal activities are observed during the ovulatory season characterized by ovulation and subsequent formation of the corpus luteum (CL) (Goodrowe et al., 2007).

The lifespan of the CL following the first ovulation is short (averaged, 4 days), resulting in a short first interovulatory interval (averaged, 8 days [McCorkell et al., unpublished data] or 10 days [Rutley and Rajamahendran, 1995]). This initial interovulatory interval is composed of only one follicular wave, while the interval of a normal estrous cycle in bison is composed of two follicular waves (Adams, 2007) resulting in an estrous cycle length of 20 to 23 days (Matsuda et al., 1996; Kirkpatrick et al., 1991; McCorkell et al., unpublished data).

In the second follicular wave, the ovulatory size of the dominant follicle has been shown to be 14 mm in diameter while the maximum size of the CL is 18 mm in diameter (McCorkell et al., unpublished data). The last ovulation occurs in early spring (March-April) ending the ovulatory season in bison (Rutley and Rajamahendran, 1995). As in other seasonal breeding species (e.g. wapiti [McCorkell et al., 2007]), a period of transition from the ovulatory season to the anovulatory season has been speculated to occur in bison (Palomino, 2015).

1.2.2. Anovulatory season

In contrast, only follicular activity is observed during the anovulatory season in wood bison. During this time, follicle development occurs in waves characterized by regular and synchronous development of a group of follicles associated with a surge in serum FSH concentration (McCorkell et al., 2013). A dominant follicle is selected 3 days after wave emergence and reaches a maximum diameter of 10 mm, whereas subordinate follicles regress. However, ovulation does not occur and progesterone levels remain below 1 ng/mL. After an interval of seven days, a new wave emerges, and the wave pattern of anovulatory follicles is repeated (McCorkell et al., 2013). The transition period from the anovulatory to the ovulatory season has been investigated in wood bison (McCorkell et al., unpublished), and photoperiod and melatonin secretion are speculated to

be involved in the resumption of the LH surge and ovulation in wood bison during the ovulatory season (Palomino, 2015).

1.3. Oogenesis and folliculogenesis

Oogenesis includes the formation, growth and maturation of the oocyte (Dunbar et al., 2012). Folliculogenesis is the process involving growth and development of ovarian follicles from primordial to preovulatory stages (Malgorzata et al., 2016), and includes the differentiation of somatic cells associated with the oocyte to form a follicular unit (Dunbar et al., 2012). The processes involved in growth and differentiation of the oocyte and its surrounding granulosa cells are highly coordinated (Salustri et al., 2003) highlighting the link between oogenesis and folliculogenesis in mammals (McGinnis et al., 2012). In cattle, the time required to complete folliculogenesis has been estimated to be 80 to 100 days (Britt, 1991).

Oogenesis and folliculogenesis in cattle begin in fetal life, when the primary oocyte (diploid), becomes surrounded by squamous granulosa cells to form the primordial follicle (Haston and Pera, 2007). The primary oocyte enters meiosis and arrests at prophase of meiosis I while the granulosa cells of the follicle transform to cuboidal cells forming a primary follicle (Haston and Pera, 2007). During puberty, the primary oocyte increases in size and a membrane, the zona pellucida, forms around it as the follicle matures (Pansky, 1982). The primary oocyte remains arrested whereas the primary follicle develops into the secondary follicle that includes multiple layers of granulosa cells surrounded by an outer layer of theca cells and a basement membrane (Haston and Pera, 2007). Under the influence of gonadotrophins (i.e., FSH), the secondary follicle develops into the tertiary follicle or antral follicle (Ergüven et al., 2012) characterized by the formation of the follicular antrum (Salustri et al., 2003). In large antral follicles, there are mural granulosa cells that line the wall of the follicle, and cumulus cells that surround the oocyte (Ergüven et al., 2012). The oocyte

grows but still remains in meiotic arrest until triggered by a follicular response to a preovulatory surge of LH (Ergüven et al., 2012). During each estrous cycle, several follicles may develop to the antral stage, but only one follicle, the dominant follicle, reaches full maturity and is selected for ovulation, while most of the others undergo atresia (Ginther et al., 1989). A few hours before the antral follicle ovulates, the oocyte completes meiosis I and becomes a secondary oocyte, which is arrested at metaphase of meiosis II (Haston and Pera, 2007). At ovulation, the released secondary oocyte is surrounded by the zona pellucida and a follicular cell layer, the corona radiate (Pansky, 1982). After ovulation, the granulosa cells and theca cells within the preovulatory follicle differentiate into luteal cells to form the corpus luteum (CL), which secretes progesterone (Channing et al., 1980). If fertilization occurs (penetration of spermatozoa), the oocyte completes meiosis II and becomes a fully competent oocyte, or mature ovum (haploid), which is able to support early embryonic growth (Haston and Pera, 2007). Approximately 1 h after fertilization, the nuclei of the sperm and ovum fuse, forming a diploid zygote (Jorde et al., 2009).

Oocytes acquire the competence for resumption of meiosis at ~100 µm in diameter, and for completion of meiotic maturation to metaphase II at ~110 µm in diameter (Fair et al., 1995). When the oocyte has reached 120-130 µm in diameter, its diameter does not change further, whereas the follicle continues to grow to reach its preovulatory size of 15–20 mm in diameter (Fair, 2003). Additionally, the oocyte within a dominant follicle undergoes ultrastructural modifications before the preovulatory LH peak, permitting the oocyte to attain full competence to develop into an embryo (Hyttel et al., 1997). In other words, oocyte developmental competence (i.e., the ability to mature, grow and develop, be fertilized and give rise to normal, healthy offspring [Duranthon and Renard, 2001]) is acquired gradually *in vivo*. However, oocytes for maturation *in vitro*, are usually obtained from follicles of different sizes and stages of development. In this case, oocytes obtained

from follicles greater than 6 mm in diameter have a greater competence to reach the blastocyst stage than those obtained from follicles of 2-6 mm (Lonergan et al., 1994).

1.4. Oocyte maturation

Oocyte maturation is the physiological process that is required for successful fertilization and subsequent embryo development (Lonergan and Fair, 2016). This process involves complex and distinct events of nuclear and cytoplasmic maturation within the oocyte (Ferreira et al 2009). The oocytes grow and undergo remodelling on cellular and molecular levels to meet all requirements for subsequent development (Sirard et al., 2006).

1.4.1. Nuclear Maturation

Nuclear maturation corresponds to the process of resumption of meiosis that results in a haploid oocyte (Voronina and Wessel, 2003). In response to the LH surge (Sirard et al., 2006), the arrested oocyte progresses from germinal vesicle (GV) stage, through germinal vesicle breakdown (GVBD), Metaphase I (MI), Anaphase I, Telophase I and rearrests at Metaphase II (MII) (Krisher 2013).

Immature oocytes are arrested in prophase I of meiosis I and are characterized by the presence of a nuclear envelope that contains a large GV with a large nucleolus, and decondensed, dispersed chromosomes (Wasserman and Albertini, 1994; Lonergan and Fair, 2016). Once maturation starts, the chromosomes begin to condense, the GV breaks down (i.e., nuclear envelope dissolves), and the nucleolus disperses (Masui and Clarke, 1979). Following GVBD, condensed chromosomes line up forming the MI plate with a visible spindle (Fulka and Fulka, 2007). This stage is followed by a short Anaphase I to Telophase I transition. During this transition, paired homologous chromosomes separate and move towards opposing spindle poles. Separation of chromosomes is

complete when they are arranged forming the MII plate (Fulka and Fulka, 2007) and half of the chromosomes are then extruded in the first polar body during cytokinesis (Maddox et al., 2012). Parallel to those changes, intracellular connections between mural granulosa cells and cumulus cells disappear (Berisha et al., 2009), so that the cumulus cells together with the enclosed oocyte detach from the follicular wall and float freely in the follicular fluid that increases markedly before ovulation (Hunter, 2003).

Shortly before ovulation, the follicular wall becomes thin and stretched, and gonadotropins stimulate the digestion of collagen of the follicular wall by proteolytic enzymes such as plasmin and collagenase (Speroff and Fritz, 2005). Subsequently, mature oocytes surrounded by the corona radiata are ovulated at MII stage of meiosis II and remain arrested at that stage until fertilization occurs. Meiosis is completed following successful fertilization visualized as the presence of the second polar body (Grondahl 2008).

1.4.2. Cytoplasmic Maturation

Cytoplasmic maturation involves the group of processes that modify the cytoplasm of the oocyte and are essential for fertilization and embryonic developmental competence (Grondahl 2008). Cytoplasmic maturation can be divided into three main events: redistribution of cytoplasmic organelles, dynamics of the cytoskeletal filaments, and molecular maturation (Ferreira et al., 2009).

Mitochondria, ribosomes, endoplasmic reticulum, cortical granules and Golgi complex reorganize and assume different positions in MII oocytes compared to GV oocytes (Ferreira et al., 2009). Organelles (except for cortical granules) move to the central area of the oocyte during maturation, forming an 'organelle-free zone' at the cortex of a mature oocyte (Sun and Schatten, 2006). The cytoskeletal filaments (i.e., microfilaments and microtubules) promote these

movements and are responsible for chromosome segregation (Ferreira et al., 2009), and cell division during cytokinesis (Li et al., 2005). Molecular maturation involves the transcription, storage and processing of maternal mRNA, and is responsible for the regulation of the cytoskeleton (Ferreira et al., 2009). Proteins derived from these mRNAs and transcription factors are required for oocyte maturation, fertilization, pronucleus formation and early embryogenesis (Watson, 2007).

Correct changes in the localization, morphology and biochemical properties of organelles and cytoskeleton must occur for the oocyte to acquire developmental competence (Mao et al 2014). To date, no method exists to measure completion of cytoplasmic maturation other than successful fertilization and embryonic and fetal development that results in healthy offspring (Krisher 2013).

1.5. Ovarian superstimulation

Ovarian superstimulation refers to stimulating the growth of multiple antral follicles. In cattle, superstimulation is commonly accomplished by administration of exogenous gonadotropins, e.g. follicle-stimulating hormone (FSH; Mapletoft et al., 2002) and equine chorionic gonadotropin (eCG; Ongaratto et al., 2015). Traditionally, the superstimulatory treatments consist of twice daily injections of FSH over a period of 4 or 5 days or a single administration of eCG (Mapletoft et al., 2002). Ovarian superstimulation is induced in donor cows to increase the number of follicles available for aspiration. Therefore, superstimulatory treatments enable the collection of a greater number of oocytes per donor per attempt (Chaubal et al. 2007; De Roovera et al. 2005) and increase the efficiency of *in vitro* embryo production (Vieira et al. 2014).

The variability of superstimulatory response in cattle, investigated by ultrasonography, was found to be associated with the status of follicular wave development at the time treatment is initiated, and the intrinsic number of follicles present at wave emergence within individuals (Singh

et al., 2004). More follicles develop when the superstimulatory treatment is initiated at the time of wave emergence than after (presence of follicles ≥5 mm in diameter; Nasser et al., 1993), and the superstimulatory response can be predicted by the number of follicles ≥2 mm at wave emergence (Singh et al., 2004). As a result of these findings, methods to synchronize follicular wave emergence before superstimulation are now used. The most common methods of synchronization are the administration of estradiol and progesterone (Bo et al., 1995) and transvaginal ultrasound-guided follicle aspiration (Bergfelt et al., 1994).

Evidence suggests that FSH starvation (i.e., also known as coasting period or FSH withdrawal) between gonadotropin stimulation and oocyte collection seem to have a positive effect on *in vitro* developmental potential of the oocytes in cattle (Blondin et al., 2002, Nivet et al., 2012). Nevertheless, contradictory results were reported by other researchers (Durocher et al., 2006; Monteiro et al., 2010) and therefore more studies are needed to investigate the effect of coasting on oocyte competence.

Unlike traditional superstimulatory protocols in cattle, methods recently developed in bison consist of a reduced number of treatment/handling events for the purpose of reducing stress in the animals. These superstimulatory protocols in bison were based on simplified protocols recently reported in cattle consisting in one or two doses of NIH-FSH-P1 (Tríbulo et al., 2011; 2012). Studies to date have shown that the most effective protocol to induce ovarian superstimulation in wood bison consists of two doses of 300 mg and 100 mg NIH-FSH-P1 (diluted in 0.5% hyaluronan) administered 2 days apart, with the first dose administered on the day of wave emergence (i.e. one day after follicular ablation). By using this superstimulatory protocol, an average of 6 and 7 oocytes were collected during the ovulatory and anovulatory seasons, respectively. (Palomino et al., 2013; Palomino et al., 2014).

1.6. *In vitro* production of embryos

In vitro production of embryos (IVP) is commonly interchangeable with the general term of *in vitro* fertilization and is referred to as the process of generating embryos in the laboratory or outside the body (Hasler and Barfield, 2014). This process includes three main steps, i.e. oocyte maturation, fertilization and zygote/embryo culture. All the steps will be described below.

An important prerequisite for IVP is the oocyte. Oocytes can be obtained from immediately deceased (slaughterhouse-derived ovaries) or living animals (by transvaginal follicular aspiration). Each of these sources have advantages and disadvantages that need to be evaluated for the study in question.

Oocytes from slaughterhouse-derived ovaries are an excellent source of material without animal welfare constraints and provide a larger number of oocytes for IVP. Furthermore, the scarcity of oocytes makes IVP in wild species challenging (Fernandez-Gonzalez et al 2015), thus ovaries from post mortem or convalescent animals are valuable sources of oocytes and female genetic material (Silva et al 2004). In commercial cattle IVP, slaughterhouse-derived ovaries are utilized extensively as they are a reliable and an inexpensive source of oocytes (Hasler and Barfield, 2014). Nevertheless, there are disadvantages of using such source of oocytes. For instance, usually the identity and health status of the donors are unknown, and there is possibility for cross-contamination as ovaries from different individuals come in contact with each other (Hansen, 2007). Moreover, collected oocytes are immature and require *in vitro* maturation that does not support high blastocyst rates. Researchers have determined that the potential of *in vitro* matured oocytes to develop to blastocysts is lower than that of *in vivo* matured oocytes (van de Leemput et al., 1999; Rizos et al., 2002; Alcoba et al., 2015).

Alternatively, collection of oocytes from live animals by transvaginal ultrasound-guided follicular aspiration offers some advantages compared to the collection of oocytes from slaughtered animals. The identity of the animal is known (Hansen, 2007), aspirated oocytes have greater developmental competence than slaughterhouse-derived oocytes (Neglia et al., 2003), oocytes can be collected repeatedly from the same animal (Fuquay et al., 2011), oocytes can be collected not only from cyclic animals but even from pregnant, post-partum or prepubertal animals (Gordon, 2004), and oocytes can be collected after inducing partial or complete maturation *in vivo* (Bordignon et al., 1997; Rizos et al., 2002). Moreover, current equipment developed to perform follicular aspiration are practical for routine use without affecting ovarian structure or function (Gordon, 2004). Importantly, the use of FSH treatments before follicular aspiration has been shown to increase the total number of oocytes collected from an animal per attempt (Garcia and Salaheddine, 1998; Ball and Peters, 2008; Presicce et al., 2011), as well as the blastocyst production rates (Vieira et al., 2015). However, when oocytes are collected from live animals there are some factors that have to be taken into consideration: technical challenges because of requirement of trained personnel and adequate equipment (Hansen, 2007), vacuum pressure affecting the oocyte yield from an individual per collection session (Bols et al., 1996), increased cost (Hansen, 2007) and animal welfare concerns (Chastant-Maillard et al., 2003).

1.6.1. In vitro maturation

In vitro maturation (IVM) corresponds to the culture of immature oocytes collected from antral follicles (Chang et al., 2014). The step of IVM is considered one of the most critical steps for the production of embryos in the laboratory (Mermillod, 2011). The most widely used medium for IVM is tissue culture medium 199 (TCM-199) with Earle's salts which contains bicarbonate buffer, minerals, glucose, glutamine, vitamins and amino acids (Gordon, 2004). This medium is

generally supplemented with LH, FSH, and bovine serum albumin or fetal calf serum (Sirard et al., 1988; Dell'Aquila et al., 2004; Sprícigo et al., 2015), which provide hormones and growth factors (Burgener and Butler, 2005).

Bovine oocytes are matured at 38.5-39.0°C, 5% CO_2 in air and high humidity for 22-24 h (Sirard et al., 1988; Prentice-Biensch et al., 2012). The presence of cumulus cells is required for oocyte maturation (Zhang et al., 1995, Shirazia et al., 2007), as intercellular communication between oocyte and cumulus cells is important for the acquisition of developmental competence (Kidder and Vanderhyden, 2010). For this reason, oocytes surrounded by several layers of compact cumulus cells are selected for IVM. During culture, expansion of the cumulus cells is a visible sign of oocyte maturation (Mermillod, 2011) and is used as a predictor of the developmental competence of oocytes matured *in vitro* (Furnus et al., 1998).

Oocyte maturation *in vitro* is still a limiting factor for IVP in mammals (Banwell and Thompson, 2008). In cattle, only about 35% of oocytes submitted to IVM become blastocysts (Rizos et al., 2002). The *in vitro* developmental potential to the blastocyst stage of *in vitro*-matured oocytes is generally lower than that of *in vivo*-matured oocytes (van de Leemput et al., 1999; Rizos et al., 2002; Alcoba et al., 2015), and this may be related to differences in protein synthesis (Kastrop et al., 1991) and in rearrangement of cortical granules (Hyttel et al., 1986) between oocytes matured *in vitro* vs. *in vivo*.

For many years, studies in several species have been oriented to improve the success of oocyte maturation *in vitro*, by investigating different media systems in an effort to increase the developmental competence of oocytes following fertilization.

1.6.2. In vivo maturation

Maturation *in vivo* corresponds to the maturation of immature oocytes within follicles in the ovaries of live animals. This process requires exposure of the COC within the follicle to the gonadotropin surge (i.e., preovulatory LH surge), which triggers breakdown of the germinal vesicle and progression to metaphase II (Krisher, 2013), and the expansion of the cumulus cells (Russell and Robker, 2007).

Because the quality of oocyte is crucial in determining the proportion of immature oocytes that form blastocysts (Rizos et al., 2002), researchers have worked on increasing oocyte competence before removing them from the follicle (Krisher, 2013). For this reason, several authors have developed protocols aimed at the collection of oocytes at metaphase II stage for the purpose of IVP.

In cattle, the administration of gonadotropin-releasing hormone (GnRH) 26 h before transvaginal follicular aspiration resulted in the collection of *in vivo*-matured oocytes (i.e. COC with expanded cumulus cells and oocytes at metaphase II) and following immediate *in vitro* fertilization resulted in a higher percentage of blastocysts than COC collected from non-GnRH-treated animals (Bordignon et al., 1997). Similarly, *in vivo* matured oocytes collected 20 h after administration of GnRH and followed by immediate fertilization, resulted in higher percentage of blastocysts than oocytes matured *in vitro* (Rizos et al., 2002). Moreover, higher blastocyst rates were obtained from immediately fertilized *in vivo* matured oocytes collected 24 h after the LH surge than from those matured *in vitro* after being collected 2 h before the LH surge (Dielemann et al., 2002). In addition, treatment with LH 24 h before COC collection resulted in *in vivo* matured bovine oocytes (Dadarwal et al., 2015).

In wood bison, COC with expanded cumulus cells were reported to be collected 24 h after administration of pLH in superstimulated animals (Palomino et al., 2013, 2014b). However, when nuclear maturation of those oocytes was evaluated, none had yet reached metaphase II

(unpublished data). This finding suggested that an *in vivo* maturation period longer than 24 h is required for bison oocytes to reach the MII stage. Therefore, more studies are needed to determine the most appropriate time interval between pLH treatment and COC collection in order to collect *in vivo* matured oocytes for IVP in bison.

1.6.3. In vitro fertilization

In vitro fertilization (IVF) consists on the co-incubation of mature oocytes with sperm in a suitable fertilization medium. In cattle, the co-incubation is commonly done in Brackett-Oliphant (BO) fertilization medium (Brackett and Oliphant, 1975) or Tyrode's albumin lactate pyruvate (TALP; Parrish et al., 1986) at 38.5-39.0°C, 5% CO_2 in air and high humidity for 18 h in a 4-well dish or in a microdrop under oil (Hasler and Barfield, 2014).

The availability of motile sperm for IVF is important to achieve success in IVP. The step of IVF can be performed using fresh or frozen semen and only after preparation for fertilization. A common method for isolating motile spermatozoa for fertilization is by centrifugation (Hasler and Barfield, 2014) through a Percoll density gradient (Parrish et al., 1995). The Percoll method is largely adopted (Parrish, 2014) because of the higher recovery rate of motile sperm from frozen thawed semen compared to the swim-up method (40% vs. 9%, respectively [Parrish et al., 1995]). The fertilization medium contains capacitation agents to support sperm capacitation *in vitro* (Parrish, 2014), and to stimulate and maintain motility (Kang et al., 2015). Capacitated sperm undergo the acrosome reaction after binding to the zona pellucida of *in vitro* matured oocytes, a process which enables sperm to penetrate into the oocyte and fertilize it (Breitbart et al., 2005).

The ability of sperm to be capacitated has been shown to vary among bulls (Sirard et al., 1984) and researchers indicate that bull to bull variation is an important factor affecting fertilization rates

in vitro (Sirard and Lambert, 1985; Hillery et al., 1990; Saeki et al., 1995; Lua and Seidel, 2004). Metabolic characteristics of sperm (Brackett and Oliphant, 1975), time required for sperm to capacitate, male age (Sirard and Lambert, 1985), quality of the ejaculate, among other factors may be responsible for male variations in fertilization rates. Therefore, it is recommended to test the fertilizing ability of sperm before performing IVP (Molnarova et al., 2006), especially when valuable oocyte are used. Moreover, a common practice in cattle IVP is the use of pooled semen as a method of limiting male variability (Demyda-Peyrás et al., 2015).

Several substances such as heparin (Parish et al., 1988), caffeine (Niwa and Oghoda, 1988), D-penicillamine, hypotaurine, epinephrine (Hasler and Stokes, 2013), and theophylline (Kang et al., 2015) have been used to enhance fertilization rates *in vitro*. To date, heparin is preferentially used in cattle IVF to stimulate sperm capacitation and improve IVP production (Parrish et al., 1988; Mendes et al., 2003; Parrish, 2014). The efficiency of heparin used to enhance the ability of sperm to fertilize oocytes depends on the dose used and synergistic effects with other substances. For instance, heparin levels added to the fertilization medium range from 0.2 to 5 µg/mL (Parrish et al., 1988), and bicarbonate and bovine serum albumin (BSA) are generally used in combination with heparin in the capacitation and fertilization media (Parrish, 2014).

1.6.4. In vitro culture

In vitro culture (IVC) refers to the process involving the culture of embryos for approximately 5 to 7 days after fertilization to the blastocyst stage in other species (Hasler and Barfield, 2014). Therefore, IVC is the last and longest step of IVP and is intended to support the development of the zygote during an specific period of time until it reaches the desired stage of development to be transferred to a recipient, cryopreserved and/or used for research. In cattle, the embryo is usually

cultured at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2 and high humidity for 7-9 days (Prentice-Biensch et al., 2012, Hasler and Barfield, 2014).

There are several media available for embryo culture *in vitro* and the most commonly used media for bovine IVC (Sagirkaya et al., 2006; Kocyigit, 2016) are SOF (synthetic oviduct fluid), KSOM (potassium simplex optimization medium), and CR1aa (Charles Rosenkran's aminoacid). In general, IVC media require a combination of various components such as minerals, energy sources, amino acids, pH buffer systems, growth factors, antioxidants, vitamins, hormones, and antibiotics (Kim et al., 2007; Kocyigit, 2016). The composition of IVC media is critical for embryo development (Gordon, 2004) and also has been shown to affect embryo cryotolerance (Rizos et al, 2003; Nedambale et al., 2004).

According to the formulation of the media, the culture systems can be classified as undefined, semi-defined and fully defined (Bavister, 2013). Undefined includes the use of serum (fetal bovine serum, bovine calf serum) and/or co-culture (epithelial cells from oviducts, cumulus and granulosa cells, etc); semi-defined includes the use of albumin (bovine serum albumin [BSA]) to replace serum and co-culture is omitted; fully defined or chemically defined includes the use of synthetic macromolecules (polyvinyl alcohol [PVA], polyvinyl pyrrolidone [PVP]) to replace albumin and is a protein-free system where all components are well characterized (Vanroose et al., 2001; Bavister, 2013).

The embryo may be cultured in either a single medium for the duration of IVC or in two or three sequential media of different composition and/or concentration which are changed at certain points during IVC (Hasler and Barfield, 2014). There is no agreement among researchers about the benefits of one type of culture system over the other on embryo development (Reed et al., 2009; Hennings et al., 2016; Werner et al., 2016), thus IVC conditions continue to be studied.

1.6.5. Experiences of IVP in bison

In cattle IVP, slaughtered-derived ovaries are routinely used with blastocyst development rates of approximately 35% (Rizos et al., 2002). In bison, there are few reports of IVP attempts, but in all cases, *in vitro* matured slaughterhouse-derived oocytes were used and the resulting blastocyst production was not comparable to that obtained in cattle. In the first study conducted in wood bison (Thundathil et al., 2007), the oocytes were fertilized with either frozen-thawed or chilled epididymal spermatozoa, and were obtained 7.5% and 10.0% blastocyst rates, respectively. Six wood bison blastocysts were produced in total in that study. In plains bison, 8% blastocyst development was reported in another study using similarly prepared semen (Aurini et al., 2009). In a subsequent study (Barfield and Seidel, 2011), improved development of plain bison oocytes fertilized with frozen-thawed epididymal sperm was demonstrated in zygotes cultured in medium supplemented with or without 5% fetal calf serum; 16% blastocyst rates. In the same study, the percentage of blastocysts increased to 20% when embryos were cultured in the medium supplemented with 5% calf serum after they reached the 8-cell stage. The low blastocyst production rates in bison IVP studies has led researchers to produce bison hybrid embryos (cattle x bison) in order to conduct further analysis of the IVP embryos (Seaby et al., 2012). However, wood bison and plains bison hybrid (x cattle) blastocysts had significantly lower cell numbers than cattle blastocysts, and wood bison hybrid blastocysts had a greater incidence of apoptosis than cattle blastocysts (Seaby et al., 2012). Not surprisingly, no pregnancies or live calves have been reported after transfer of *in vitro*-produced bison embryos. Unfortunately, improvement in IVP efficiency in bison is limited not only by the availability of the biological material needed to perform this technology but also by the lack of basic knowledge about the reproductive biology of this species. Therefore, further investigation is required to achieve an efficient production of *in vitro*-produced embryos in wood bison.

1.7. Alternatives for wood bison conservation

Reclamation of the wood bison population at Wood Buffalo National Park has been very complicated due to the on-going presence of cattle diseases (Gates et al., 2001; Shury et al., 2015). Some efforts to mitigate this problem were initiated in the 1960s and 1970s with the introduction of disease-free wood bison to the Mackenzie Bison Sanctuary in the Northwest Territories and the establishment of a disease-free herd at the Elk Island National Park (EINP) in Alberta (Gates et al., 2001; McFarlane et al., 2006). Since then, only the EINP has been a source of healthy wood bison to repopulate different areas in Canada, the United States, and even Russia (Environmental Assessment Panel, 1990, Gates et al., 2001). However, translocation of wood bison from the EINP to other herds has resulted in the spread of its less variable genetic diversity (Gates et al., 2001; Nishi et al., 2002). Therefore, it is important to rescue the genetic diversity of wood bison population at the Wood Buffalo National Park to avoid the founder effect that is occurring with the dissemination of the EINP genetics (McFarlane et al., 2006).

Vaccines against brucellosis have been developed with encouraging results in bison (Olsen et al., 2002, Clapps et al., 2011). However, there are no studies describing vaccines that have demonstrated efficacy for tuberculosis in bison. In any event, the success of these vaccines will depend on the efficacy of the method of delivery of the vaccine to protect the animals against cattle diseases (Olsen et al., 2002). Likewise, the number of doses, booster vaccinations, and adverse effects of the vaccines constitute additional challenges that any vaccination program will face in the eradication of cattle diseases in Canada (Shury et al., 2015).

Reproductive technologies are being developed in wood bison as a means for the salvage of their genetics (Shury et al., 2015). These technologies have been used successfully to preserve endangered wild species (Loskutoff et al., 1995; Comizzoli et al., 2000; Solti et al., 2000). Artificial insemination (AI) is currently the most extensively applied reproductive technology in

non-domestic species around the world (Comizzoli, 2015) due to the increased potential for success compared to other techniques. In wood bison, AI has been applied successfully to produce pregnancies and live calves (Adams et al., 2010; Adams et al., 2016; Mastromonaco, personal communication). Likewise, *in vitro* and *in vivo* embryo production along with gamete/embryo cryopreservation have allowed the establishment of genetic resource banks to store and maintain the genetic diversity of a variety of endangered species around the world (Wild, 1992; Holt et al., 1999; Comizzoli and Holt, 2014). In wood bison, superovulation and embryo collection have been developed recently resulting in an increased number of freezable embryos (a total of 11 transferable embryos from 20 bison: Palomino et al., 2016) in comparison with previous reports (a total of 6 transferable embryos from 20 bison: Toosi et al., 2013). Successful pregnancy has also been obtained by transfer of *in vivo*-derived embryos resulting in live birth of two calves (Toosi et al., 2013). Other technologies such as interspecies somatic cell nuclear transfer (iSCNT) have been attempted in wood bison, and researchers suggest that iSCNT may provide a possible alternative for embryo production and genetic preservation in wood bison (Kumar et al., 2009; Seaby et al., 2013; González-Grajales et al., 2015). Additionally, a limited number of studies involving the use of IVP have been reported and all have resulted in low embryo production rates (Thundathil et al., 2007; Seaby et al., 2012) as summarized previously in this thesis.

CHAPTER 2. GENERAL HYPOTHESIS AND OBJECTIVES

General Hypothesis

The developmental competence of wood bison oocytes is influenced by maturational environment and time (*in vivo* vs *in vitro*) and morphologic characteristics of the cumulus-oocyte complex.

General Objective

To develop efficient protocols for the collection of competent oocytes and the production of disease-free wood bison embryos by *in vitro* fertilization and culture.

Specific objectives

1. To determine the optimal interval of time after treatment of superstimulated wood bison with human chorionic gonadotrophin (hCG) required for *in vivo* maturation of COC
2. To determine the developmental competence of oocytes after maturation *in vitro* vs. *in vivo*.
3. To improve cleavage and blastocyst production rates for oocytes collected from superstimulated bison.
4. To evaluate the efficacy of embryo washing procedures to remove *Brucella abortus* from *in vitro*-produced wood bison embryos following *in vitro* exposure to the pathogen.

CHAPTER 3

IN VIVO AND *IN VITRO* MATURATION OF OOCYTES COLLECTED FROM SUPERSTIMULATED WOOD BISON (*Bison bison athabascae*) DURING THE ANOVULATORY AND OVULATORY SEASONS

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3.1. Abstract

Experiments were done to compare the *in vivo* and *in vitro* maturational characteristics of cumulus-oocyte complexes (COC) collected from live wood bison. In Experiment 1 (anovulatory season), follicular ablation was done to synchronize follicle wave emergence among bison on Day -1, and FSH diluted in hyaluronan was given on Days 0 (300 mg) and 2 (100 mg). Bison were then assigned to 5 groups (n=5/group) in which COC were collected by transvaginal follicle aspiration on Day 4 and either fixed immediately with no maturation (control), matured *in vitro* for 24 or 30 h, or collected by follicle aspiration on Day 5 after *in vivo* maturation for 24 or 30 h (i.e., after hCG treatment). In Experiment 2 (ovulatory season), bison were treated as described for Experiment 1, but PGF2α (cloprostenol) was given to control the luteal phase on Days -9 and 3. In both experiments, cumulus cell expansion was more extensive following *in vivo* than *in vitro* maturation, and the percentage of fully expanded COC was highest in the *in vivo* 30 h groups. Nuclear maturation occurred more rapidly *in vitro*; 60-70% of oocytes were at the MII stage after 24 h of *in vitro* maturation while only 25-27% of oocytes had reached the MII stage after 24 h of *in vivo* maturation. In conclusion, nuclear maturation occurred more rapidly during *in vitro* vs. *in vivo* maturation, but was associated with less cumulus expansion than *in vivo* maturation. *In vivo* oocyte nuclear maturation was more complete at 30 vs. 24 h after hCG treatment. Season had no effect on the maturational capacity of wood bison oocytes.

3.2. Introduction

The free-ranging wood bison (*Bison bison athabascae*) population in Wood Buffalo National Park (WBNP) in northern Alberta, Canada, is threatened by endemic disease (Joly and Messier, 2004). Brucellosis and tuberculosis hamper population growth and there is a risk of losing

sufficient genetic diversity to sustain this native species. The WBNP population, Canada's largest and most diverse, has declined by nearly 70% since 1971, from 16,000 bison (Mitchell and Gates, 2002) to approximately 5,000 bison today (Rosano, 2013). Reproductive technologies have previously been used in the conservation of genetic diversity of similarly threatened and endangered animals (Wildt et al., 1993; Holt and Pickard, 1999), and have been proposed recently as a strategy specifically for Canadian wood bison (MacPhee et al., 2016).

In vitro embryo production has some advantages over *in vivo* embryo production in that it circumvents the need to control and induce ovulation in the donor animal, it has the potential for producing a greater number of embryos, and it enables the salvage of genetic material from dead or dying animals (Loskutoff et al., 1995; Rao et al., 2010; Stoops et al., 2011). In the only report on *in vitro* maturation and embryo production in wood bison to-date, less than10% of oocytes that were fertilized and cultured *in vitro* developed into blastocysts (Thundathil et al., 2007).

Technical development of *in vitro* embryo production in bison is limited by the lack of biological material and a dearth of information about oocyte maturation in this species. Abattoir-derived bison ovaries are scarce and availability is inconsistent, but in other wild species, oocytes have been collected from live donors in both captive (Wildt et al., 1993; Hermes et al., 2009) and wild settings (Berlinguer et al., 2008). Recently, we have collected cumulus-oocyte complexes (COC) from live donors in a captive herd of wood bison by transvaginal ultrasound-guided follicle aspiration (Palomino et al., 2013, 2014b). Oocyte collections were done after ovarian superstimulation, during both the ovulatory and anovulatory seasons. Collection rates (COC collected/follicles aspirated) were 50 - 60% with 6 to 7 COC per bison per attempt.

Oocyte maturation is a complex process that is necessary for attainment of developmental competence (Ferreira et al., 2009). Final maturation of the oocyte is triggered by a preovulatory LH surge which causes the expansion of the cumulus cells and resumption of meiosis in the oocyte

(Russell and Robker, 2007). Cumulus cell expansion is part of the process whereby the COC separates from the follicle wall prior to ovulation (Gordon, 2003). An immature oocyte arrested at prophase I of the first meiotic division contains a nucleus of mostly decondensed chromosomes, a large nucleolus, and an intact nuclear membrane (germinal vesicle; GV). During the final stages of maturation, the chromosomes begin to condense, the nucleolus disperses, and the nuclear envelope disappears (GV breakdown; Masui and Clarke, 1979). In most mammals, a mature oocyte is characterized by the presence of a first polar body and chromosomes arranged on a meiotic spindle at metaphase of the second meiotic division. Oocytes in this stage are expected to have the capacity for fertilization and embryonic development (Cha and Chian, 1998). There is evidence that *in vivo*-matured oocytes have greater developmental competence than oocytes matured *in vitro* (Leibfried-Rutledge et al., 1987; van de Leemput et al., 1999; Dieleman et al., 2002), but the characteristics of oocyte maturation in bison (*in vivo* or *in vitro*) have not been critically examined.

Treatment with GnRH or LH 24 h before COC collection has been reported to induce *in vivo* maturation of bovine oocytes (Laurincik et al., 1993; Bordignon et al., 1997; Dadarwal et al., 2015). A similar effect was observed in superstimulated wood bison where more than 70% COC were expanded when collected 24 h after LH treatment (Palomino et al., 2013, 2014b). Whether the expanded COC were developmentally competent to permit immediate *in vitro* fertilization was not tested. Using anti-lamin AC/DAPI staining, we examined the nuclear status of oocytes of expanded COC collected from wood bison the day after LH treatment and found that none had yet reached metaphase II. Even after an additional 8 h of *in vitro* maturation, the oocytes had not reached metaphase II (unpublished data), suggesting that bison oocytes require an extended period (i.e., >24 h) to reach a mature stage.

The objectives of the present study were to 1) determine the optimal interval of time after hCG treatment required for *in vivo* oocyte maturation in wood bison, 2) compare the maturational characteristics of COC after *in vitro* vs. *in vivo* maturation, and 3) compare the maturational capacity of bison oocytes collected during the anovulatory vs. ovulatory seasons.

3.3. Materials and methods

3.3.1. Animals

Twenty-five adult female wood bison, 6 to 11years of age, were used in two experiments. Experiment 1 was done during the anovulatory season (May - June) and Experiment 2 was done during the ovulatory season (October - November), using the same individuals (n = 25) in both seasons. In both experiments, the bison were confined to corrals at the Native Hoofstock Centre, University of Saskatchewan, with free access to fresh water and hay to maintain an average body condition score of 3.5 (scale of 1 to 5; Vervaecke et al., 2005). The experimental protocol was approved by the University of Saskatchewan's Animal Research Ethics Board, and done in accordance with the guidelines of the Canadian Council on Animal Care.

3.3.2. Experiment 1 - Anovulatory season

Follicular wave emergence was induced among bison (n = 25) by transvaginal ultrasound-guided aspiration of all follicles ≥ 5 mm in diameter (follicular ablation), as previously described (Bergfelt et al., 1994; Palomino et al., 2014a). The procedure was performed using a 5 MHz transvaginal probe (ALOKA SSD-900, Tokyo, Japan) equipped with a disposable 18-ga x 1 ½" vacutainer needle (BD, Mississauga, Ontario, Canada) attached to a 6 mL syringe by silicon tubing 60 cm long x 1.14 mm internal diameter (Cole-Palmer, Montreal, Quebec, Canada). Follicular wave emergence (Day 0) was expected to occur the day after follicular ablation.

Ovarian superstimulation was induced with a total dose of 400 mg NIH-FSH-P1 (Folltropin-V, Vetoquinol Canada Inc., Lavaltrie, QC, Canada) im diluted in 10 mL of hyaluronan (5 mg/ml, MAP-5, Vetoquinol Canada Inc.) given on Day 0 (300 mg) and Day 2 (100 mg), as previously described in wood bison (Palomino et al., 2013). The bison were assigned randomly (lottery method) to 5 groups (n=5 bison per group) in which COC were collected by transvaginal ultrasound-guided follicle aspiration on Day 4 and fixed and stained immediately (no maturation, control) or after *in vitro* maturation for 24 h or 30 h, or collected on Day 5 after *in vivo* maturation for 24 h or 30 h (Table 1). *In vivo* maturation was induced by a single dose of 2000 IU of hCG (Chorulon, Merck Animal Health, Kirkland, Quebec, Canada) im on Day 4 in the *in vivo* maturation groups.

3.3.3. Experiment 2 - Ovulatory season

Synchronization of the follicular wave emergence and ovarian superstimulation was performed as described for Experiment 1. However, since the bison were ovulatory and all had a corpus luteum, a luteolytic dose of 500 µg cloprostenol (Estrumate, Merck Animal Health, Kirkland, Quebec, Canada) was given im on Day -9 and Day 3 (Day 0 = day of wave emergence, one day after follicular ablation). The bison were assigned randomly to 5 groups (n=5 per group), as described for Experiment 1 (Table 1).

3.3.4. COC collection

Collection of COC was done by transvaginal ultrasound-guided aspiration of all follicles ≥5 mm in diameter, as previously described (Palomino et al., 2013, 2014b). Collection was performed using a custom-made disposable 18-ga x 2” short-bevel needle (Misawa Medical Industry Ltd., Edogawa-Ku, Tokyo, Japan) connected to a 50mL conical Falcon tube via silastic tubing (length

50 cm; internal diameter 1.14 mm; Cole Palmer, Montreal, Quebec, Canada). Follicular contents were aspirated using a regulated vacuum pump set at a flow rate of 20 mL/min (Brogliatti et al., 1996). The collection medium consisted of Dulbecco's phosphate-buffered saline (DPBS, Gibco, Grand Island, NY, USA), 0.15% ET Surfactant (Vetoquinol Canada Inc.), and 200 IU/L of heparin (heparin sodium injection USP, Sandoz, Boucherville, Quebec, Canada). Follicular aspirates were poured from the Falcon tube into an ova/embryo filter (Emcon filter; Agtech, Manhattan, Kansas, USA). The COC were rinsed from the filter using collection medium without surfactant, and poured into a 90 mm Petri dish for searching procedures. The temperatures in the collection area, surrounding the Falcon tube and silastic tubing, was maintained at 22- 25°C using a portable liquid propane forced-air heater (Dyna-Glo Pro; GHP Group Inc, Morton Grove, IL, USA), while the temperature of the COC searching room was maintained at 25 - 30°C.

At the time of collection, COC were classified by stereomicroscopy according to the appearance of the cumulus cell layers and the appearance of the oocyte cytoplasm (ooplasm). The cumulus layer was classified as compact (at least one complete layer of granulosa cells tightly surrounding the oocyte), expanded (cumulus cells expanded or partially dissociated), or denuded (oocyte without cumulus cells). Oocytes with pyknotic granulosa cells and vacuolated ooplasm were classified as degenerate (Ratto et al., 2007) and discarded from further processing. For assessment of nuclear status, compact COC were used in the *in vitro* maturation groups, expanded COC in the *in vivo* maturation groups, and all COC categories were used in the control group.

3.3.5. In vitro maturation

The medium used for *in vitro* maturation was TCM-199 with Earle's salts (Gibco, Grand Island, NY, USA) supplemented with 5% (vol/vol) bovine calf serum (Sigma-Aldrich; Oakville, ON, Canada) heat inactivated, 5 µg/mL LH (Lutropin-V; Vetoquinol Canada Inc.), 0.5 µg/mL FSH

(Folltropin-V; Vetoquinol Canada Inc.), and 0.05 µg/mL gentamicin sulfate (Sigma-Aldrich; Oakville, ON, Canada). Compact COC were washed three times in maturation medium and were placed in four-well dishes (NUNC, Thermo Fisher Scientific, Rochester, NY, USA) containing 1 mL/well of maturation medium (4 to 6 COC/well), within 4 h of collection. The COC were maintained in maturation medium at 38.5°C, 5% CO_2 in air and high humidity for 24 h or 30 h.

3.3.6. Evaluation of cumulus cell expansion and nuclear maturation

After maturation (*in vitro* or *in vivo*), cumulus expansion was assessed subjectively under a stereomicroscope as partially expanded or fully expanded (Fig. 1). After assessment of cumulus cell expansion, the COC were denuded by pipetting with 0.3% hyaluronidase (wt/vol) in Ca^{++} and Mg^{+} free DPBS (Invitrogen, Burlington, ON, Canada), and the oocytes were fixed in 4% paraformaldehyde in DPBS for 30 min at room temperature. All types of COC (expanded, compact and partially denuded) in the control group were also completely denuded and fixed as described. After fixation, oocytes were washed three times with 0.2% Polyvinyl alcohol (PVA) in DPBS (wt/vol), permeabilized with 0.5% Triton X-100 (BIO-RAD, Hercules, CA, USA) in DPBS (vol/vol) for 30 min and with 0.05% Tween-20 (BIO-RAD, Hercules, CA, USA) in DPBS (vol/vol) for 30 min, followed by blocking with 2% bovine serum albumin in DPBS (wt/vol) for 60 minutes. Oocytes were then incubated with primary antibody (mouse anti-Lamin AC, Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1:300 in blocking buffer for 60 min, followed by 3 washes in DPBS and incubation with secondary antibody (Alexa Fluor 488 labeled anti-mouse IgG, Invitrogen) 1:200 in blocking buffer for 60 min (Prentice-Biench et al., 2012). Oocytes were mounted onto glass microscope slides (n=1 to 2 oocytes per slide) in Vectashield Mounting Medium containing DAPI (H-1200; Vector Laboratories Inc., Burlington, ON, Canada), protected with a coverslip (using paraffin-vaseline on each corner of the coverslip), sealed with nail polish

(NYC New York Color, Coty US LLC, Sanford, NC, USA) and examined using an epifluorescence laser microscope (Zeiss Axioskop 5 Carl Zeiss Ltd., Toronto, ON, Canada). The stage of nuclear maturation was classified as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), or metaphase II (MII) (Fig. 2).

3.3.7 Statistical analyses

The number of COC in each category were compared among groups by one-way analysis of variance and Tukey's post hoc tests. Proportional data (collection rate, cumulus cell expansion rate and proportion at each stage of nuclear maturation) were compared by 2-tailed Chi-square or 2-tailed Fisher's exact test using Proc Frequency procedure (SAS, Enterprise Guide 4.2, Statistical Analysis System Institute Inc., Cary, NC, USA). Values are expressed as mean ± SEM, and P values of < 0.05 were considered significant.

Notwithstanding a 5-month separation in time between Experiments 1 and 2, end points were compared between experiments to determine the effects of season. The same 25 bison were used in both experiments, but were assigned randomly (i.e., lottery method) to treatment groups in each experiment; i.e., no attempt was made to assign bison to the same group, and observations were considered independent from one experiment to the next. Data (COC collection, nuclear maturation stages) were analyzed with season (anovulatory vs. ovulatory) and maturation type (*in vitro* vs. *in vivo*) as fixed factors using the generalized linear model procedure in SAS with a binomial error distribution and a logit link function. The effects of maturation type (*in vivo* vs. *in vitro*) and season (anovulatory vs. ovulatory) on COC morphology were compared by two-way analysis of variance.

3.4. Results

3.4.1. Experiment 1 – Anovulatory season

A mean (±SEM) of 7.6 ± 0.6 COC were collected per bison, with no differences among groups. The overall COC collection rate (COC collected/follicles aspirated) was 60.4% (189/313) and did not differ among groups. At the time of collection, more COC were classified as expanded in the hCG-treated groups than in the non-treated groups [5.9 ± 1.5(n=10) vs. 0.8± 0.2 (n=15); P<0.05; Table 2], with a greater proportion of expanded COC in the 30-h vs. 24-h post-hCG groups (86% [42/49] vs. 50% [17/34], respectively; P=0.001). A greater proportion of COC were fully expanded in the *in vivo* vs. *in vitro* maturation group (92% vs. 74%, respectively; P<0.05), and the proportion of fully expanded COC was greatest in the *in vivo* 30 h group (98%; P<0.05; Table 3).

A total of 16 COC (n=1 to 9 per group) were lost during the fixation and staining procedures; data on the remaining oocytes were used for statistical comparison of nuclear maturation among groups (Table 4). None of the COC in the control (0 h) group was at the MII stage. The proportion of MII stage oocytes was greater in the *in vitro* maturation groups than in the *in vivo* groups, but did not differ between 24 h vs. 30 h.

3.4.2. Experiment 2 – Ovulatory season

One animal in the control group was excluded because she did not respond to superstimulatory treatment and COC collection was not attempted. A mean (±SEM) of 7.6 ± 1.1 COC were collected per bison, with no differences among groups. The overall COC collection rate was 68.2% (182 COC/267 follicles aspirated) and did not differ among groups. As in Experiment 1, treatment with

hCG (vs. no hCG) resulted in a greater number (P<0.05) of expanded COC per bison at the time of collection (6.1 ± 1.8 vs. 0.5± 0.2, respectively; Table 5). Similarly, a greater proportion of expanded COC was collected at 30 vs. 24 h after hCG treatment (88% [35/40] vs. 53% [10/19], respectively; P<0.05). Cumulus cell expansion was more extensive following *in vivo* than *in vitro* maturation, and the proportion of fully expanded COC was greatest in the *in vivo* 30 h group (88%; P<0.05; Table 6).

A total of 18 COC (n=1 to 8 per group) were lost during the fixation and staining procedures; data on nuclear maturation of the remaining oocytes were analyzed and are summarized in Table 7. As in Experiment 1, no COC in the control group (0 h) reached the MII stage. Likewise, maximal nuclear maturation was achieved *in vitro* by 24 h, with no difference in the proportion of MII-stage COC at 24 vs. 30 h. However, there tended to be a greater proportion of MII-stage COC in the 30- vs. 24-h *in vivo* maturation groups (P = 0.06).

3.4.3. Seasonal comparison (anovulatory season vs. ovulatory season)

The number of follicles ≥5 mm on the day of COC collection was similar in bison during the anovulatory versus ovulatory season (15.4 ± 0.9 vs. 13.3 ± 1.2, respectively; P=0.17), and the number of COC collected (all morphological categories) did not differ between seasons (7.6 ± 0.6 vs. 7.6 ± 1.1, respectively; P=0.99). Results for COC collection rate, COC morphological characteristics after maturation (*in vitro* and *in vivo*) and nuclear maturation stages during the anovulatory versus ovulatory season are summarized in Tables 8 and 9. No effect of season was found for any endpoint. When data from the two seasons were combined, the number of fully expanded COC per bison tended to be greater (P=0.06) in the *in vivo* vs. the *in vitro* maturation groups, whereas the proportion of MII-stage oocytes was greater (P<0.01) in the *in vitro* vs. *in vivo*

maturation groups. The collection efficiency (COC collected/follicle aspirated) tended to be greater in the hCG-treated groups (P=0.07; Table 8).

3.5. Discussion

Canadian wood bison are threatened by a lack of genetic diversity and reproductive strategies have been proposed to address the issue (MacPhee et al., 2016). Experiments in the present study were done as an initial step in answering the question: Can oocytes be collected from live wood bison as a tool for retaining genetic diversity through *in vitro* embryo production? Results were affirmative. We collected an average of 7.6 COC per bison, 3.9 (50.2%) of which were fully expanded after either *in vitro* or *in vivo* maturation. Furthermore, results demonstrate that oocytes can be collected by transvaginal ultrasound-guided follicular aspiration from live bison throughout the year, i.e., during both the anovulatory and ovulatory seasons.

Importantly, results document the capability of bison oocytes to undergo maturation *in vitro* as well as *in vivo*. At 0 h, in both the anovulatory and ovulatory seasons, all COC collected were at either the GV or GVBD stage. Following maturation, *in vitro* or *in vivo*, more than 80% of the oocytes were at the MI or MII stage. Results also demonstrated that whereas nuclear maturation was maximal after 24 h of *in vitro* culture, the majority (≥50%) of oocytes matured *in vivo* did not reach the MII stage until 30 h after hCG treatment. In a previous study in wood bison (unpublished data), none of the oocytes collected at 24 h after LH treatment had reached the MII stage even after incubation *in vitro* in maturation medium for an additional 8 h. The reason for the lack of oocyte maturation in the initial study is not clear, but may be associated with the use of LH rather than hCG to induce maturation. Recently, we found hCG treatment more effective than LH at the doses used for inducing ovulation in both unstimulated (Palomino et al., 2015a) and superstimulated

wood bison (Palomino et al., 2016). The greater ovulatory effect was attributed to the longer half-life of hCG compared to LH (Matzuk et al., 1990). The same characteristic may be responsible for the greater maturational effect observed in the present study compared to the previous study in which LH was used for *in vivo* maturation prior to oocyte aspiration.

In the present study, delaying collection from 24 to 30 h after hCG treatment resulted in more than a 20% increase in the proportion of expanded COC and more than a 25% increase in the proportion of fully expanded COC. Although the absolute number of MII-stage oocytes was greater at 30 h than at 24 h after hCG treatment, the difference was not as marked as that of cumulus expansion. Developmentally, nuclear maturation (i.e., resumption of meiosis and attainment of metaphase II) occurs after cumulus cell expansion (Bézard et al., 1997; Chen et al., 2001). We suspect that additional maturational time would enable completion of nuclear maturation in COC that had expanded but not yet achieved metaphase II, similar to that reported in monkeys and humans where additional *in vitro* incubation for 2 to 11 h resulted in an increase in the proportion of MI-stage oocytes that reached the MII stage (Lanzendorf et al., 1990, Vanhoutte et al., 2005). An increase in the dose of hCG or an extended period of *in vivo* maturation may increase the proportion of fully mature oocytes collected from wood bison, as observed in humans (Hourvitz et al., 2010).

The degree of expansion of the cumulus oophorous is thought to be a reflection of physiologic normality (Gordon, 2003), and optimal expansion of the cumulus is essential for ovulation and embryo development (Chen et al., 1993). Hence, cumulus expansion was the goal in the different maturation groups in the present study, but the high degree of expansion, particularly in the *in vivo* maturation groups, was surprising. The highest percentage of fully expanded COC was found in the *in vivo* 30-h groups in Experiments 1 and 2 (combined, 76/82; 93%). Our finding that cumulus cell expansion was more extensive *in vivo* than *in vitro* is consistent with reports in horses

(Grondahl and Hyttel, 1996) and hamsters (Kito and Bavister, 1997). Presumably, full cumulus cell expansion that occurs *in vivo* is triggered by factors present in the follicular environment (Kito and Bavister, 1997). In cattle, cumulus cell expansion was a predictor of the developmental competence of oocytes matured *in vitro* (Furnus et al., 1998) and *in vivo* (Aardema et al., 2013). Only COC with full cumulus cell expansion at the time of collection developed into blastocysts after immediate *in vitro* fertilization (Aardema et al., 2013). If the degree of cumulus cell expansion observed in wood bison COC is associated with developmental competence, then results provide rationale for the hypothesis that *in vivo* matured wood bison COC collected 30 h after hCG treatment are more capable of developing into blastocysts, following *in vitro* fertilization, than those matured *in vitro* or *in vivo* for a lesser period of time.

Nuclear maturation of bison oocytes occurred more rapidly during *in vitro* than *in vivo* maturation in the present study. In both experiments (anovulatory and ovulatory seasons), the proportion of oocytes that reached the MII stage was maximal after 24 h of *in vitro* maturation, and was greater than in either the 24- and 30-h *in vivo* maturation groups. Similar results were reported in nonhuman primates; nuclear status of *in vitro* matured oocytes was more advanced than in *in vivo* matured oocytes within 24 h (Nyholt de Prada et al., 2009). The time to complete nuclear maturation *in vitro* varies according to culture conditions (Roberts et al., 2002) and among species (i.e., pig oocytes within 36 to 42 h [Song and Lee, 2007] and bovine oocytes within 20 to 24 h [Critser et al., 1986]). Cytoplasmic maturation has been described as a gradual capacitation that corresponds to the acquisition of critical cellular functions by the oocyte which are important for fertilization and early embryonic development (Ferreira et al., 2009). The cytoplasmic implications of more rapid resumption of meiosis *in vitro* than *in vivo* on later embryonic development in wood bison remain unknown. In cattle, the *in vitro* developmental potential of *in vivo* matured oocytes was twice as high as that of *in vitro* matured oocytes (Van der Leemput et

al., 1999), and may be related to differences in protein synthesis (Kastrop et al., 1991) and in rearrangement of cortical granules (Hyttel et al., 1986) between oocytes matured *in vivo* vs *in vitro*.

Season had no effect on the number of follicles ≥5 mm in the ovaries at the time of COC collection or the number of COC collected per superstimulated bison. Likewise, no effect of season was evident on the morphological characteristics of matured COC or on the dynamics of nuclear maturation. Results are in agreement with previous studies in wood bison where the number of COC collected per superstimulated bison was 7.4 during both the anovulatory and ovulatory seasons (Palomino et al., 2013; 2014b), and support the notion that oocytes may be obtained from wood bison for the purpose of *in vitro* embryo production at any time of the year.

In summary, the ovarian superstimulatory response in wood bison was similar in the anovulatory and ovulatory seasons, as was the COC collection efficiency (65% of follicles aspirated), number of COC collected (7.6 per collection attempt), and the number of fully expanded COC after maturation *in vitro* (2.9 per bison) or *in vivo* (5.0 per bison). Treatment with hCG effectively induced *in vivo* maturation, unlike previous attempts using LH, and tended to increase COC collection efficiency. *In vivo* maturation was associated with more extensive cumulus cell expansion and a slower onset of the resumption of oocyte meiosis than *in vitro* maturation. The implications of these nuclear maturational characteristics on oocyte competence to develop into embryos remains to be investigated, but results support the feasibility of developing a year-around *in vitro* embryo production system for wood bison.

3.6. Acknowledgements

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Table 3.1. Experimental design to compare characteristics of the cumulus-oocyte complexes (COC) after *in vitro* vs. *in vivo* maturation in wood bison (n = 5bison per group) during the anovulatory and ovulatory seasons (Experiments 1 and 2, respectively). The only difference in treatment between experiments was that a luteolytic dose of PGF2α given on Day -9 and Day 3 in the ovulatory season to control the corpus luteum.

	Control	*In vitro* maturation		*In vivo* maturation	
Day	0hour	24 h	30 h	24 h	30 h
-1	Transvaginal ultrasound-guided follicle ablation				
0	Day of follicular wave emergence and first administration of FSH (300 mg)				
2	Second administration of FSH (100 mg)				
4	COC collected, fixed &stained	COC collected, IVM	COC collected, IVM	hCG (2000 IU)	hCG (2000 IU)
5		Fixed & stained at 24 h	Fixed & stained at 30 h	COC collected 24 h after hCG, fixed &stained	COC collected 30 h after hCG, fixed & stained

IVM: *in vitro* maturation

Table 3.2. Morphologic characteristics (mean ± SEM per bison) of cumulus-oocyte complexes (COC) at the time of collection from superstimulated wood bison before maturation(i.e., control and *in vitro* maturation groups combined) vs. after *in vivo* maturation (anovulatory season, Experiment 1).

	No maturation	*In vivo* maturation[1]	
		24 h	30 h
Number of bison	15	5	5
COC collected per bison	7.1±0.6[a]	6.8±1.3[a]	9.8±1.9[a]
Compact COC	4.9±0.5[a]	2.4±1.0[b]	1.2±0.4[b]
Expanded COC	0.8±0.2[a]	3.4±1.5[ab]	8.4±2.1[b]
Denuded COC	1.0±0.2[a]	0.4±0.2[a]	0.2±0.2[a]
Degenerated COC	0.3±0.1[a]	0.6±0.2[a]	0.2±0.2[a]

[1] COC collected 24 hours or 30 hours after treating bison with 2000 IU hCG

[abc]Within rows, values with no common superscripts are different ($P<0.05$)

Table 3.3. Status of expansion of cumulus-oocyte complexes (COC) collected from superstimulated wood bison (n=5 bison per group) after *in vitro* vs. *in vivo* maturation (anovulatory season, Experiment 1).

	In vitro maturation			*In vivo* maturation[1]		
	24 h	30 h	subtotal	24 h	30 h	subtotal
Total expanded COC	18	24	42	17	42	59
Partially expanded	6/18[a]	5/24[a]	11/42[x]	4/17[a]	1/42[b]	5/59[y]
	(33%)	(21%)	(26%)	(24%)	(2%)	(8%)
Fully expanded	12/18[a]	19/24[a]	31/42[x]	13/17[a]	41/42[b]	54/59[y]
	(67%)	(79%)	(74%)	(76%)	(98%)	(92%)

[1] COC collected 24 hours or 30 hours after treating bison with 2000 IU hCG

[ab] Within rows, values with no common superscripts are different ($P<0.05$)

[xy]Within rows, values with no common superscripts are different ($P<0.05$)

Table 3.4. Nuclear status of oocytes collected from superstimulated wood bison (n=5 bison per group) after no maturation (Control), or after *in vitro* vs. *in vivo* maturation (anovulatory season, Experiment 1).

	Control	*In vitro* maturation		*In vivo* maturation	
	0 h	24 h	30 h	24 h	30 h
Total COC collected	34	32	40	34	49
Expanded COC	3	18	24	17	42
Stained oocytes[1]	25	17	23	15	33
GV	21/25[a] (84%)	0/17[b] (0%)	0/23[b] (0%)	0/15[c] (0%)	0/33[c] (0%)
GVBD	4/25[a] (16%)	0/17[b] (0%)	0/23[b] (0%)	3/15[a] (20%)	2/33[ab] (6%)
MI	0/25[a] (0%)	5/17[b] (29%)	6/23[b] (26%)	6/15[b] (40%)	15/33[b] (45%)
MII	0/25[a] (0%)	12/17[b] (71%)	17/23[b] (74%)	6/15[c] (40%)	16/33[c] (48%)

COC: Cumulus-oocyte complexes, GV: Germinal vesicle, GVBD: Germinal vesicle breakdown, MI: Metaphase I, MII: Metaphase II

[1]A total of 16 COC (n=1 to 7 per group) were lost during the fixation and staining procedures

[abc]Within rows, values with no common superscripts are different (P<0.05)

Table 3.5. Morphologic characteristics (mean ± SEM per bison) of cumulus-oocyte complexes (COC) at the time of collection from superstimulated wood bison before maturation (i.e., control and *in vitro* maturation groups combined) vs. after *in vivo* maturation (ovulatory season, Experiment 2).

	No maturation	*In vivo* maturation[1]	
		24 h	30 h
Number of bison	14	5	5
COC collected per bison	6.5 ± 1.0^{a}	8.0 ± 3.0^{a}	10.2 ± 3.4^{a}
Compact COC	5.2 ± 0.8^{a}	2.8 ± 1.0^{ab}	1.8 ± 0.8^{b}
Expanded COC	0.5 ± 0.2^{a}	4.2 ± 1.8^{ab}	8.0 ± 2.5^{b}
Denuded COC	0.6 ± 0.3^{a}	1.0 ± 0.6^{a}	0.2 ± 0.2^{a}
Degenerated COC	0.2 ± 0.1^{a}	0.0 ± 0.0^{a}	0.2 ± 0.2^{a}

[1] COC collected 24 hours or 30 hours after treating bison with 2000 IU hCG

[abc]Within rows, values with no common superscripts are different (P<0.05)

Table 3.6. Status of expansion of cumulus-oocyte complexes (COC) collected from superstimulated wood bison (n=5 bison per group) after *in vitro* vs. *in vivo* maturation (ovulatory season, Experiment 2).

	In vitro maturation			*In vivo* maturation[1]		
	24 h	30 h	subtotal	24 h	30 h	subtotal
No. of expanded COC	16	25	41	19	40	58
Partially expanded	$6/16^{a}$	$9/25^{a}$	15/41	$9/19^{a}$	$5/40^{b}$	14/58
	(37%)	(36%)	(37%)	(47%)	(12%)	(24%)
Fully expanded	$10/16^{a}$	$16/25^{a}$	26/41	$10/19^{a}$	$35/40^{b}$	45/58
	(63%)	(64%)	(63%)	(53%)	(88%)	(78%)

[1] COC collected 24 hours or 30 hours after treating bison with 2000 IU hCG

[ab] Within rows, values with no common superscripts are different (P<0.05)

Table 3.7. Nuclear status of oocytes collected from superstimulated wood bison (n=5 bison per group) with no maturation (Control), or after *in vitro* vs. *in vivo* maturation (ovulatory season, Experiment 2).

	Control	*In vitro* maturation		*In vivo* maturation[1]	
	0 h	24 h	30 h	24 h	30 h
Total COC collected	26	29	36	40	51
Expanded COC	0	16	25	19	40
Stained oocytes[2]	20	15	24	16	31
GV	$19/20^{a}$ (95%)	$0/15^{b}$ (0%)	$0/24^{b}$ (0%)	$0/16^{b}$ (0%)	$0/31^{b}$ (0%)
GVBD	$1/20^{a}$ (5%)	$0/15^{a}$ (0%)	$0/24^{a}$ (0%)	$0/16^{a}$ (0%)	$0/31^{a}$ (0%)
MI	$0/25^{a}$ (0%)	$6/15^{b}$ (40%)	$9/24^{b}$ (37%)	$12/16^{b}$ (75%)	$13/31^{b}$ (42%)
MII	$0/25^{a}$ (0%)	$9/15^{b}$ (60%)	$15/24^{bc}$ (63%)	$4/16^{bd}$ (25%)	$18/31^{b}$ (58%)

COC: cumulus-oocyte complexes, GV: Germinal vesicle, GVBD: Germinal vesicle breakdown, MI: Metaphase I, MII: Metaphase II

[1] COC collected 24 hours or 30 hours after treating bison with 2000 IU hCG

[2] A total of 18 COC (n=1 to 8 per group) were lost during the fixation and staining procedures

[abcd]Within rows, values with no common superscripts are different ($P<0.05$)

Table 3.8. Effect of season (anovulatory vs. ovulatory) and maturation type (*in vitro* vs. *in vivo*) on morphologic characteristics of wood bison cumulus-oocyte complexes (COC) collected from superstimulated wood bison (n=20 bison per season).

	Anovulatory season	Ovulatory season	Total
COC collection rate (COC collected/follicles aspirated)[1]			
in vitro	72/129 (56%)	65/105 (62%)	137/234 (59%)
in vivo[3]	83/124 (67%)	91/120 (76%)	174/244 (71%)
Total	155/253 (61%)	156/225 (69%)	311/478 (65%)
Fully expanded COC/bison (mean ±SEM)[2]			
in vitro	3.1±0.6	2.6±0.6	2.9±0.4
in vivo[3]	5.4±1.5	4.5±1.3	5.0±1.0
Total	4.3±0.8	3.6±0.7	3.9±0.5

[1]Anovulatory vs. Ovulatory (P = 0.91), *in vitro* vs. *in vivo* (P = 0.07), Interaction(P = 0.63).

[2]Anovulatory vs. Ovulatory (P = 0.51), *in vitro* vs. *in vivo* (P = 0.06), Interaction(P = 0.85).

[3] COC collected 24 hours or 30 hours after treating bison with 2000 IU hCG

Table 3.9. Effect of season (anovulatory vs. ovulatory) and maturation type (*in vitro* vs. *in vivo*) on nuclear maturation of wood bison oocytes collected from superstimulated wood bison (n=20 bison per season).

	Anovulatory season	Ovulatory season	Total
Germinal vesicle[1]			
in vitro	0/40 (0%)	0/39 (0%)	0/79 (0%)
in vivo[5]	0/48 (0%)	0/47 (0%)	0/95 (0%)
Total	0/88 (0%)	0/86 (0%)	0/174 (0%)
Germinal vesicle breakdown[2]			
in vitro	0/40 (0%)	0/39 (0%)	0/79 (0%)
in vivo[5]	5/48 (10%)	0/47 (0%)	5/95 (5%)
Total	5/88 (6%)	0/86 (0%)	5/174 (3%)
Metaphase I[3]			
in vitro	11/40 (27%)	15/39 (38%)	26/79 (33%)
in vivo[5]	21/48 (44%)	25/47 (53%)	46/95 (48%)
Total	32/88 (36%)	40/86 (46%)	72/174 (41%)
Metaphase II[4]			
in vitro	29/40 (73%)	24/39 (62%)	53/79[a](67%)
in vivo[5]	22/48 (46%)	22/47 (47%)	44/95[b](46%)
Total	51/88 (58%)	46/86 (54%)	97/174 (56%)

[1]Anovulatory vs. Ovulatory (P = 0.99), *in vitro* vs. *in vivo* (P = 0.99), Interaction (P = 0.99).
[2]Anovulatory vs. Ovulatory (P = 0.99), *in vitro* vs. *in vivo* (P = 0.99), Interaction (P = 0.99).
[3]Anovulatory vs. Ovulatory (P = 0.56), *in vitro* vs. *in vivo* (P = 0.12), Interaction (P = 0.85).
[4]Anovulatory vs. Ovulatory (P = 0.32), *in vitro* vs. *in vivo* (P = 0.01), Interaction (P = 0.40).
[5] COC collected 24 h or 30 h after treating bison with 2000 IU hCG

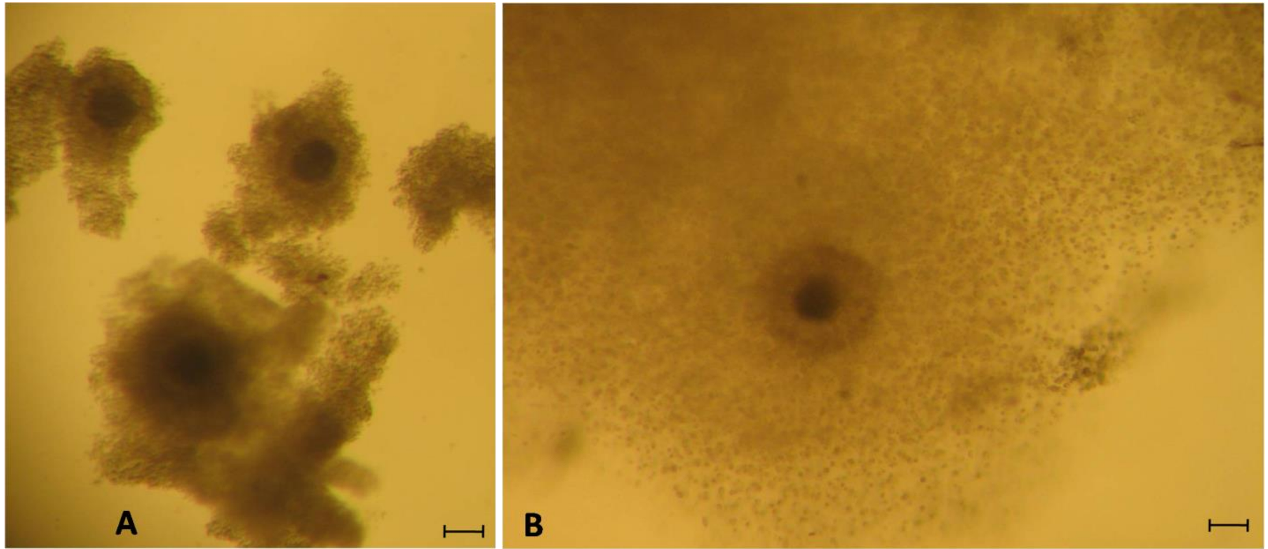


Fig. 3.1. Wood bison cumulus-oocyte complexes (COC) collected by transvaginal ultrasound-guided follicle aspiration. Compact (A)and fully expanded COC (B). Scale bar represents 100 µm.

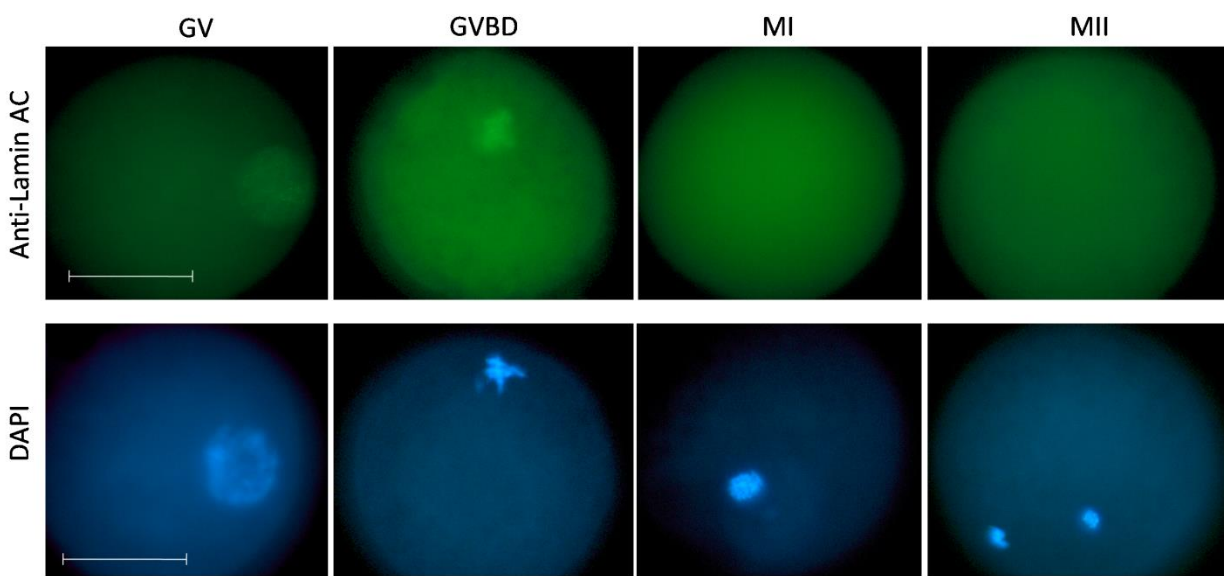


Fig. 3.2. Wood bison oocytes at different stages of nuclear maturation: germinal vesicle (GV), germinal vesicle break down (GVBD), metaphase I (MI) and metaphase II (MII). Oocytes were stained with anti-Lamin AC/DAPI. Note that the nuclear envelope (only seen in GV and GVBD stages) is visualized with green fluorescence after staining with Anti-Lamin AC, while DNA is visualized with blue fluorescence after staining with DAPI (Diaminophenylindole). Scale bar represents 50 µm.

CHAPTER 4

*IN VITRO EMBRYO PRODUCTION IN WOOD BISON (*Bison bison athabascae*) USING IN VITRO MATURED CUMULUS-OOCYTE COMPLEXES*

4.1 Abstract

Two experiments were done to test the hypothesis that morphologic characteristics of wood bison cumulus-oocyte complexes (COC) are reflective of the ability of the oocyte to develop to an advanced embryonic stage after *in vitro* maturation, fertilization and culture, and to determine the effect of prolonging the interval from the end of superstimulation treatment to oocyte collection (FSH starvation period). Experiments were done during the anovulatory season. Follicular wave synchronization was induced by follicular aspiration prior to ovarian superstimulation with two doses of FSH given at 48 h intervals during the anovulatory season. In Experiment 1, COC were collected 3 days (72 h) after the last dose of FSH by follicular aspiration from 10 bison, and classified as compact, expanded or denuded. The COC were matured *in vitro* for 24 h before fertilization *in vitro* (Day 0). Embryo development was assessed on Days 3, 7 and 8. The blastocyst rate was 7/34, 2/10 and 0/3 in COC classified as compact, expanded and denuded, respectively (P>0.05); however, only compact COC resulted in embryos that reached the expanded blastocyst stage. In Experiment 2, COC were collected at either 3 or 4 days (72 or 96 h) after the last dose of FSH (n=16 bison/group) to determine the effect of the duration of FSH starvation on oocyte competence. The COC were classified as compact good (>3 layers of cumulus cells), compact regular (1-3 layers of cumulus cells), expanded or denuded, and oocytes were matured, fertilized and cultured *in vitro*. Although follicles were larger (P<0.05) in the 4-day FSH starvation group, there was no effect of starvation period on the distribution of COC morphology; overall, 112/194 (57.7%) were compact, 29/194 (26.3%) were expanded, 39/194 (20.1%) were denuded, and 14/194 (7.2%) were degenerated (P<0.05). Similarly, there was no effect of starvation period on embryo development. Compact good COC had the highest cleavage (88%) and blastocyst rate (54%; P<0.05). Compact regular COC tended to have a higher blastocyst rate (25%, P=0.08) than expanded COC and had a higher blastocyst rate than denuded COC. Expanded and denuded COC

had similarly low cleavage (40% vs. 59%, respectively) and blastocyst rates (5% vs. 8%, respectively). In conclusion, morphologic characteristics of wood bison COC are reflective of the ability of the oocyte to develop into an embryo *in vitro*. Importantly, oocytes collected from superstimulated bison cows were competent to develop to the blastocyst stage following *in vitro* maturation, fertilization and culture.

4.2. Introduction

Wood bison (*bison bison athabascae*) are the largest terrestrial mammal in North America. This species is listed as Threatened under Schedule I of the Canadian Species at Risk Act (SARA; Environment and Climate Change Canada, 2016). The population in Wood Buffalo National Park in Canada represents the largest, most genetically diverse reserve of wood bison in the world (McCormack, 1992; McFarlane et al., 2006), but has an on-going disease prevalence of 30-40% for brucellosis and tuberculosis (10 of the 12 free-ranging herds of wood bison in Canada are endemically infected; Environment and Climate Change Canada, 2016). To retain the genetic diversity of wood bison and to mitigate the effects of endemic disease, the use of reproductive technologies was recommended in a recent report as an effective strategy to preserve the genetic material (gametes and embryos) of wood bison (MacPhee et al., 2016).

In vitro production of embryos (IVP) is used worldwide in a variety of species (e.g. cattle [Sirard and Blondin, 1996], llamas [Trasorras et al., 2014], deer [Comizzoli et al., 2001]) and has been proposed as a means of rescuing the genetics of wood bison (reviewed in Cervantes et al., 2016a). Collection of potentially competent oocytes is the first step for successful *in vitro* embryo production (Merton et al., 2003). Immature oocytes are matured *in vitro* after collection as a standard procedure in various species (e.g, cattle [Goodhand et al., 1999], human [Smith et al.

2000], river buffalo [Manjunatha et al., 2008]). Importantly, immature oocytes may be obtained from a variety of donors including those that are pregnant [Meintjes et al., 1995], prepubertal [Bernal et al., 2015], or that have recently died [Sambasiva Rao et al., 2010]). While the use of immature oocytes for *in vitro* embryo production provides an opportunity to rescue biological material for conservation purposes, fewer than 10% of immature oocytes derived bison ovaries obtained following slaughter developed into blastocysts in the only study reported to-date (Thundathil et al., 2007). Because slaughterhouse-derived ovaries are not readily available for bison, and because ovarian status may influence oocyte competence, we developed a practical and effective method of collecting cumulus oocyte complexes (COC) from live wood bison by transvaginal ultrasound-guided follicle aspiration (Palomino et al., 2013, 2014). By using this approach, results of a recent study revealed that the proportion of wood bison oocytes that reached the MII stage of development was maximal after 24 h of *in vitro* maturation (Cervantes et al., 2016a).

Results of several other studies support the notion that morphology of the COC is related to the *in vitro* developmental potential of immature oocytes in different species (e.g., cattle [Shioya et al., 1988; Hazeleger et al., 1995; Boni et al., 2002; Madison et al., 1992; Bakri et al., 2016]; goat [Katska-Ksiazkiewicz et al., 2007]; sheep [Kelly et al. 2007; Dadashpour Davachia et al., 2012]; water buffalo [Singh et al., 2012]). In cattle, immature oocytes are routinely selected for IVP on the basis of the appearance of the ooplasm and the characteristics of cumulus cells surrounding the oocyte (i.e., compactness and number of cell layers; Gordon, 2004). In bison, the morphologic characteristics of immature oocytes and their relationship to *in vitro* embryo development have not been reported.

Aside from the *in vitro* environment itself, the ability of oocytes to develop into viable embryos (oocyte competence) has been reported to be associated with the physiologic status of the follicle from which it came (reviewed in Dias et al., 2014). For instance, the period of FSH starvation between the end of superstimulatory treatment and the time of oocyte collection in cattle (i.e. also referred to as the FSH withdrawal or coasting period) impacts the follicle diameter and maturity, and in turn, the *in vitro* developmental potential of the oocytes (Blondin et al. 2002, 2012). During FSH starvation, granulosa cells undergo transcriptomic changes related to post-LH surge maturation and, depending on the duration of FSH starvation, these changes may increase oocyte competence (reviewed in Dias et al., 2014; Sirard et al., 2006). A 48-h period of FSH starvation, compared to 24 and 72 h, resulted in increased production of bovine embryos *in vitro* (Blondin et al., 1997). In another study in cattle, oocyte competence was highest after an FSH starvation period of between 44 and 68h, and was lower after 92 h of starvation (Nivet et al., 2012). The effects of FSH starvation on the competence of wood bison oocytes has not been reported.

The objectives of the present study were to test the hypothesis that morphologic characteristics of wood bison cumulus-oocyte complexes (COC) are reflective of the ability of the oocyte to develop to an advanced embryonic stage after *in vitro* maturation, fertilization and culture (Experiments 1 and 2), and to determine the effect of prolonging the interval from the end of superstimulation treatment involving two doses of FSH diluted in hyaluronan given 48 h apart to oocyte collection (FSH starvation period) on follicular response, oocyte morphologic indicators, and blastocyst development (Experiment 2).

4.3. Materials and methods

4.3.1. Animals

The experiments were done in March (Experiment 1) and May to June (Experiment 2); i.e., both during the anovulatory season in wood bison. Mature (6 to 11 years old) non-lactating female wood bison (n = 32), with an average body condition score of 3.5 (scale of 1 to 5; Vervaecke et al., 2005), were confined to corrals with free access to fresh water and alfalfa-brome grass hay at the Native Hoofstock Centre, University of Saskatchewan. The experimental protocol was approved by the University of Saskatchewan's Animal Research Ethics Board, and done in accordance with the guidelines of the Canadian Council on Animal Care.

4.3.2. Experiment 1: In vitro embryo production (pilot study)

Follicular wave emergence was induced among bison (n=10; 5 bison per day on 2 separate days) by transvaginal ultrasound-guided aspiration of all follicles ≥5 mm in diameter (follicular ablation) as reported previously (Palomino et al., 2014a). Briefly, follicles were aspirated using a 5-MHz transvaginal probe (ALOKA SSD-900, Tokyo, Japan) equipped with a disposable 18-ga x 1 ½" vacutainer needle attached to a 6 mL syringe by silicon tubing 60 cm long x 1.14 mm internal diameter. On the day after follicle ablation (i.e., day of a new wave emergence; Bergfelt et al., 1994, Palomino et al., 2014a), bison were treated with 300 mg of NIH-FSH-P1 (Folltropin-V, Vetoquinol Canada Inc., Lavaltrie, QC, Canada) i.m. diluted in 0.5% hyaluronan (5 mg/mL, MAP-5, Vetoquinol Canada Inc.) and again 2 days later with 100 mg of NIH-FSH-P1 to induce ovarian superstimulation, as previously described (Palomino et al., 2013).

Three days (72 h) after the second dose of FSH (Fig. 1), COC were collected by transvaginal ultrasound-guided aspiration of all follicles ≥5 mm in diameter, as previously described (Palomino et al., 2013, 2014b). In brief, a short-beveled 18-ga x 2" disposable needle (Misawa Medical Industry Ltd., Edogawa-Ku, Tokyo, Japan) connected to a 50 mL conical Falcon tube by silastic tubing (internal diameter 1.14 mm; Cole Palmer, Montreal, Quebec, Canada) was used for COC

collection. Follicular contents were aspirated using a regulated vacuum pump set at a flow-rate of 20 mL/min. The temperature in the collection area, surrounding the Falcon tube and silastic tubing, was kept at 22-25°C using a portable liquid propane forced-air heater (Dyna-Glo Pro; GHP Group Inc, Morton Grove, IL, USA).

The collection medium consisted of Dulbecco's phosphate-buffered saline (DPBS, Gibco, Grand Island, NY, USA), supplemented with 0.15% ET Surfactant (Vetoquinol Canada Inc.) and 200 IU/L of heparin (heparin sodium injection USP, Sandoz, Boucherville, Quebec, Canada). The contents of the Falcon tube were poured through an ova/embryo filter with a 75 µm mesh (Emcon filter; Agtech, Manhattan, Kansas, USA), and the filter was rinsed using D-PBS medium without surfactant, and gently poured into a 90 mm Petri dish to search for COC using a stereomicroscope (SMZ 1000, Nikon Instrument Inc., Melville, NY, USA) at 10x magnification. The temperature of the COC searching room was maintained at 25 to 30°C.

The isolated COC were washed three times with holding medium (D-PBS + 5% calf serum) and morphologically classified according to the number of cumulus cell layers and the appearance of the cumulus cells and ooplasm. Compact COC were those with two or more layers of granulosa cells tightly surrounding the oocyte, expanded COC were those with expanded or partially dissociated cumulus cells, denuded COC were those with no cumulus cells or an incomplete layer of cumulus cells, and degenerated COC were those with pyknotic cumulus cells and vacuolated ooplasm (adapted from Ratto et al., 2007). Degenerated COC were discarded from further processing.

The number of follicles ≥5 mm was determined on the day of COC collection by transrectal ultrasonography using a 7.5-MHz probe (MyLab 5 VET, Esaote NA, IN, USA). Treatments were scheduled so that collection of COC was done in two replicates (n=5 bison/replicate).

4.3.3. In vitro maturation, fertilization, and culture

All chemicals and reagents used for maturation, fertilization and embryo culture were purchased from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated. Maturation medium consisted of TCM-199 (Gibco, Grand Island, NY, USA) supplemented with 5% (v/v) calf serum (CS), 5 µg/mL pLH (Lutropin-V, Vetoquinol Canada Inc.), 0.5 µg/mL pFSH (Folltropin-V, Vetoquinol Canada Inc.) and 0.05 µg/mL gentamicin. The COC from each morphologic category were washed three times in maturation medium, then incubated in four-well dishes (NUNC, Thermo Fisher Scientific, Rochester, NY, USA) containing 1 mL/well of maturation medium at 38.5°C, 5% CO_2 in air and high humidity.

After 24 h of *in vitro* maturation, semen from two wood bison bulls, frozen in Triladyl extender (Hussain et al., 2011), was thawed, pooled and used for *in vitro* fertilization. Motile sperm were selected using the Percoll density gradient method (45% and 90%), as described previously (Parrish et al., 1995). Progressive motility (i.e., the percentage of spermatozoa that had directional movement), as assessed by conventional light microscopy at 400x magnification was 60 to 75% for all replicates. Sperm were re-suspended to a final concentration of 5×10^6 sperm/mL in Brackett-Oliphant fertilization medium (Brackett and Oliphant, 1975). Oocytes were washed three times in fertilization medium supplemented with 10% BSA and transferred to four-well dishes containing 500 uL/well of the sperm suspension. Oocytes and sperm were co-incubated at 38.5°C in 5% CO_2 in air and high humidity.

After 18 h co-incubation, the presumptive zygotes were mechanically denuded of cumulus cells by gentle pipetting, then washed three times in *in vitro* culture medium (Charles Rosenkran's aminoacid [CR1aa] medium with 5% calf serum containing amino acids, L-Glutamic acid, BSA

and gentamicin, Prentice et al., 2011) and cultured in four-well dishes (n=3 to 13 zygotes/well) containing 500 µL/well of *in vitro* culture medium in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C, as reported (Cervantes et al., 2016b).

Cleavage was evaluated after 56 h in culture. Embryos with ≥2 cells were separated from the unfertilized oocytes, washed and transferred to fresh *in vitro* culture medium. Embryo development was assessed on Days 7 and 8 (Day 0 = day of *in vitro* fertilization), and classified according to guidelines established by the International Embryo Transfer Society (IETS Manual, 2010) for *in vivo*-derived embryos as compact morula (embryo undergoing the compaction process where individual blastomeres are difficult to discern from one another), early blastocyst (embryo has formed a fluid-filled blastocele occupying ≤50% of the volume of the embryo), blastocyst (blastocele occupies >50% of the volume of the embryo, visual differentiation between the trophoblast and the compact inner cell mass), expanded blastocyst (overall diameter of embryo increases, zona pellucida becomes thinner), hatching-hatched blastocyst (embryo partially or completely free of the zona pellucida).

4.3.4. Experiment 2: Effect of FSH starvation period on in vitro embryo production from in vitro matured oocytes in wood bison

Ovarian follicular synchronization and superstimulation treatments were done in female wood bison (n = 32, 6 to 10 years old) as described in Experiment 1 (Fig. 1). Treatments were scheduled so that collection of COC was done in four replicates (n=8 bison/replicate). The COC were collected transvaginally, as described in Experiment 1, but at either 3 days (72 h) or 4 days (96 h) after the second dose of FSH (n=16 bison/group). The COC were located under a stereomicroscopy at 10x magnification, washed three times in holding medium (D-PBS + 5% calf serum), and classified morphologically according to the characteristics and the number of cumulus cell layers

and the appearance of the oocyte cytoplasm (adapted from Seneda et al., 2001) as follows: compact good (more than three layers of unexpanded cumulus cells, and with homogeneous ooplasm), compact regular (one to three layers of unexpanded cumulus cells, and with homogeneous ooplasm), denuded (partially or not covered by cumulus cells), expanded (expanded cumulus cells), and atretic/degenerated (dark cumulus oophorus and signs of cytoplasmic degeneration such as irregular/heterogeneous cytoplasm). The COC from the latter category were discarded from further processing.

As in Experiment 1, the COC were placed in maturation medium for 24 h and then co-incubated (*in vitro* fertilized) with bison sperm for 18 h. Presumptive zygotes were placed in culture medium and evaluated after 56 h for cleavage. Cleaved zygotes were transferred to fresh culture medium, and development was evaluated on Days 7 and 8, as described in Experiment 1. In this experiment, blastocysts were also graded morphologically according to the grading system established by the IETS for *in vivo*-derived embryos as Grade 1 (symmetrical and spherical cell mass, individual blastomeres uniform in size, color and density, and minor irregularities), Grade 2 (moderate irregularities in the overall shape of the embryonic mass, or in size, color, and density of individual cells), Grade 3 (major irregularities in shape of the embryonic mass, or in size, color, and density) and Grade 4 (degenerated/dead), as described (IETS Manual, 2010).

4.3.5. Statistical analyses

In both experiments, cleavage and blastocyst rates (based on the total number of COC submitted to *in vitro* maturation) were compared by 2-tailed Chi-square or 2-tailed Fisher's exact test using Proc Frequency procedure (SAS, Enterprise Guide 4.2, Statistical Analysis System Institute Inc., Cary, North Carolina, USA). In Experiment 2, cleavage, morula and blastocyst rates were compared using the generalized linear model procedure in SAS with a binomial error

distribution and a logit link function to determine the effects of FSH starvation (3-day vs. 4-day) and COC morphological category (compact good, compact regular, expanded, denuded). The number and size of follicles available for aspiration (i.e., ≥5 mm) and the number of COC collected were compared between the 3-day and 4-day FSH starvation groups by Student's t-test, and COC collection rate was compared by 2-tailed Chi-square. Values are expressed as a proportion or a mean ± SEM; P-values of <0.05 were considered significant.

4.4. Results

4.4.1. Experiment 1: In vitro embryo production (pilot study)

The number of follicles ≥5 mm per bison on the day of COC collection was 10.8 ± 1.3. A total of 50 COC were collected from 108 follicles aspirated (46% collection rate) in 10 superstimulated wood bison. Of the COC collected, 34 (68%) had compact cumulus cells, 12 (24%) had expanded cumulus, 3 (6%) were partially or completed denuded of cumulus cells, and 1 (2%) was degenerated. The degenerated COC was discarded from further processing, and two expanded COC were lost during washing and handling.

No effect of replicate was detected for any end point; therefore, data from the two replicates were combined for further analyses. The proportion of oocytes undergoing cleavage by 56 h, and the proportion that developed to the blastocyst stage on Day 7 and Day 8 did not differ among morphologic categories (Table 4.1). However, none of the denuded COC developed into a blastocyst. Three embryos derived from compact COC reached the expanded blastocyst stage, but none from expanded COC.

4.4.2. Experiment 2: Effect of FSH starvation period on in vitro embryo production using in vitro matured oocytes in wood bison

The ovarian response and results of COC collection are summarized in Table 4.2. In the 4-day FSH starvation group, the number of follicles ≥10 mm tended to be greater (P = 0.09) and the mean diameter of follicles ≥5 mm was greater than in the 3-day group. The COC collection rate was higher in the 3-day vs. 4-day FSH starvation groups, but the total number of COC collected per bison did not differ between groups.

A total of 98 and 96 COC were collected from superstimulated wood bison in the 3-day vs. 4-day FSH starvation groups, respectively. From these COC, 55 (56.1%) and 57 (59.4%) had compact cumulus in each group, respectively, and did not differ between groups. The number of COC in other morphologic categories was also similar between the groups (Table 4.3). Twelve compact COC and nine expanded COC were lost during handling, washing and transport.

Extending the FSH starvation period from 3 to 4 days was not associated with an increase in cleavage rate or blastocyst rate (including all COC morphologic categories; Table 4.4, Fig. 4.2). Cleavage and blastocyst rates were highest for compact good COC (Table 4, Fig. 4.3). No effect of FSH starvation period was detected for morphological grading of blastocysts; therefore, data from the two starvation groups were combined for further analyses. The proportion of blastocysts classified as Grade 1 and 3 differed between compact good COC and compact regular COC (Table 4.5). Although embryo development beyond Day 8 was not examined critically, six hatching/hatched blastocysts and one hatching/hatched blastocyst were detected between Days 9 and 11 (Fig. 4.3), from the compact good COC and compact regular COC, respectively.

4.5. Discussion

Results of the present study support the hypothesis that the morphologic characteristics of wood bison COC at the time of collection are reflective of oocyte developmental competence. A greater proportion of bison oocytes with compact cumulus cells than partially denuded or completely

denuded oocytes developed to the blastocyst stage. This is in agreement with previous studies in cattle (Hazeleger et al., 1995) and water buffalo (Warriach and Chohan, 2004) where morphologic criteria were used to select COC with a higher potential for blastocyst development. Results of the present study also demonstrate a developmental advantage of oocytes with more than three layers of cumulus cells (i.e. good compact) over those with fewer cumulus cells (i.e., regular compact), consistent with findings in other species (e.g. cat [Wood and Wildt, 1997]; cattle [Kelly et al., 2007]; sheep [Kelly et al., 2007; Dadashpour Davachia et al., 2012]).

The present study is the first to demonstrate that immature COC collected by follicle aspiration from live bison are competent to develop to the blastocyst stage following *in vitro* maturation, fertilization and culture. In an earlier study of the characteristics of *in vitro* maturation of bison oocytes (Cervantes et al., 2016a), 60-70% of the oocytes were at the metaphase-II stage after 24 h of *in vitro* culture. In the present study, 20% of compact COC developed to the blastocyst stage in Experiment 1, and about 40% in Experiment 2. Improved blastocyst production in Experiment 2 may be attributed to a greater number of compact COC with more than three layers of cumulus cells collected in this experiment, and which is consistent with the concept that the number of cumulus cell layers surrounding the oocyte is related to subsequent embryo development. Interestingly, wood bison oocytes matured *in vivo* were found to be surrounded by multiple layers of fully expanded cumulus cells (Cervantes et al., 2016a), and were competent to develop to the blastocyst stage after immediate *in vitro* fertilization (Cervantes et al., 2016b). Importantly, over 50% of blastocyst produced from compact good COC in the present study were Grade 1 (excellent or good), which was higher than the 16% from compact regular COC. In cattle, Grade 1 embryos at the blastocyst stage were shown to yield the highest pregnancy rates when transferred either fresh or frozen-thawed (*in vitro*-produced embryos: [Hasler et al., 1987; Hasler et al., 1995]; *in vivo*-derived embryos: [Scenna et al., 2008]).

The percentage of compact COC that developed to the blastocyst stage in the present study was similar to that reported in FSH-treated cows (53%, Vieira et al., 2014) and FSH-treated goats and ewes (50% and 62%, respectively; Cox and Alfaro, 2007). This represents a remarkable improvement in blastocyst production compared to previous reports in bison involving the use of ovaries from slaughtered animals (i.e., 8 to 16% blastocyst production; Thundathil et al., 2007; Aurini et al., 2009; Barfield and Siedel, 2011). A similar difference was reported in water buffalo where oocytes collected by follicular aspiration from live animals had greater developmental competence than slaughterhouse-derived oocytes (Neglia et al., 2003). Oocytes collected from excised ovaries often include those aspirated from small follicles (≤3 mm) which have been shown to produce the lowest proportion of blastocysts in cattle (Karami Shabankareh et al., 2014). These findings highlight the advantage of being able to differentially collect oocytes from follicles of a particular size (i.e., ≥5 mm) by transvaginal follicular aspiration, resulting in a greater proportion of competent oocytes. In addition, oocytes collected from FSH superstimulated cows were shown to result in higher number of high-quality blastocysts in comparison with those collected from non-superstimulated animals (Chaubal et al., 2006). In FSH superstimulated cows, follicles of medium size (6-10 mm) increase and those may contain oocytes with improved developmental competence *in vitro* (Goodhand et al., 2000).

The period of FSH starvation evaluated in the present study resulted in larger follicles at the time of COC collection, similar to studies in cattle (Blondin et al., 2002; Nivet et al., 2012), but had no effect on the total number and types of COC collected. Furthermore, the proportion of oocytes developing to the blastocyst stage was unaffected by extending the FSH starvation period by 24 h. This is in contrast to previous results in cattle (Blondin et al., 2002) where 48 h compared to 33 h of FSH starvation following multiple injections of FSH (diluted in saline), increased blastocyst production. Further experiments are needed to determine the effect of duration of the

FSH starvation period and/or FSH dosing schedule on *in vitro* embryo production in bison, as was reported in cattle.

In summary, morphologic characteristics of wood bison COC were associated with the potential of oocytes to develop to advanced embryo stages after *in vitro* maturation, fertilization and culture. Compact COC with >3 layers of cumulus cells collected by transvaginal ultrasound-guided follicle aspiration from superstimulated wood bison had the highest competence to develop to the blastocyst stage following *in vitro* maturation, fertilization and culture. Extending the FSH starvation period from 3 to 4 days did not increase blastocyst production in wood bison. The blastocyst production rate of 20-30% of all COC collected offers promise for efficient propagation of genetically valuable bison for both conservation and production purposes, but further studies are needed to evaluate techniques to preserve the embryos for future use, as well as evaluate the potential of these embryos to complete full-term pregnancy after transfer to recipients.

4.6. Acknowledgements

This study was supported by grants from the University of Saskatchewan and the Toronto Zoo. The authors gratefully acknowledge Elk Island National Park as the original source of the wood bison used in the study, and Dr. Murray Woodbury (Agriculture and Agri-Food Canada Specialized Livestock Chair, University of Saskatchewan) for fostering the bison research program. The authors gratefully acknowledge Dr. Rodrigo Carrasco, Dr. Serena Caunce, and Mrs. Mary Von der Porten for help with data collection, and the farm staff at the Native Hoofstock Centre, University of Saskatchewan for maintaining the animals used in the study. We also thank Vetoquinol Canada Inc. for providing Folltropin-V, MAP-5, and supplies for oocyte collection and handling.

Table 4.1. *In vitro* development of wood bison embryos produced from different morphologic categories of cumulus-oocyte complexes (COC) collected from superstimulated wood bison (n=10) by transvaginal ultrasound-guided follicular aspiration. Oocytes were matured, fertilized and cultured *in vitro* (Day 0 = day of *in vitro* fertilization, IVF; Experiment 1).

	Morphologic category		
End point[1]	Compact COC	Expanded COC	Denuded COC
COC submitted to IVF (n)	34	10	3
Cleaved oocytes	19/34 (56.0%)	6/10 (60.0%)	1/3 (33.0%)
Blastocysts on Day 7	6/34 (17.6%)	1/10 (10.0%)	0/3 (0%)
Blastocysts on Day 8	7/34 (20.6%)	2/10 (20.0%)	0/3 (0%)

[1]No significant differences among COC morphologic categories for any end point.

Table 4.2. Ovarian response and collection of cumulus-oocyte complexes (COC) in superstimulated wood bison after FSH starvation (collection 3 vs. 4 days after the last treatment of FSH; mean ±SEM; Experiment 2).

End point	3-day FSH starvation	4-day FSH starvation
Number of bison	16	16
Number of follicles ≥5 mm of day of COC collection	12.4 ± 1.0	13.3 ± 1.2
Number of follicles ≥10 mm on day of COC collection	4.8 ± 0.7^{x}	6.9 ± 1.0^{y}
Mean diameter of all follicles ≥5 mm (day of COC collection)	9.3 ± 0.21^{a}	10.0 ± 0.22^{b}
Number of follicles aspirated per bison	9.4 ± 0.8	11.4 ± 1.1
COC collection rate (COC collected/follicle aspirated)	98/151^{a} (64.9%)	96/182^{b} (52.7%)
Number of COC collected per bison	6.1 ± 0.9	6.0 ± 1.0

^{ab} Within rows, values with different superscripts are different ($P<0.05$)

^{xy} Within rows, values with different superscripts tended to differ ($P\leq0.09$)

Table 4.3. Distribution of morphologies of cumulus-oocyte complexes (COC) collected from superstimulated wood bison after FSH starvation (3 vs. 4 days after the last treatment of FSH; n = 16 bison per group). The COC were classified 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), expanded, or denuded (Experiment 2).

End point	3-day FSH starvation[1]	4-day FSH starvation[1]	Total
Compact COC	55/98 (56.1%)	57/96 (59.4%)	112/194[a] (57.7%)
Compact good COC	29/98 (29.6%)	32/96 (33.3%)	61/194[b] (31.4%)
Compact regular COC	26/98 (26.5%)	25/96 (26.0%)	51/194[bd] (26.3%)
Expanded COC	17/98 (17.3%)	12/96 (12.5%)	29/194[c] (14.9%)
Denuded COC	20/98 (20.4%)	19/96 (19.8%)	39/194[cd] (20.1%)
Degenerated COC	6/98 (6.1%)	8/96 (8.3%)	14/194[e] (7.2%)

[1] No significant differences between groups for any end point.

[abcde] In the last column (total), values with different superscripts are different ($P<0.05$).

Table 4.4. *In vitro* development of wood bison embryos produced from cumulus-oocyte complexes (COC) collected from superstimulated bison by transvaginal ultrasound-guided follicular aspiration after FSH starvation (3 vs. 4 days after the last treatment of FSH; n=16 bison per group). At the time of collection, COC were categorized 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), expanded, or denuded. Oocytes were matured, fertilized and cultured *in vitro* (Day 0 = day of *in vitro* fertilization, IVF; Experiment 2).

End point	3-day FSH starvation	4-day FSH starvation	Total
Cleaved oocytes[1]			
Compact good COC	24/29 (82.8%)	22/23 (95.7%)	46/52[a] (88.5%)
Compact regular COC	19/25 (76.0%)	16/23 (69.6%)	35/48[ac] (72.9%)
Expanded COC	6/13 (46.2%)	2/7 (28.6%)	8/20[b] (40.0%)
Denuded COC	12/20 (60.0%)	11/19 (57.9%)	23/39[bc] (59.0%)
Total	61/87 (70.1%)	51/72 (70.8%)	112/159 (70.4%)
Morulas on Day 7[2]			
Compact good COC	6/29 (20.7%)	5/23 (21.7%)	11/52 (21.2%)
Compact regular COC	3/25 (12.0%)	7/23 (30.4%)	10/48 (20.8%)
Expanded COC	1/13 (7.7%)	0/7 (0%)	1/20 (5.0%)
Denuded COC	2/20 (10.0%)	4/19 (21.1%)	6/39 (15.4%)
Total	12/87 (13.8%)	16/72 (22.2%)	28/159 (17.6%)
Blastocysts on Day 7[3]			
Compact good COC	13/29 (44.8%)	11/23 (47.8%)	24/52[a] (46.2%)
Compact regular COC	6/25 (24.0%)	8/23 (34.8%)	14/48[ab] (29.2%)
Expanded COC	1/13 (7.7%)	0/7 (0%)	1/20[bc] (5.0%)
Denuded COC	2/20 (10.0%)	2/19 (10.5%)	4/39[c] (10.3%)
Total	22/87 (25.3%)	21/72 (29.2%)	43/159 (27.0%)
Blastocysts on Day 8[4]			
Compact good COC	14/29 (48.3%)	14/23 (60.9%)	28/52[a] (53.8%)
Compact regular COC	5/25 (20.0%)	7/23 (30.4%)	12/48[b] (25.0%)
Expanded COC	1/13 (7.7%)	0/7 (0%)	1/20[bc] (5.0%)
Denuded COC	2/20 (10.0%)	1/19 (5.3%)	3/39[c] (7.7%)
Total	22/87 (25.3%)	22/72 (30.6%)	44/159 (27.7%)

[1] FSH starvation group (P = 0.46), COC morphology (P = 0.03), Interaction (P = 0.46).
[2] FSH starvation group (P = 0.90), COC morphology (P = 0.25), Interaction (P = 0.55).

[3] FSH starvation group (P = 0.57), COC morphology (P = 0.004), Interaction (P = 0.84).
[4] FSH starvation group (P = 0.15), COC morphology (P = 0.003), Interaction (P = 0.27).
[abc] For each embryonic stage, values with no common superscript in the final column are different (P<0.05).

Table 4.5. Morphological grading of wood bison blastocysts produced *in vitro* from compact cumulus-oocyte complexes (COC) collected from superstimulated bison by transvaginal ultrasound-guided follicular aspiration (n=32 bison). The compact COC were categorized as compact good (> 3 layers of unexpanded cumulus cells and homogeneous ooplasm) or compact regular (1 to 3 layers of unexpanded cumulus cells and homogeneous ooplasm) at the time of collection. Oocytes were matured, fertilized and cultured *in vitro* (Day 0 = day of *in vitro* fertilization, IVF; Experiment 2).

End point	Compact good COC	Compact regular COC
Blastocysts on Day 7 (n)	24	14
Grade 1	14/24 (58.3%)^{a}	3/14 (21.4%)^{b}
Grade 2	6/24 (25.0%)	4/14 (28.6%)
Grade 3	4/24 (16.7%)^{x}	7/14 (50.0%)^{y}
Blastocysts on Day 8 (n)	28	12
Grade 1	16/28 (57.1%)^{a}	2/12 (16.7%)^{b}
Grade 2	6/28 (21.4%)	3/12 (25.0%)
Grade 3	6/28 (21.4%)^{a}	7/12 (58.3%)^{b}

Grade 1*:* embryos with symmetrical and spherical cell mass, uniform blastomeres

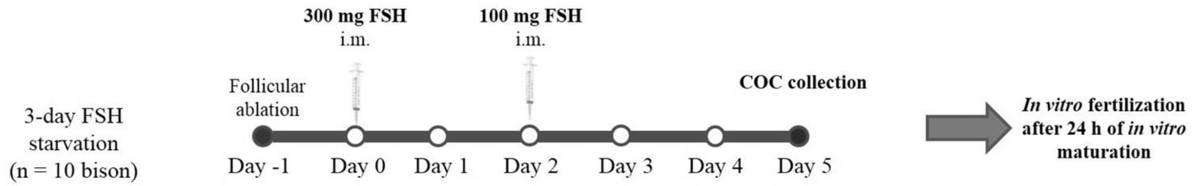
Grade 2: embryos with moderate irregularities in size, shape, color and density of cell mass

Grade 3: embryos with major irregularities in size, shape, color and density of cell mass

[ab] Within rows, values with different superscripts are different (P<0.05)

[xy] Within rows, values with different superscripts tended to differ (P≤0.06)

Experiment 1



Experiment 2

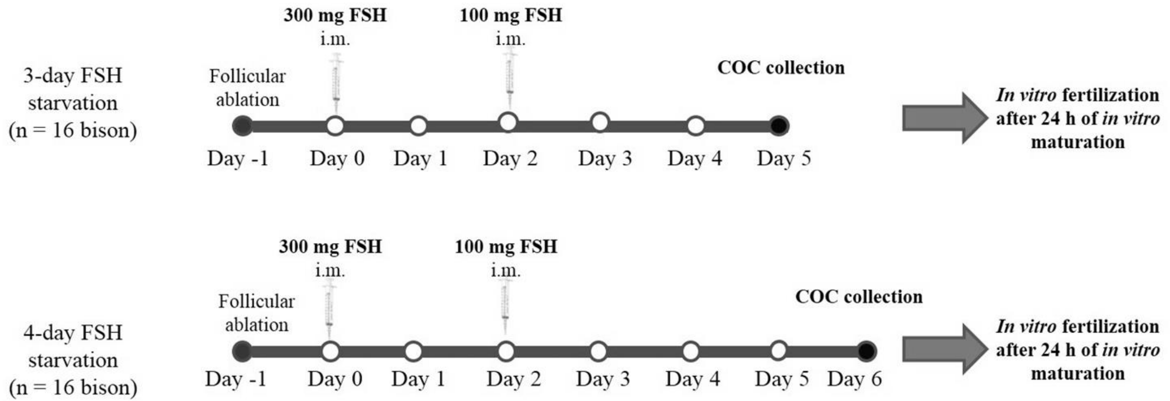


Fig. 4.1. Experimental design including the superstimulation protocol for the purpose of *in vitro* embryo production in wood bison to determine the developmental competence of cumulus-oocyte complexes (COC) of different morphologic categories collected by transvaginal ultrasound-guided follicular aspiration. In Experiment 2, COC collection was performed either 3 days or 4 days after the last dose of follicle stimulating hormone (FSH).

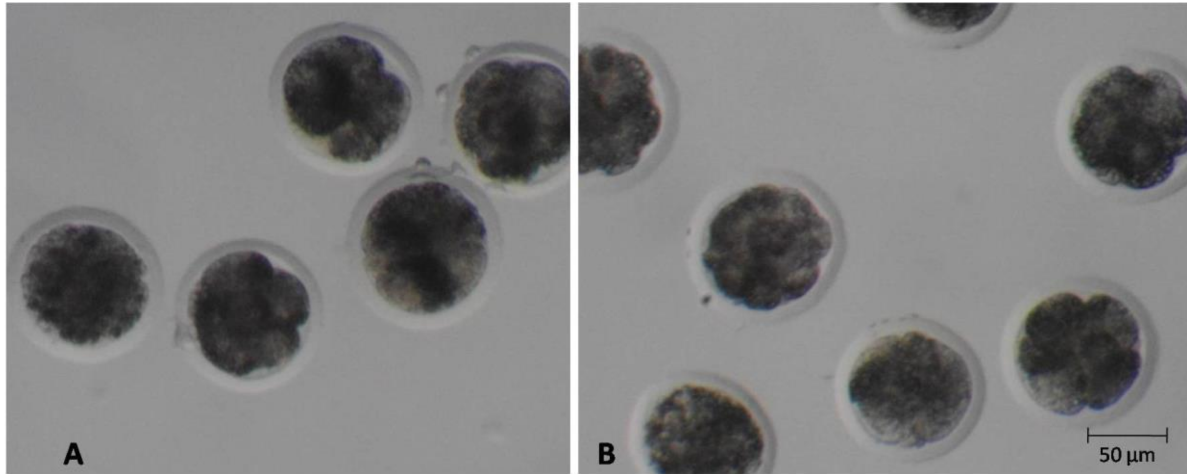


Fig. 4.2. *In vitro*-produced wood bison embryos from *in vitro* matured oocytes at cleavage stages (56 h after *in vitro* fertilization). The oocytes were collected from superstimulated bison after a 3-day (A) or 4-day (B) FSH starvation period (Experiment 2).

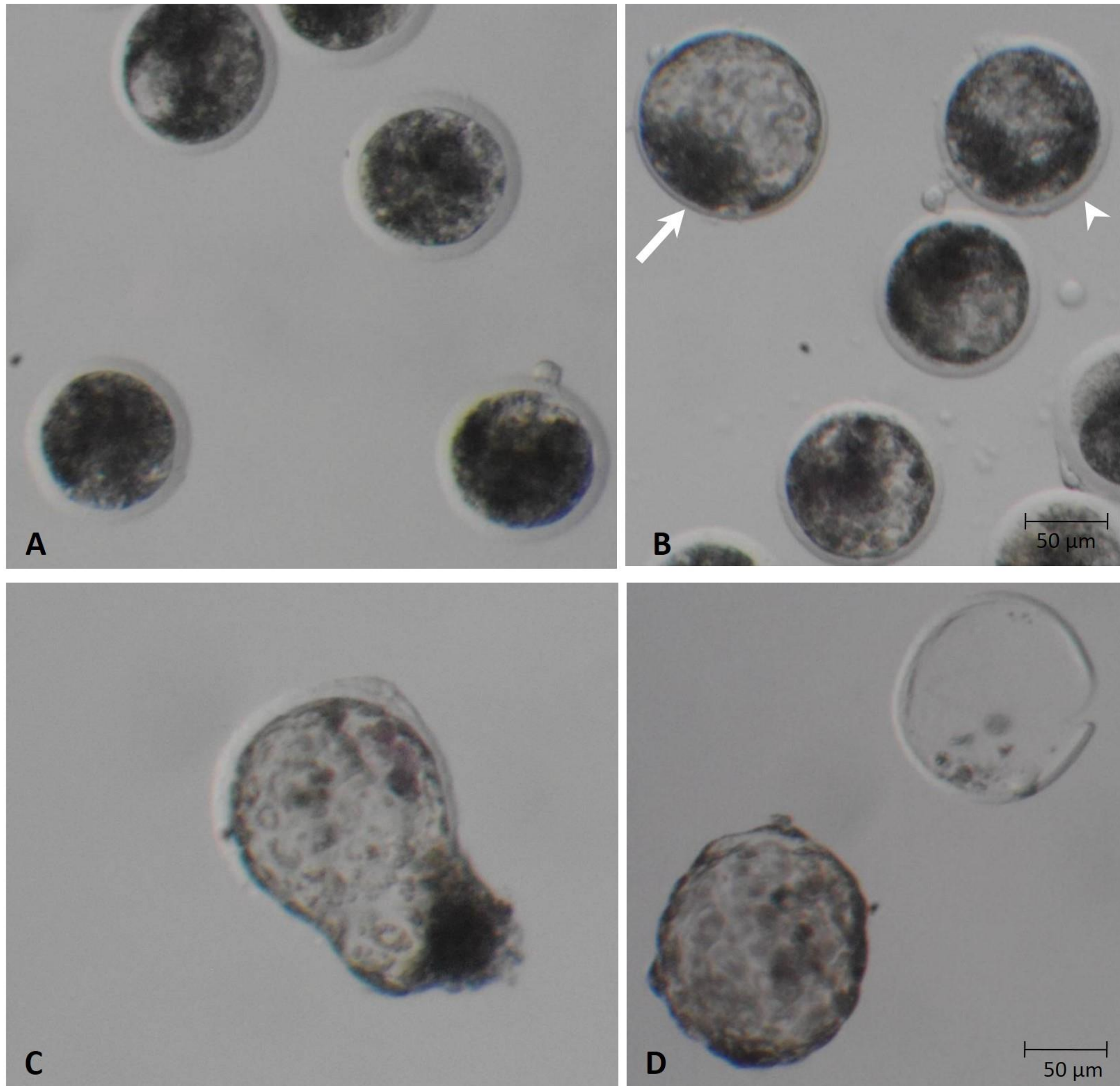


Fig. 4.3. *In vitro*-produced wood bison embryos from *in vitro* matured oocytes at A) the early blastocyst stage on Day 7, and B) the blastocyst (arrowhead) and expanded blastocyst (arrow) stages on Day 8, C) the hatching and D) hatched blastocysts between Days 9 and 11 of *in vitro* culture in Charles Rosenkran's aminoacid (CR1aa) medium with 5% calf serum containing amino acids, L-Glutamic acid, BSA and gentamicin (Day 0=day of *in vitro* fertilization).

CHAPTER 5

IN VITRO EMBRYO PRODUCTION IN WOOD BISON (Bison bison athabascae) USING IN VIVO MATURED CUMULUS-OOCYTE COMPLEXES

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5.1. Abstract

Experiments were conducted in wood bison to determine the effect of additional 4 h of maturation time on embryo development of *in vivo* matured oocytes. In Experiment 1, cumulus-oocyte complexes (COC) were collected 30 h after hCG treatment in superstimulated wood bison, and expanded COC were fertilized immediately or after 4 h of additional *in vitro* maturation. Embryo development was assessed on Days 3, 7 and 8 (Day 0= day of fertilization). No difference in cleavage rate was detected (55.3 vs. 60.5%, P= 0.82), but the Day 8 blastocyst rate was higher after an additional 4 h of *in vitro* maturation time (44.7 vs. 18.4 %, P=0.03). In Experiment 2, COC were collected at either 30 h or 34 h after hCG treatment. Expanded COC from the 30 h group were fertilized after 4 h of *in vitro* maturation, while those from the 34 h group were fertilized immediately. A higher cleavage rate (74.3 vs. 57.0%) and blastocyst rate (54.1 vs. 37.2%) was found in the 34 h group vs. the 30 h group (P<0.05). In conclusion, an additional short period of *in vitro* maturation, or an extended period of *in vivo* maturation are beneficial for *in vitro* embryo production in wood bison.

5.2. Introduction

Endemic diseases (i.e., brucellosis and tuberculosis) have infected wild wood bison (*Bison bison athabascae*) herds in Canada, and represent a risk to remaining healthy wood bison populations and neighbouring domestic livestock (Mitchell et al., 2000). Eradication of infected bison has been suggested (Environment Assessment Panel, 1990), but carries with it the risk of non-compensable loss of genetic diversity in this threatened population. In a recent report on the threat posed by the loss of genetic diversity, the use of reproductive technologies was

recommended as an effective strategy to preserve the genetic material (gametes and embryos) of wood bison (MacPhee et al., 2016).

In vitro production of embryos is one such technique for rescuing the genetics of wood bison. The technique has been developed successfully in cattle largely through the use of abattoir-derived ovaries (Sirard and Blondin, 1996). However, bison oocytes available for research are scarce, consequently few studies on *in vitro* embryo production using abattoir-derived ovaries have been reported, and have resulted in low blastocyst production (Thundathil et al., 2007; Aurini et al., 2009; Barfield and Seidel 2011). In cattle, collection of cumulus-oocyte complexes (COC) in live animals by transvaginal follicular aspiration has become an important source of genetic material for *in vitro* embryo production (Pieterse et al., 1991). Since *in vitro* culture conditions do not yet faithfully mimic the intrafollicular environment (Hunter, 1998), we developed a transvaginal collection technique in live wood bison as a method of harvesting oocytes for *in vitro* embryo production (Palomino et al., 2013, 2014a, 2014b). By using live wood bison as a source of oocytes, we now have an opportunity to optimize oocyte competence by inducing maturation *in vivo*.

In a recent study (Cervantes et al., 2016a), we found that nuclear maturation occurred more rapidly *in vitro* vs. *in vivo*, as has been reported in pigs (Motlik and Fulka, 1976), but was associated with lesser cumulus expansion than *in vivo* maturation. *In vivo* oocyte maturation was more complete at 30 h than 24 h after treatment of superstimulated wood bison with hCG; more than one-third of oocytes collected 30 h post-hCG were at the MII stage and had fully expanded cumulus cells. Another third of the oocytes had fully expanded cumulus cells suggesting that nuclear maturation to the MII stage was imminent. The results provided rationale for the hypothesis that additional maturation time is required for expanded wood bison COC at the MI stage to complete maturation. Whether expanded COC collected 30 h after hCG administration

can be used for immediate *in vitro* fertilization (IVF), or whether an additional period of maturation is beneficial for fertilization and embryo development, has not been investigated in wood bison.

The objectives of the present study were to determine the effect of an additional 4 h of *in vitro* maturation on embryo development of *in vivo* matured oocytes collected 30 h after hCG treatment (Experiment 1), and to determine if extending the interval between hCG treatment and COC collection from 30 to 34 h will improve *in vitro* embryo production (Experiment 2). The study also provided the opportunity to compare the effect of season (ovulatory vs. anovulatory) on the number of follicles ≥ 5 mm available for aspiration at the time of COC collection, the number of expanded COC collected after inducing *in vivo* maturation, and on the production of wood bison embryos *in vitro*.

5.3. Materials and methods

5.3.1. Animals

The study was performed with mature (6 to 11 years old) non-lactating female wood bison during the ovulatory season (September to November, Experiment 1; n = 24) and anovulatory season (April to May, Experiment 2; n = 28). The bison were part of the research herd maintained on pasture at the Native Hoofstock Centre, University of Saskatchewan. For the period extending from 10 days before the experiments to the end of the experiments, the bison were confined to corrals with free access to fresh water and alfalfa-brome grass hay to maintain an average body condition score of 3.5 (scale of 1 to 5; Vervaecke et al., 2005). The experimental protocol was approved by the University of Saskatchewan's Animal Research Ethics Board, and done in accordance with the guidelines of the Canadian Council on Animal Care.

5.3.2. Experiment 1: Additional in vitro maturation

Ovarian synchronization was induced among bison (n = 24) using an intramuscular dose of 500 µg cloprostenol (Estrumate, Merck Animal Health, Kirkland, Quebec, Canada) followed 8 days later by transvaginal ultrasound-guided aspiration of all follicles ≥5 mm in diameter (follicular ablation),as described previously (Palomino et al., 2014a). Briefly, for follicular aspiration, bison were restrained in a squeeze chute and caudal epidural anesthesia was induced by administration of 3–5mL of 2% lignocaine hydrochloride (Bimeda-MTC, Animal Health Inc., Cambridge, Ontario, Canada) between the first intercoccygeal joint. The vulva was washed with detergent and disinfectant before the transvaginal probe was introduced into the vagina and placed in the fornix. Follicular ablation was performed using a 5-MHz transvaginal probe (ALOKA SSD-900, Tokyo, Japan) equipped with a disposable 18-ga x 1 ½" needle (Vacutainer, BD, Mississauga, Ontario, Canada) attached to a 6 mL syringe by silicon tubing 60 cm long x 1.14 mm internal diameter. On the day after ablation (i.e., expected day of follicular wave emergence; Day 0), bison were treated intramuscularly with 300 mg of pFSH (Folltropin-V, Vetoquinol NA Inc., Lavaltrie, Québec, Canada) diluted in 0.5% hyaluronan (5 mg/ml, MAP-5, Vetoquinol NA Inc.) and an additional 100 mg pFSH in hyaluronan 2 days later, as described previously (Palomino et al., 2013). A luteolytic dose of 500 µg cloprostenol (Estrumate, Merck Animal Health, Kirkland, Quebec, Canada) was given on Day 3 and an intramuscular dose of 2500 IU of hCG (Chorulon, Merck Animal Health, Summit, NJ, USA) was administered on Day 4 to induce oocyte maturation *in vivo* (Fig. 1).

At 30 h after hCG treatment, COC were collected by transvaginal ultrasound-guided aspiration of all follicles ≥5 mm in diameter, as described (Palomino et al., 2013, 2014b). The COC were collected using a disposable 18-gax 2" short-bevel needle (Misawa Medical Industry Ltd., Edogawa-Ku, Tokyo, Japan) connected to a 50 ml conical Falcon tube via silastic tubing (internal diameter 1.14 mm; Cole Palmer, Montreal, Quebec, Canada), and a regulated vacuum pump set at

a flow-rate of 20 mL/min. The collection medium consisted of Dulbecco's phosphate buffered saline (D-PBS), 0.15% (vol/vol) ET Surfactant (Vetoquinol NA Inc.), and 200 IU/L of heparin (heparin sodium injection USP, Sandoz, Boucherville, Quebec, Canada). The follicular aspirate was poured through an embryo filter (Emcon filter; Agtech, Manhattan, Kansas, USA), and the COC were rinsed from the filter into a 90 mm Petri dish using collection medium without surfactant. The COC were located under a stereomicroscopy at 10x magnification, washed three times in holding medium (D-PBS + 5% calf serum), and morphologically classified according to the number of cumulus cell layers and the appearance of the oocyte cytoplasm. Compact COC were those with three or more layers of granulosa cells tightly surrounding the oocyte, expanded COC were those with expanded or partially dissociated cumulus cells, and denuded or degenerated oocytes were those without cumulus cells or with pyknotic cumulus cells and vacuolated ooplasm (Ratto et al., 2007).

The number of follicles ≥5 mm was determined on the day of COC collection by transrectal ultrasonography using a 7.5-MHz probe (MyLab5; Esaote, Ajax, Ontario, Canada). Treatments were scheduled so that collection of COC was done in four replicates (n=6 bison/replicate). Only expanded COC were used for the purposes of this experiment, and were pooled among bison in a given replicate. The pooled COC were divided randomly into two groups for either immediate fertilization or for additional *in vitro* maturation before fertilization.

5.3.3. In vitro maturation, fertilization & culture

All chemicals and reagents used for maturation, fertilization and embryo culture were purchased from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated below. Maturation medium consisted of TCM-199 (Gibco, Grand Island, NY, USA) supplemented with 5% (vol/vol) calf serum, 5µg/mL pLH (Lutropin-V; Vetoquinol NA Inc.), 0.5µg/mL (Folltropin-V; Vetoquinol

NA Inc.), and 0.05µg/mL gentamicin. In the additional *in vitro* maturation group, the expanded COC (n = 38) were washed three times in maturation medium, and incubated in four-well dishes (NUNC, Thermo Fisher Scientific, Rochester, NY, USA) containing 1 mL/well of maturation medium for 4 h at 38.5°C, 5% CO_2 in air and high humidity. After 4 h of maturation, the COC were submitted to the *in vitro* fertilization procedure.

Semen from two wood bison bulls, already frozen in Triladyl extender (Hussain et al., 2011), was thawed, pooled and used for *in vitro* fertilization of COC. Motile sperm were selected by placing pooled semen on a Percoll gradient (45% and 90%), as described previously (Parrish et al., 1995). Progressive motility (i.e, the percentage of spermatozoa that had directional movement) was 60 to 75% for all replicates, as assessed by conventional light microscopy at 400x magnification. Sperm were re-suspended to a final concentration of 5 x 10^6 sperm/mL in Brackett-Oliphant (BO) fertilization medium (Brackett and Oliphant, 1975). The COC were washed three times in BO medium supplemented with 10% BSA and transferred to 4-well dishes containing 500 uL/well of sperm suspension. The COC and sperm were co-incubated for 18 h at 38.5°C in 5% CO_2 in air and high humidity.

The presumptive zygotes were mechanically denuded of cumulus cells by gentle pipetting, then washed three times in *in vitro* culture medium (Charles Rosenkran's aminoacid [CR1aa] medium with 5% calf serum containing amino acids, L-glutamic acid, BSA and gentamicin; Prentice et al., 2011) and cultured in four-well dishes (n=8 to 11 zygotes/well) containing 500µL/well of *in vitro* culture medium at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2 and high humidity. The cleavage rate was recorded after 56 h of culture (Fig. 2). Embryos with ≥2 cells were separated from the unfertilized oocytes, placed in fresh *in vitro* culture medium and cultured to Day 8 (Day 0 = day of fertilization). Blastocyst formation was assessed on Days 7 and 8 (Fig. 5.3). Blastocysts were graded morphologically according to the grading system established by the

IETS for *in vivo*-derived embryos as Grade 1 (symmetrical and spherical cell mass, individual blastomeres uniform in size, color and density, and minor irregularities), Grade 2 (moderate irregularities in the overall shape of the embryonic mass, or in size, color, and density of individual cells), Grade 3 (major irregularities in shape of the embryonic mass, or in size, color, and density) and Grade 4 (degenerated/dead), as described (International Embryo transfer Society, 2010).

5.3.4. Experiment 2: Extending the in vivo maturation period

Ovarian follicular synchronization, superstimulation and hCG treatments were done in female wood bison (n = 28, 6 to 10 years old) as described in Experiment 1 (Fig. 5.1). The COC were collected transvaginally, as described in Experiment 1, but at either 30 h or 34 h after hCG treatment (n=14 bison per group). Expanded COC in the 30 h-group were fertilized after 4 h of *in vitro* maturation, while expanded COC in the 34 h-group were fertilized immediately. The number and size of follicles were determined on the day of hCG treatment and on the day of COC collection by transrectal ultrasonography. The experiment was done in four replicates (n = 7 bison per replicate), and expanded COC from the 30- and 34-h groups were pooled respectively. Presumptive zygotes were cultured *in vitro*, and embryos were assessed morphologically on Days 3, 7 and 8, as described in Experiment 1.

5.3.5. Statistical analyses

In both experiments, cleavage and blastocyst rates (based on the total number of COC used in each treatment group) were compared by Fisher's exact test or Chi-square test using Proc Frequency procedure *(SAS, Enterprise Guide 4.2, Statistical Analysis System Institute Inc., Cary, North Carolina, USA).*

In Experiment 2, the number and size of follicles available for aspiration (i.e., ≥5 mm) was compared between the 30 h and 34 h group by Student's t-test. Binomial data (COC collection rate and ovulation rate) were compared by 2-tailed Chi-square test, and the proportion of bison that ovulated was compared using Fisher's exact test. The occurrence of pre-collection ovulation was detected on the day of COC collection by the disappearance of follicles ≥10 mm that had been present in the ovaries on the day of hCG treatment. Values are expressed as a proportion or a mean ± SEM.

Notwithstanding a 5-month separation in time between Experiments 1 and 2, end points were compared between experiments to determine the effects of season. The 24 bison used in Experiment 1 were used in Experiment 2, but were assigned randomly (i.e., lottery method) to treatment groups in each experiment; i.e., no attempt was made to assign bison to the same group, and observations were considered independent from one experiment to the next. Data regarding the number of follicles ≥5 mm on the day of COC collection, total number of COC collected, number of expanded COC collected from the 30 h post-hCG groups were combined within season for seasonal comparison (ovulatory vs. anovulatory season) using Student's t-tests. Only data from the 30 h *in vivo* + 4 h *in vitro* groups in each experiment were used for seasonal comparisons of cleavage, morula, and blastocyst rates of embryo development, using Chi-square tests.

5.4. Results

5.4.1. Experiment 1: Additional in vitro maturation

One bison did not respond to superstimulatory treatment (i.e., no follicles ≥5 mm) and COC collection was not attempted; her data were not included in any of the statistical analyses. On the day of COC collection, the number of follicles ≥5 mm (i.e., aspiratable) was 10.9 ± 1.3 per bison, with a mean diameter of 8.6 ± 0.18 mm. The pre-collection ovulation rate (number of ovulations

detected on the day of collection/number of follicles≥10 mm on the day of hCG treatment) was 23/97 (23.7%). A total of 128 COC were collected from 228 follicular aspirations in 23 bison (56.1% collection rate, 5.6 COC per bison). Of the COC collected, 82 (64.1%) had an expanded cumulus layer and were used in the study. Six expanded COC were lost during washing and handling; therefore, 38 expanded COC were assigned to each group (30 h *in vivo* maturation, and 30 h *in vivo* + 4 h *in vitro* maturation) and submitted to *in vitro* fertilization. No effect of replicate was detected for any end point; therefore, data from the four replicates were combined for further analyses. The proportion of oocytes that underwent cell cleavage by 56 hours, and the proportion that developed to the morula and blastocyst stages on Day 7 did not differ between groups (Table 1). However, the proportion of COC that reached the blastocyst stage on Day 8 was greater in those exposed to 4 h of additional *in vitro* maturation than in those with no additional maturation time (Table 1).

On Days 7 and 8, the proportion of blastocysts at each developmental stage (early, mid-, expanded) was similar between the 30 h *in vivo* vs. 30 h *in vivo* + 4 h *in vitro* groups (Table 2). When data were combined between groups, no statistical difference was detected between Days 7 and 8 in the proportion of blastocysts at each stage (early, 12/14 [85.7%] vs. 14/24 [58.3%], P=0.15; mid-, 2/12 [16.7%] vs. 6/18 [33.3%], P=0.68; expanded, 0/14 [0%] vs. 4/20 [20%], P=0.27). Similarly, the proportion of blastocysts classified as Grade 1, 2 or 3 did not differ between groups; the majority of blastocysts were Grade 1 (Table 3).

5.4.2. Experiment 2: Extending the in vivo maturation period

The number of follicles ≥5 mm and ≥10 mm at the time of COC collection was numerically higher in the 30 h vs. 34 h group (19.0 ± 1.4 vs. 17.4 ± 2.4, and 9.5 ± 1.2 vs. 7.7 ± 1.8), and the average size of follicles ≥5 mm did not differ (9.9 ± 0.2 vs. 9.8 ± 0.2 mm). Similarly, the number

of follicles aspirated was numerically higher in the 30 h vs. 34 h group (16.4 ± 1.4 vs. 13.4 ± 2.1). The numerical difference between groups in the number of large follicles and the number of follicles aspirated was the result of a lower pre-collection ovulation rate (number of ovulations detected on the day of collection/number of follicles ≥10 mm on the day of hCG treatment) in the 30 h vs. 34 h group (12/89 [13.5%] vs. 47/147, [32.0%]; P = 0.003). Accordingly, the proportion of bison in which ovulation was detected was lower in the 30 h vs. 34 h group (3/14 vs. 10/14, P = 0.02). The COC collection rate was lower in the 30 h vs. 34 h group (64.3% vs. 78.2%; P = 0.003), but the total number of COC collected per bison was similar (10.6 ± 1.7 vs. 10.5 ± 1.5).

Of the total number of COC collected, the proportion that were expanded at the time of collection was similar between the 30 h and 34 h groups (104/147 [70.7%] and 92/148 [62.2%]. Eight expanded COC had a degenerated oocyte and were discarded, and 28 expanded COC were lost during searching, washing and handling. Therefore, the number of expanded COC submitted to *in vitro* fertilization was 86 and 74 in the 30 h- and 34 h-groups, respectively. Extending the *in vivo* maturation period from 30 h to 34 h was associated with an increase in the percentage of oocytes that cleaved, and that developed into blastocysts on Day 7 and Day 8 (P<0.05; Table 4), as well as the proportion that reached the expanded blastocyst stage on Day 8 (P<0.05; Table 5). The proportion of blastocysts classified as Grade 1, 2, or 3 did not differ between the two groups (Table 6). Although blastocyst development was not assessed critically after Day 8, hatching was detected on Day 9 in both groups (30 h, n=3 and 34 h, n=5; Fig. 4).

5.4.3. Seasonal comparison (ovulatory season vs. anovulatory season)

The number of follicles ≥5 mm on the day of COC collection was higher in bison during the anovulatory versus ovulatory season (P=0.0004), as was the total number of COC collected (P=0.001), the number of expanded COC collected (P=0.006), and the collection efficiency (P

=0.0003; Table 7). No effect of season was detected for any end point related to *in vitro* developmental capacity (Table 8). The mean number of blastocysts (total number of blastocysts at Day 8/number of bison) was 1.0 during the ovulatory season and 2.3 during the anovulatory season.

5.5. Discussion

In the present study, the developmental competence of *in vivo* matured COC collected 30 h after hCG treatment from superstimulated wood bison improved after an additional 4 h of *in vitro* maturation. Importantly, oocyte competence improved even further when the interval between hCG treatment and COC collection (*in vivo* maturation time) was extended from 30 to 34 h. To our knowledge, this is the first study to report the *in vitro* production of blastocysts from *in vivo* matured oocytes collected from live wood bison.

In Experiment 1, an additional 4 h of *in vitro* maturation beyond the 30 h of *in vivo* maturation had a positive effect on embryo development suggesting that a significant number of COC collected 30 h after hCG had not become developmentally competent. Similar findings have been reported in monkeys where 8 h of additional *in vitro* maturation allowed COC in MI stage to reach the mature MII stage (Lanzendorf et al., 1990), and in humans where an additional 2 to 11 h of *in vitro* maturation for COC in the MI stage increased their capacity to be fertilized and to develop to embryos (Vanhoutte et al., 2005; De Vos et al., 1999). In Experiment 2, extending the interval between hCG treatment and COC collection from 30 to 34 h (extending the period of *in vivo* maturation) was associated with a greater capacity to develop to the blastocyst stage following *in vitro* fertilization and culture. These findings agree with results in women where prolonging the interval between hCG and oocyte collection resulted in a greater number of fertilized oocytes and embryos (Bokal et al., 2005). Likewise, a greater cleavage rate was observed in oocytes collected from women when the interval between hCG treatment and oocyte collection was extended by 4

h (De Vits et al., 1994). A developmental advantage conferred by *in vivo* maturation vs. *in vitro* maturation has been reported previously in cattle (Rizos et al., 2002; Dieleman et al., 2002), and results of Experiment 2 in the current study in bison corroborates these findings; an extra 4 h of *in vivo* maturation was associated with a significant increase in embryo production compared to an extra 4 h of *in vitro* maturation.

The percentage of blastocyst development in the present study represents a notable advancement in *in vitro* embryo production in wood bison. The blastocyst rate (i.e., number of blastocysts/number of oocytes submitted to IVF) was 54% in the present study, in comparison to 8 to 16% in previous reports in bison (Thundathil et al., 2007; Aurini et al., 2009; Barfield and Seidel 2011). Differences may be attributed to the source of oocytes (live bison vs. abattoir-derived ovaries) and differences in culture conditions. The blastocyst rate from *in vivo*-matured oocytes in the present study is comparable to that reported in cattle (70%, Rizos et al., 2002; 41%, Dieleman et al., 2002; 52%, Hendriksen et al., 2000).

The success of pregnancy in other species has been related to the overall quality of the transferred embryo; i.e., chances of successful pregnancy are greater after transfer of high-quality embryos (Hasler et al., 1995; Scenna et al., 2008). Over 70% of blastocysts produced in the present study were Grade 1 (excellent or good). In cattle, embryos at the blastocyst stage graded as excellent and good (Grade 1) have yielded the highest pregnancy rates when transferred either fresh or even frozen-thawed (*in vitro*-produced embryos: Hasler et al., 1987; Hasler et al.,1995; *in vivo*-derived embryos: Scenna et al., 2008). Moreover, only Grade 1 embryos are selected for cryopreservation in most studies (Massip et al., 1995a; Nicacio et al., 2012; Romão et al., 2013) because of their greater cryo-tolerance (Han et al., 1994; Massip et al., 1995b; Gustafsoon et al., 2001). Embryo culture conditions have a critical effect on blastocyst quality (Negrin Pereira et al., 1997; Rizos et al., 2002; Lonergan et al., 2003a; Lonergan et al., 2003b). In particular, culture

media used for presumptive zygotes from Day 3 onwards affects blastocyst quality (Negrin Pereira et al., 1997).

The highest cleavage rate in the current study (60%) was similar to that reported for a study in plains bison (63%) involving the use of abattoir-derived ovaries (Barfield and Siedel, 2011), but was lower than that reported for other studies in wood bison (95%, Thundathil et al., 2007), plains bison (73%, Aurini et al., 2009; 72%; Barfield and Siedel 2011), and cattle (83%, Bordingon et al., 1997). Factors such as sperm preparation, sperm concentration, COC origin, oocyte maturation and culture media may account for the differences in cleavage rate among the studies. For instance, serum was not added to the *in vitro* culture media used in two previous studies (Thundathil et al., 2007; Aurini et al., 2009). It appears that the cleavage rate is a limiting factor for *in vitro* embryo production in wood bison, and future attempts to improve production efficiency may focus not only on maturational status, but on specific effects of culture conditions during fertilization.

There was no seasonal effect on the developmental capacity of oocytes in the present study; i.e., a similar percentage of COC developed into blastocysts during both the ovulatory and anovulatory seasons. However, the absolute number of embryos produced during the anovulatory season was more than double that of the ovulatory season. The numerical difference was attributed to a nearly two-fold increase in the number of follicles ≥5 mm at the time of COC collection and in the number of COC collected during the anovulatory season. The reason for a greater superstimulatory response during the anovulatory season is unknown, but may be related to a greater number of small follicles at the beginning of FSH treatment, as previously reported in bison (Palomino, 2015). In ewes, the number of follicles at wave emergence was significantly greater during the anovulatory season, and the total number of follicles present after progesterone sponge removal in superstimulated animals was nearly twice as high in anestrous compared to cyclic ewes (Barret et al., 2004). The mean number of COC collected per bison in the present study is consistent

with that of previous studies in superstimulated wood bison (Palomino et al., 2013; 2014b). The positive relationship between the number of follicles available for aspiration and the number of COC collected is well documented in cattle (Goodhand et al., 2000; Durocher et al., 2006).

In summary, mature oocytes collected 34 h after hCG treatment in superstimulated wood bison were more competent to develop into blastocysts following *in vitro* fertilization and culture than those collected 30 h after hCG treatment. An additional 4 h of *in vitro* maturation of oocytes collected 30 h after hCG treatment resulted in improved competence of oocytes to develop to the blastocyst stage, but to a lesser extent than an additional 4 h of *in vivo* maturation. *In vivo* matured oocytes are a valuable source of genetic material for *in vitro* production of wood bison embryos and offer an interesting alternative for the conservation of this species.

5.6. Acknowledgements

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Table 5.1. *In vitro* development of wood bison embryos produced from expanded cumulus-oocyte complexes collected at 30 h after hCG treatment and immediate fertilization (30 h *in vivo*) or following additional 4 h of *in vitro* maturation before fertilization (30 h *in vivo* + 4 h *in vitro*). Day 0= day of *in vitro* fertilization (Experiment 1, n=24 bison).

End point	30 h *in vivo* maturation	30 h *in vivo* + 4 h *in vitro* maturation
Oocytes submitted to *in vitro* fertilization (n)	38	38
Cleaved oocytes	21/38 (55.3%)	23/38 (60.5%)
Morulas on Day 7	15/38 (39.5%)	12/38 (31.6%)
Blastocysts on Day 7	5/38 (13.2%)	9/38 (23.7%)
Blastocysts on Day 8	7/38[a] (18.4%)	17/38[b] (44.7%)
Blastocysts on Day 8/cleaved embryos	7/21[a] (33.3%)	17/23[b] (73.9%)

[ab]Within rows, values with no common superscripts are different ($P<0.05$, Chi-square test)

Table 5.2. Blastocyst development of cultured wood bison zygotes produced from expanded cumulus-oocyte complexes collected at 30 h after hCG treatment and immediate fertilization (30 h *in vivo*) or following additional 4 h of *in vitro* maturation before fertilization (30 h *in vivo* + 4 h *in vitro*). Day 0= day of *in vitro* fertilization (Experiment 1, n=24 bison).

End point	30 h *in vivo* maturation	30 h *in vivo* + 4 h *in vitro* maturation
Blastocyst stage at Day 7		
Early blastocyst	5/38 (13.2%)	7/38 (18.4%)
Blastocyst	0/38 (0%)	2/38 (5.3%)
Expanded blastocyst	0/38 (0%)	0/38 (0%)
Blastocyst stage at Day 8		
Early blastocyst	4/38 (10.5%)	10/38 (26.3%)
Blastocyst	2/38 (5.3%)	4/38 (10.5%)
Expanded blastocyst	1/38 (2.6%)	3/38 (7.9%)

Percentages based on the number of cumulus-oocyte complexes submitted to *in vitro* fertilization

No significant differences were found between groups for any end point

Table 5.3. Morphological grading of wood bison blastocysts produced *in vitro* from expanded cumulus-oocyte complexes collected at 30 h after hCG treatment and immediate fertilization (30 h *in vivo*) or following additional 4 h of *in vitro* maturation before fertilization (30 h *in vivo* + 4 h *in vitro*). Day 0= day of *in vitro* fertilization (Experiment 1, n = 24 bison).

End point	30 h *in vivo* maturation	30 h *in vivo* + 4 h *in vitro* maturation
Blastocysts on Day 7 (n)	5	9
Grade 1	4/5 (80.0%)	8/9 (88.9%)
Grade 2	---	1/9 (11.1%)
Grade 3	1/5 (20.0%)	---
Blastocysts on Day 8 (n)	7	17
Grade 1	5/7 (71.4%)	12/17 (70.6%)
Grade 2	1/7 (14.3%)	3/17 (17.6%)
Grade 3	1/7 (14.3%)	2/17 (11.8%)

Grade 1: embryos with symmetrical and spherical cell mass, uniform blastomeres

Grade 2: embryos with moderate irregularities in size, shape, color and density of cell mass

Grade 3: embryos with major irregularities in size, shape, color and density of cell mass

No significant differences were found between groups for any end point

Table 5.4. *In vitro* development of expanded wood bison cumulus-oocyte complexes (COC) collected at 30 h after hCG treatment and matured for additional 4 h *in vitro* before fertilization (30 h *in vivo* + 4 h *in vitro*), or 34 h after hCG treatment and fertilized immediately (34 h *in vivo*). Day 0= day of *in vitro* fertilization (Experiment 2, n = 14 bison per group).

End point	30 h *in vivo* + 4 h *in vitro* maturation	34 h *in vivo* maturation
COC submitted to *in vitro* fertilization (n)	86	74
Cleavage	49/86[a] (57.0%)	55/74[b] (74.3%)
Morulas on Day 7	23/86 (26.7%)	18/74 (24.3%)
Blastocysts on Day 7	9/86[a] (10.5%)	25/74[b] (33.8%)
Blastocysts on Day 8	32/86[a] (37.2%)	40/74[b] (54.1%)
Blastocysts on Day 8/cleaved embryos	32/49 (65.3%)	40/55 (72.7%)

[ab]Within rows, values with different superscripts are different ($P<0.005$)

Table 5.5. Blastocyst development of cultured wood bison zygotes produced from expanded cumulus-oocyte complexes collected at 30 h after hCG treatment and matured for additional 4 h *in vitro* before fertilization (30 h *in vivo* + 4 h *in vitro*), or 34 h after hCG treatment and fertilized immediately (34 h *in vivo*). Day 0= day of *in vitro* fertilization (Experiment 2, n = 14 bison per group).

End point	30 h *in vivo* + 4 h *in vitro* maturation	34 h *in vivo* maturation
Blastocyst stage at Day 7		
Early blastocyst	9/86[a] (10.5%)	19/74[b] (25.7%)
Blastocyst	0/86 (0%)	3/74 (4.1%)
Expanded blastocyst	0/86 (0%)	3/74 (4.1%)
Blastocyst stage at Day 8		
Early blastocyst	14/86 (16.3%)	21/74 (28.4%)
Blastocyst	13/86 (15.1%)	5/74 (6.8%)
Expanded blastocyst	4/86[a] (4.7%)	14/74[b] (18.9%)
Hatched blastocyst	1/86 (1.2%)	0/74 (0%)

Percentages based on the number of cumulus-oocyte complexes submitted to *in vitro* fertilization

[ab]Within rows, values with different superscripts are different ($P<0.005$)

Table 5.6. Morphological grading of wood bison embryos produced *in vitro* from expanded cumulus-oocyte complexes collected at 30 h after hCG treatment and matured for additional 4 h *in vitro* before fertilization (30 h *in vivo* + 4 h *in vitro*), or 34 h after hCG treatment and fertilized immediately (34 h *in vivo*). Day 0= day of *in vitro* fertilization (Experiment 2, n = 14 bison per group).

End point	30 h *in vivo* + 4 h *in vitro* maturation	34 h *in vivo* maturation
Blastocysts on Day 7 (n)	9	25
Grade 1	6/9 (66.7%)	22/25 (88.0%)
Grade 2	3/9 (33.3%)	3/25 (12.0%)
Blastocysts on Day 8 (n)	32	40
Grade 1	22/32 (68.8%)	27/40 (67.5%)
Grade 2	7/32 (21.9%)	9/40 (22.5%)
Grade 3	3/32 (9.4%)	4/40 (10.0%)

Grade 1:embryos with symmetrical and spherical cell mass, uniform blastomeres
Grade 2: embryos with moderate irregularities in size, shape, color and density of cell mass
Grade 3: embryos with major irregularities in size, shape, color and density of cell mass
No significant differences were found between groups for any end point.

Table 5.7. Effect of season (ovulatory vs. anovulatory) on the ovarian response and collection efficiency of cumulus-oocyte complexes (COC) in superstimulated wood bison (mean ± SEM; Experiments 1 and 2).

End point	Ovulatory season	Anovulatory season
Bison (n)	24	28
Follicles ≥5 mm on day of COC collection (n)	10.8 ± 1.1 [a]	17.5 ± 1.3 [b]
Total COC collected (n)	5.6 ± 0.8 [a]	10.5 ± 1.3 [b]
Expanded COC collected (n)	3.6 ± 0.7 [a]	7.0 ± 0.9 [b]
Collection efficiency (COC/follicles aspirated)	128/228[a] (56.1%)	295/418[b] (70.6%)

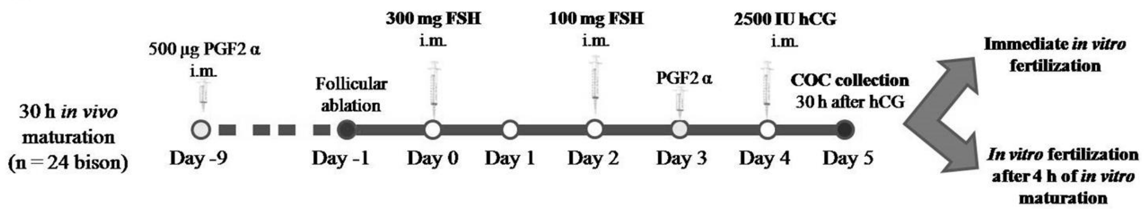
[ab]Within rows, values with different superscripts are different ($P<0.01$)

Table 5.8. Effect of season (ovulatory vs. anovulatory) on *in vitro* developmental capacity of wood bison embryos. The embryos were produced from oocytes collected from superstimulated bison 30 h after treatment with hCG and matured for an additional 4 h *in vitro* (30 h *in vivo* + 4 h *in vitro* maturation; Experiments 1 and 2).

End point	Ovulatory season	Anovulatory season	Total
Bison (n)	12	14	26
Cleavage rate	23/38 (60.5%)	49/86(57.0%)	72/124 (58.1%)
Morula rate at Day	12/38 (31.6%)	23/86 (26.7%)	35/124 (28.2%)
Blastocyst rate at Day 7	9/38 (23.7%)	9/86 (10.5%)	18/124 (14.5%)
Blastocyst rate at Day 8	17/38 (44.7%)	32/86(37.2%)	49/124 (39.5%)

No significant differences were found between seasons for any end point

Experiment 1



Experiment 2

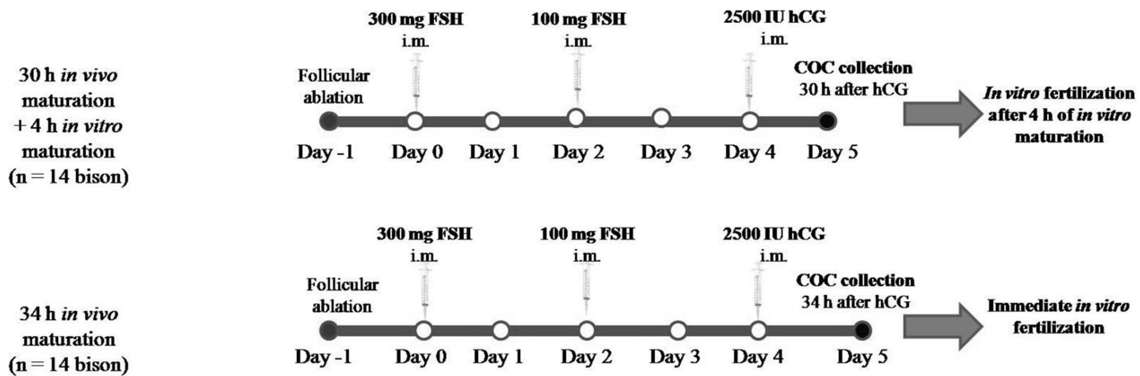


Fig. 5.1. Experimental design including the superstimulation protocol for the purpose of *in vitro* embryo production in wood bison to compare the developmental competence of the cumulus-oocyte complexes (COC) collected after inducing *in vivo* oocyte maturation with hCG (COC collected 30 h or 34 h after treating bison with 2500 IU hCG). The main difference in treatment of bison between Experiment 1 (ovulatory season) and 2 (anovulatory season) was a luteolytic dose of prostaglandin F2α given on Day -9 and Day 3 to control the corpus luteum on the ovulatory season.

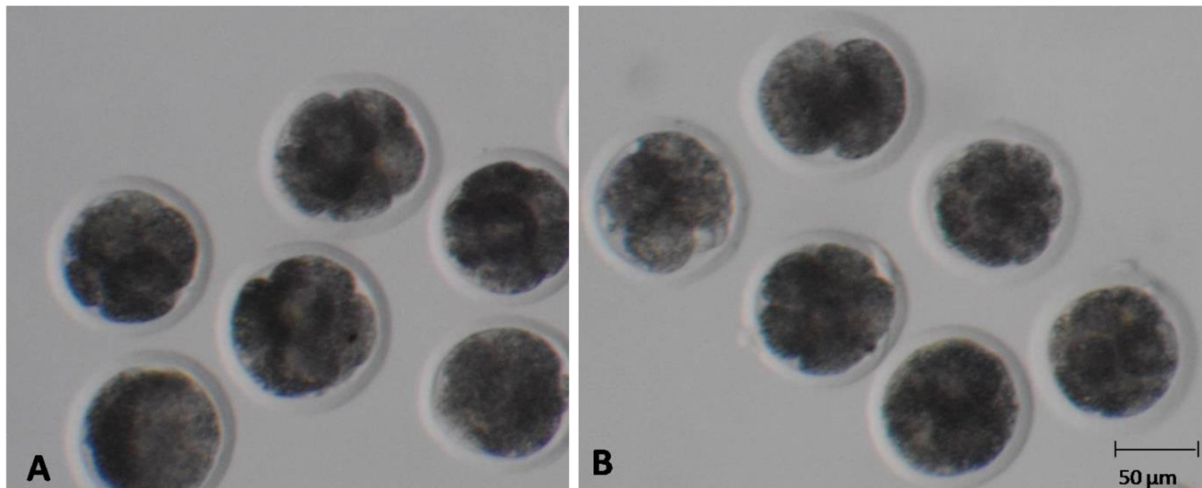


Fig. 5.2. *In vitro*-produced wood bison embryos after 56 h of *in vitro* culture. A) COC were collected 30 h after hCG treatment in superstimulated bison, and matured for an additional 4 h *in vitro* before fertilization (30 h *in vivo*+ 4 h *in vitro*),B) COC collected 34 h after hCG and immediate fertilization (34 h *in vivo*).

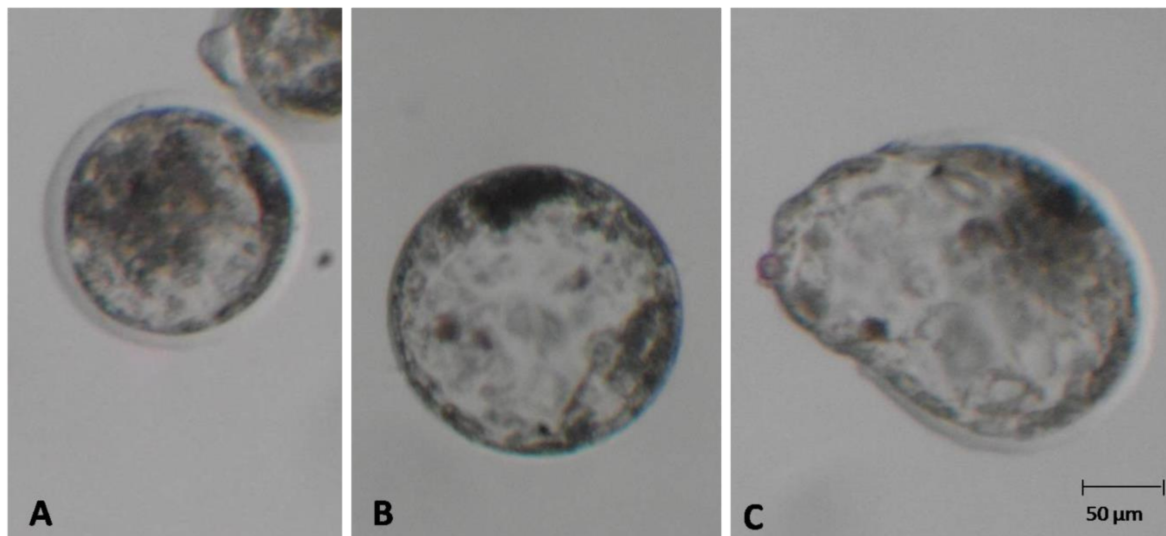


Fig. 5.3. Day 8 *in vitro*-produced wood bison embryos from *in vivo* matured oocytes at the A) blastocyst stage, B) expanded blastocyst stage, and C) hatching blastocyst stage (Day 0=day of *in vitro* fertilization).

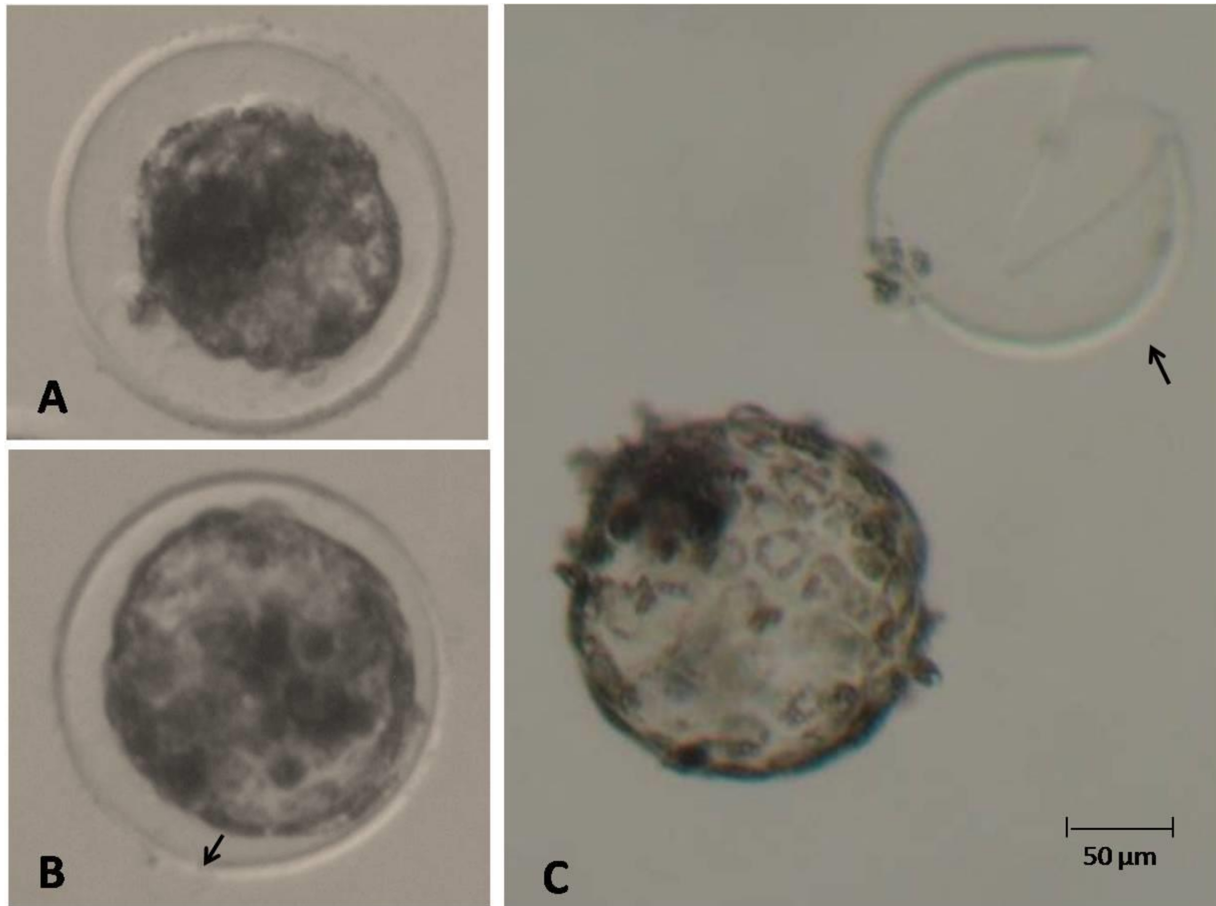


Fig. 5.4. *In vitro*-produced wood bison embryos at blastocyst stage that had *collapsed* (A)*, re-expanded (B),*and hatched (C) on Day 9 of *in vitro* culture (D0=day of *in vitro* fertilization). The arrow points to an opening in the zona pellucida (B) and the empty zona pellucida (C).

CHAPTER 6

EFFICACY OF WASHING PROCEDURES FOR REMOVING BRUCELLA ORGANISMS FROM *IN VITRO*-PRODUCED WOOD BISON (*BISON BISON ATHABASCAE*) EMBRYOS

6.1. Abstract

The primary objective was to determine the effectiveness of washing procedures for removing *Brucella* bacteria from *in vitro*-produced wood bison embryos; a secondary objective was to compare the ovarian response to two superstimulation protocols. Follicle wave emergence among bison was synchronized during the anovulatory season by follicular ablation. One day later, bison were assigned randomly to groups (14 bison/group) and given either a constant or decreasing two-dose regimen of FSH. Bison were given hCG one day after the last FSH treatment to induce *in vivo* maturation of oocytes. The ovarian response was evaluated by ultrasonography on the day of collection of cumulus-oocyte complexes (COC), 34 h after hCG treatment. Expanded COC were fertilized *in vitro* immediately (Day 0) and cultured *in vitro* for 7 days. Zona pellucida-intact embryos were transported in holding medium to a biocontainment Level 3 laboratory and placed in a minimum of two 30 mm Petri dishes (≤ 10 embryos/dish) containing 2.7 mL of holding medium. *Brucella abortus biovar 1* (approximately $1x10^9$ CFU/mL in 0.3 mL) was added to each dish and incubated for 2 h at 37ºC in 8% CO_2. In each replicate, a sample of the holding medium was cultured for bacterial growth before (negative control) and after incubation (positive control). After incubation, embryos were subjected to a 10-step washing procedure using medium (PBS + 0.4% BSA) without antibiotics or with antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin). A sample of medium was cultured at wash steps 1, 3, 6, and 9. After the tenth wash, embryos were cultured individually after breaking the zona pellucida. No differences were detected between the constant vs. decreasing superstimulatory treatment protocols in the number of follicles ≥5 mm at the time of COC collection (17.1±1.7 vs 15.1±1.6) or the number of COC collected per bison (10.1±1.9 vs 7.6±1.0). A total of 128 expanded COC were submitted to *in vitro* fertilization and culture, and 84 embryos were used to test the effectiveness of washing procedures with or without antibiotics (n=42/wash group). *Brucella abortus* was not detected in media after

the third wash in either wash group. All embryos were culture-negative (0/84). In summary, the constant and decreasing two-dose treatment regimens of FSH induced a similar ovarian response and COC collection rate, and *Brucella abortus* was removed from 100% of *in vitro*-produced embryos washed either with or without antibiotics.

6.2. Introduction

Brucella abortus is a facultative, intracellular, gram-negative bacteria with marked affinity for reproductive organs in domestic animals (Poester et al., 2013). *Brucella abortus* causes brucellosis in cattle, but other species, including humans and wildlife, can also contract the disease and play a role in brucellosis persistence and transmission (Diaz Aparicio, 2013). Bison and elk are considered reservoirs for the disease in North America (Rhyan et al., 2013). Brucellosis is typically associated with reproductive losses (abortions, stillbirths, calf mortality) and reduced fertility, resulting in potentially devastating economic effects on livestock producers (McDermott et al 2013). *Brucella* is transmitted from animal to animal by contact following an abortion, or through contaminated pastures by ingestion, inhalation, skin contamination or conjunctival inoculation (Corbel 2006).

Wood bison (*Bison bison athabascae*) are a species listed as Threatened under Schedule I of the Canadian Species at Risk Act (SARA; Environment and Climate Change Canada, 2016), and 10 of the 12 free-ranging herds of wood bison in Canada are endemically infected with bovine brucellosis. The population in Wood Buffalo National Park in Canada represents the largest, most genetically diverse reserve of wood bison in the world (McCormack, 1992; McFarlane et al., 2006), but has an on-going disease prevalence of 30-40% for brucellosis and tuberculosis (Environment and Climate Change Canada, 2016). Diseased herds in the Park are a reservoir for

infection of healthy bison and other species of animals in and around the park (McFarlane et al., 2006; Government of Alberta, 2013).

Reproductive technologies are being investigated as a method to preserve the genetic material of the threatened Canadian wood bison population and to mitigate disease transmission. Proposed techniques for rescuing the genetics of wood bison include the production of embryos after *in vivo* or *in vitro* fertilization (Toosi et al. 2013; Palomino et al., 2016; Cervantes et al. 2016b). Embryos produced using reproductive technologies may become a valuable source of genetic diversity for the purposes of conservation and restoration of threatened populations. For instance, disease-free *in vitro*-produced embryos transferred to healthy bison recipients may produce healthy individuals to be reintroduced into the wild.

The International Embryo Transfer Society (IETS) has described procedures for handling and disinfecting potentially infected embryos to reduce the risk of disease transmission when using the embryo transfer technology (Stringfellow and Givens, 2010). *In vivo*-derived embryos with intact ZP collected from infected donors, were transferred safely after proper washing procedures in cattle (Hare et al., 1985; Acree et al., 1993) and sheep (Venter et al., 2011). There is, however, evidence that some pathogens are not removed with the washing procedures from infected embryos of pigs (Bielanski et al., 2004) and goats (Oseikria et al., 2016). Furthermore, the washing procedures may not be as effective in disinfection of *in vitro*-produced embryos (Bielanski, 2007). For instance, certain pathogens (e.g., bovine viral diarrhea virus) have persisted on *in vitro*-produced bovine embryos despite washing (Stringfellow and Givens, 2000). Hence, the efficacy of washing procedures involving a specific pathogen or a particular species may not be extrapolated to different pathogens or species (Stringfellow and Wrathall, 1995).

Effective disinfection of *in vivo*-produced embryos exposed *in vitro* to *Brucella abortus* has been reported in cattle (Stringfellow et al., 1986) and more recently in wood bison (Palomino et

al., 2015b). In the latter study where embryos were exposed *in vitro* to *Brucella abortus biovar 1*, washing procedures removed *Brucella* from 89% vs. 100% of embryos using medium without vs. with antibiotics. Whether these procedures are effective in disinfecting *in vitro*-produced wood bison embryos has not been investigated.

The objective of the present study was to test the hypothesis that embryo washing procedures are effective for removing *Brucella* bacteria from *in vitro*-produced wood bison embryos. The study was designed to determine the effectiveness of washing procedures with vs. without antibiotics for removing *Brucella abortus* from zona pellucida-intact, *in vitro*-produced wood bison embryos previously exposed *in vitro* to the bacteria. The experiment also provided the opportunity to compare the ovarian superstimulatory response to two different FSH treatment protocols.

6.3. Materials and methods

6.3.1. Ovarian superstimulation

The study was performed with healthy wood bison cows (n = 28) from the Native Hoofstock Centre, University of Saskatchewan, Saskatoon, during May to June (anovulatory season). Ovarian follicular wave emergence was synchronized among bison by transvaginal ultrasound-guided aspiration of all follicles ≥5 mm in diameter (follicular ablation), as described previously (Palomino et al., 2014a). On the day after ablation (i.e., day of follicular wave emergence), bison were assigned randomly (lottery method) to 2 superstimulatory groups (n=14 bison per group). Superstimulatory treatment involved intramuscularly administration of two doses of NIH-FSH-P1 (Folltropin-V, Vetoquinol Canada Inc., Lavaltrie, QC, Canada) diluted in 10 mL of 0.5% hyaluronan (5 mg/mL, MAP-5, Vetoquinol Canada Inc.) given at an interval of 48 h beginning on the day after ablation. Bison in the respective groups were given either a constant dose (200 mg

and 200 mg) or a decreasing dose (300 mg and 100 mg). A single dose of 2500 IU of hCG (Chorulon, Merck Animal Health, Summit, NJ, USA) was given intramuscularly 48 h after the last dose of FSH to induce oocyte maturation *in vivo*. (Fig. 6.1). The number of follicles ≥5 mm was determined on the day of oocyte collection by transrectal ultrasonography using a 7.5-MHz probe (MyLab 5 VET, Esaote NA, IN, USA). The experimental protocol was approved by the University of Saskatchewan's Animal Research Ethics Board, and done in accordance with the guidelines of the Canadian Council on Animal Care.

6.3.2. Oocyte collection and handling

Cumulus-oocyte complexes (COC) were collected 34 h after hCG treatment in 4 replicates (n=7 bison/replicate) by transvaginal follicular aspiration, as reported previously (Cervantes et al., 2016b). Follicular fluid was collected in 50 mL conical Falcon tubes via silastic tubing (internal diameter 1.14 mm; Cole Palmer, Montreal, Quebec, Canada) connected to a regulated vacuum pump set at a flow-rate of 20 mL/min. The aspiration medium consisted of Dulbecco's phosphate buffered saline (D-PBS) supplemented with 0.15% ET Surfactant (Vetoquinol NA Inc.), and 200 IU/L of heparin (heparin sodium injection USP, Sandoz, Boucherville, Quebec, Canada). The follicular aspirate was poured through an ova/embryo filter (Emcon filter; Agtech, Manhattan, Kansas, USA), and the COC were rinsed from the filter into a 90 mm Petri dish using aspiration medium without surfactant. The COC were identified and classified under stereomicroscopy at 10X magnification according to the characteristics of cumulus cell layers and the appearance of the oocyte cytoplasm, as previously described (Ratto et al. 2007). Only expanded COC (those with expanded or partially dissociated cumulus cells) were used for further processing.

6.3.3. In vitro fertilization and culture

Procedures for *in vitro* fertilization and embryo culture were as previously described (Cervantes et al., 2016b). Briefly, expanded COC were fertilized with motile sperm from two bulls (pooled semen) selected through Percoll gradient (45% and 90%, Parrish et al. 1995) and resuspended at a final concentration of 5 x 10^6 sperm⁄mL in Brackett-Oliphant (BO) fertilization medium (Brackett and Oliphant, 1975). Oocytes and sperm were co-incubated for 18 h at 38.5°C in 5% CO_2 in air and high humidity. The presumptive zygotes were mechanically denuded of cumulus cells by gentle pipetting, then washed three times in *in vitro* culture medium (CR1aa with 5% calf serum containing amino acids, L-Glutamic acid, BSA and gentamicin; Prentice et al. 2011) and cultured in four-well dishes (n=8 to 11 zygotes/well) containing 500 µL/well of IVC medium at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2 and high humidity.

After seven days in culture, zona pelucida (ZP)-intact embryos (from the 8-cell stage to blastocyst stage) were pooled, then divided as equally as possible into two groups and transported from the IVF laboratory to the biocontainment Level 3 laboratory in 2 mL graduated plastic cryo-vials containing holding medium (D-PBS + 2% calf serum).

6..3.4. Preparation of Brucella suspension and embryo exposure

All procedures involving *Brucella* were performed in a biocontainment Level 3 laboratory facility (Intervac/VIDO, University of Saskatchewan) and, unless otherwise stated, sample preparation was performed within a class II biosafety cabinet. *Brucella abortus biovar 1* (most common strain of *Brucella abortus* isolated from bison found dead in and around WBNP [Tessaro et al., 1990]) was obtained from the Canada Food Inspection Agency (Ottawa Fallowfield Laboratory) and processed, as previously described (Palomino, 2015). Briefly, the bacteria were streaked on a 5% sheep blood agar plate (Fisher Scientific, #OXMP0105) and incubated at 37ºC in 8% CO_2 for at least 48 h. Growth from the Brucella agar plate was transferred a sterile 50 mL

tube with 5 mL of embryo holding medium. The *Brucella* suspension was diluted with holding medium to a concentration of approximately $1x10^9$ CFU/mL in 0.3mL.

For each of 4 replicates, embryos (n= 12 to 30/replicate) were transferred from the cryo-vials into a minimum of two 30 mm Petri dishes (≤10 embryos per dish) containing 2.7 mL of holding medium. The *Brucella* suspension (approximately $1x10^9$ CFU/mL in 0.3mL) was added to each dish and incubated with the embryos for 2 h at 37ºC in 8% CO_2. In each replicate, a sample of embryo holding medium was taken before and after incubation with *Brucella* for bacterial culture as negative and positive controls .

6.3.5. Embryo washing and bacterial culturing procedures

After incubation, embryos from the two Petri dishes were subjected to a 10-step washing procedure under stereomicroscopy according to the IETS guidelines (Stringfellow and Givens, 2010) using wash medium (PBS + 0.4% BSA) without antibiotics or with antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin, as reported previously (Palomino et al., 2015b). In brief, embryos (≤10/wash) were gently drawn from the Petri dish in a total volume of 15 µL of medium using a 20 µL pipette and placed into the first well of a 6-well dish (Partnar Animal Health, Ilderton, Ontario, Canada) containing 1.5 mL/well of holding (wash) medium (Dilution 1:100). Embryos and wash medium were gently drawn in and out of the pipette several times over a period of 10 seconds. Using a new pipette containing 5 µL of fresh wash medium from the second well, the embryos were transferred to the second well and washed as described in the first well. The process was repeated to the 10th well in a second 6-well dish. A sample (100 µL) of wash medium from wash-steps 1, 3, 6, and 9 were submitted for bacterial culture. After the 10th and final wash, each embryo was cultured individually for *Brucella* organisms after ZP breakage, as reported previously (Palomino et al., 2015b). In a 100 µL drop of wash medium, the ZP of each embryo

was physically ruptured using a glass pipette with a narrow tip, and the entire drop (100 µL) containing the broken ZP and embryo was cultured.

Brucella growth was determined by culturing the samples in a commercial *Brucella* agar plate with 5% sheep blood (Fisher Scientific #OXMP0105), as described (Palomino, 2015). Briefly, by using a sterile loop carrying the sample (i.e. holding medium, wash medium, embryos), the first third of the plate was streaked. The other two thirds were streaked in the conventional manner by dragging the loop once through the previous streak and across the surface of the non-streaked agar back and forth in a zigzag motion. Plates were incubated at 37ºC in 8% CO_2 for 5 days and examined. *Brucella* colonies visible as punctuate, non-pigmentated and non-hemolytic colonies (Dahouk et al., 2009) were determined and counted.

6.3.6. Statistical analyses

The number of follicles ≥5 mm at the time of COC collection, number of COC collected, and number of COC of different morphological characteristics were compared between the two superstimulatory treatment groups by Student's t-test. The proportion of *Brucella*-positive samples from bacterial culture of wash medium and embryos was compared between wash treatment groups (with or without antibiotics) by 2-tailed Fisher's exact test using Proc Frequency procedure (SAS, Enterprise Guide 4.2, Statistical Analysis System Institute Inc., Cary, North Carolina, USA). Values are expressed as a proportion or mean ± SEM, and P-values of <0.05 were considered significant.

6.4. Results

The number of follicles ≥5 mm at the time of COC collection, and the number of COC collected per bison were similar between the two superstimulatory FSH treatments groups (constant dose, 200 mg-200 mg vs. decreasing dose, 300 mg-100 mg), as was the number of COC in each morphological category (Table 6.1). A total of 249 COC, of which 139 were expanded, were collected from 28 superstimulated wood bison cows. Of the expanded COC, 76 (54.7%) were fully expanded and the remainder were partially expanded. Fully and partially expanded COC were used for *in vitro* production of embryos. Eleven of the COC were lost during transport and processing, thus, 128 COC were submitted to *in vitro* fertilization and culture.

A total of 84 ZP-intact *in vitro*-produced embryos were used for *Brucella* infection and washing procedures. The stage of embryo development and their distribution in each group (wash medium with and without antibiotics) are presented in Table 6.2.

Results of *Brucella* culture are shown in Table 6.3. No bacterial colony-forming units (CFU) grew in the negative control samples from either treatment group (wash medium with or without antibiotics), and all positive control samples in both treatment groups were positive for *Brucella*. All wash media samples up to and including the third wash were positive for *Brucella* in both treatment groups (with or without antibiotics), while wash samples four to ten were negative for the pathogen. Following 10 washes and ZP breakage, none of the embryos (0/84) were positive for *Brucella*. No differences were found between treatment groups for any endpoint.

6.5. Discussion

Results of the present study support the hypothesis that embryo washing procedures are effective for removing *Brucella* bacteria from *in vitro*-produced wood bison embryos exposed *in*

vitro to the pathogen. *Brucella* was not isolated from any of the embryos (0/84) after washing with medium with or without antibiotics. Others have reported similar findings for *in vivo*-derived wood bison (Palomino, 2015), pigs (Jacques et al., 2004) and cattle (Stringfellow et al., 1984) embryos. In the present study, *Brucella* was not isolated from the wash medium after the third wash, and the successive washing steps may have eliminated the bacteria or reduced the bacterial numbers to undetectable levels, as suggested previously (Bielanski, 2007).

Although Canada is considered free of brucellosis in domestic animals (Diaz Aparicio, 2013), bison herds in the Wood Buffalo National Park area are infected endemically with the disease (Mitchell and Gates, 2002). Evidence exists for the ability of *Brucella abortus biovar 1* to be transmitted to healthy *Bos taurus* cattle (Forbes and Tessaro, 1996); hence, there is a risk of the spread of brucellosis from infected bison to cattle as well as other healthy bison herds in Canada (Tessaro et al., 1990). Transmission of brucellosis from captive bison to cattle was reported in North Dakota, USA (Flagg 1983 cited by National Research Council, 1998), but no reports yet on direct transmissions of brucellosis from wild bison to cattle (Kamath et al., 2016). There are, however, evidence of transmission of brucellosis from elk to cattle (Rhyan et al., 2013; The Western Producer, 2014) and bison (Rhyan et al., 2013), as well as from bison to elk (Kamath et al., 2016).

Producing disease-free embryos in the laboratory is an important advancement for future control of disease transmission in this species primarily because processing procedures for *in vitro*-produced embryos in an established laboratory with competent personnel provides biosecurity measures appropriate for embryo production (Thibier, 2010). However, whether offspring resulting from transfer of *in vitro*-produced, disinfected wood bison embryos are free of Brucellosis remains to be tested.

These findings are important, as other researchers have demonstrated that washing procedures do not compromise survival and development of embryos from human and farm animals (Bielanski, 2007). For instance, when *in vivo*-produced bovine embryos contaminated with bovine viral diarrhea virus (BVDV) were washed according to the recommendation of the IETS, and then transferred to disease-free recipients, the resulting calves and recipients were free of BVDV and did not seroconvert after 2 years (Bielanski et al., 2013). Nevertheless, disinfection procedures are considered less effective in removing viruses from *in vitro*- vs. *in vivo*-derived embryos in cattle (Bielanski et al., 1997; Bielanski et al., 1998; D'angelo et al., 2009). One suggested reason for the lower washing efficiency is that viruses may adhere more readily to the ZP of *in vitro*-produced embryos (Bielanski et al., 2003) because of the differences in ZP ultrastructure in *in vitro* compared to *in vivo*-derived embryos (Vanroose et al., 2000). In a recent study in wood bison (Roberts et al., unpublished data), the ultrastructure of wood bison embryos produced by *in vitro* maturation and fertilization was associated with a significant increase in porosity and surface complexity compared to *in vivo*-derived embryos. It appears, however, that *Brucella abortus* does not adhere to the ZP of bovine embryos (reviewed in Stringfellow and Wright, 1989), although the number of studies using bacterial pathogens and *in vitro*-produced embryos is very limited (Perry et al., 2006).

In the present study, penicillin (100 IU/mL) and streptomycin sulfate (100 mg/mL) were used in the washing medium as recommended by the IETS guidelines (Stringfellow and Givens, 2010), and the washing medium with antibiotics disinfected 100% of *in vitro*-produced embryos exposed to *Brucella abortus.* Similar results were reported previously for *in vivo*-derived embryos from wood bison (Palomino, 2015) and cattle (Stringfellow et al., 1984). These findings confirm that the addition of antibiotics in the washing medium assures that embryos will be bacterial pathogen-free (Bielanski, 2007; Stringfellow and Givens, 2000).

In the present study, the ovarian response in supertimulated wood bison cows was similar for both FSH superstimulatory protocols. The constant-dose regimen of 200 mg or decreasing-dose regimen of 300 mg and 100 mg of pFSH did not affect the number of ovarian follicles ≥5 mm available for aspiration or the number of COC collected, nor did it influence the number of COC in each morphological category. Whether administration of a higher or lower total dose of FSH would be beneficial in wood bison for increasing the follicular response and the number of COC collected remains to be investigated.

We conclude that embryo washing procedures are effective in removing *Brucella* from *in vitro*-produced bison embryos exposed *in vitro* to the pathogen. Results suggest that washing procedures may be an effective strategy for preventing *Brucella* transmission by wood bison embryos produced *in vitro* and will reduce the disease risks associated with the transfer of *in vitro*-produced bison embryos. Whether the transfer of disinfected *in vitro*-produced bison embryos to healthy recipients will result in the birth of disease-free wood bison calves remains to be determined.

6.6. Acknowledgements

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Table 6.1. Morphologic characteristics (mean ± SEM per bison) of cumulus-oocyte complexes (COC) at the time of collection after inducing *in vivo* maturation (i.e., collected 34 h after hCG treatment) from wood bison superstimulated with two different protocols (constant and decreasing doses of FSH).

End point[1]	Constant (200 mg-200 mg)	Decreasing (300 mg-100 mg)
Number of bison	14	14
Number of follicles ≥ 5 mm[2]	17.1±1.7	15.1±1.6
COC collected per bison	10.1±1.9	7.6±1.0
Compact COC	2.0±0.6	1.3±0.4
Expanded COC	5.3±0.9	4.6±0.9
Denuded COC	1.9±1.0	0.9±0.3
Degenerated COC	0.7±0.4	0.6±0.3

[1] No differences were detected for any endpoint.

[2] On day of COC collection

Table 6.2. *In vitro*-produced embryos from *in vivo*-matured cumulus-oocyte complexes (COC) collected from superstimulated wood bison and their distribution based on experimental replicate (I to IV), developmental stage, and wash group (with vs without antibiotics). Each replicate involved COC collection and in vitro embryo production from 7 superstimulated wood bison (n=28 bison). Embryos were used on Day 7 (Day 0 = day of *in vitro* fertilization).

	Zona pellucida-intact embryos							
	Washed without antibiotics				Washed with antibiotics			
Replicate	Early[1]	Morula	Blastocyst	Total	Early[1]	Morula	Blastocyst	Total
I	2	4	4	10	1	5	4	10
II	1	5		6	1	5		6
III	4	7	4	15	4	6	5	15
IV	1	6	4	11	2	5	4	11
Total	8	22	12	42	8	21	13	42

[1]Early stage: 8 - 16 cells

Table 6.3. *Brucella abortus biovar 1* isolation from wash media and *in vitro*-produced wood bison embryos exposed to the bacteria *in vitro*. Each replicate involved COC collection and in vitro embryo production from 7 superstimulated wood bison (n=28 bison). Embryos were used on Day 7 (Day 0 = day of *in vitro* fertilization).

Replicate	Exposure dose of *Brucella* (CFU/mL)	Wash 1	Wash 3	Wash 6	Wash 9	Washed embryos	Number of culture-positive embryos
Wash medium without antibiotics							
I	6.1×10^6	P	P	N	N	10	0
II	2.1×10^7	P	P	N	N	6	0
III	2.7×10^7	P	P	N	N	15	0
IV	2.5×10^7	P	P	N	N	11	0
Total						42	0
Wash medium with antibiotics[1]							
I	6.5×10^6	P	P	N	N	10	0
II	2.6×10^7	P	P	N	N	6	0
III	2.8×10^7	P	P	N	N	15	0
IV	3.0×10^7	P	P	N	N	11	0
Total						42	0

CFU = Colony-forming units
P = Sample positive for *Brucella* bacteria after culturing.
N = Sample negative for *Brucella* bacteria after culturing.
[1]Antibiotics: 100 IU/mL penicillin and 100 μg/mL streptomycin sulfate

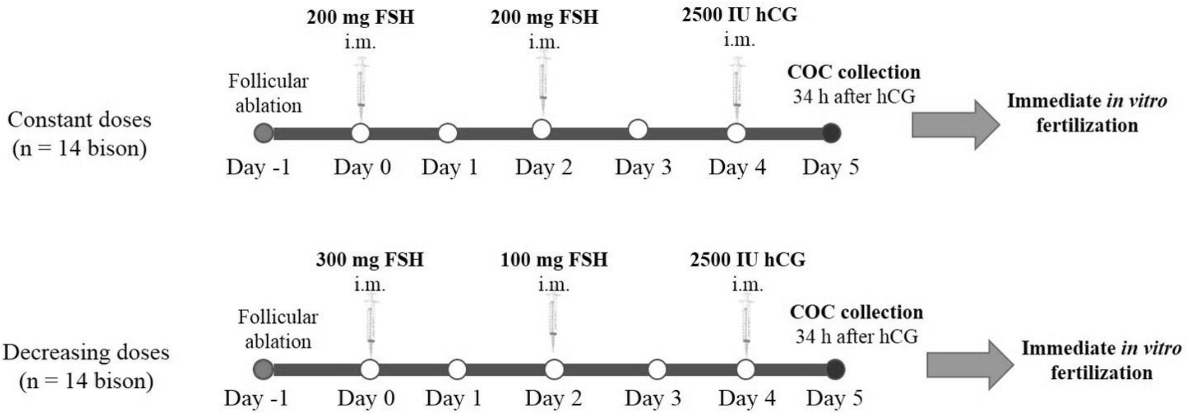

Fig. 6.1. Experimental design including the two superstimulatory protocols used for the purpose of *in vitro* embryo production in wood bison. The superstimulatory protocols differ in that the total dose of 400 mg FSH was administered in either two constant doses of 200 mg each or two decreasing doses of 300 mg and 100 mg in 0.5% hyaluronan.

CHAPTER 7. GENERAL DICUSSION

The main objective of this thesis was to develop efficient protocols for the collection of competent oocytes to produce wood bison embryos following *in vitro* fertilization and culture, and for the disinfection of these embryos from *Brucella abortus*. Few researchers have reported on successful *in vitro* production of embryos (IVP) in bison, and the blastocyst development rates have been low. Likewise, there are no data related to the factors influencing the development of *in vitro*-produced bison embryos. Therefore, investigation of each of the steps involved in bison IVP will importantly contribute to improve the efficiency of this technology, and subsequently make IVP a reliable tool for bison conservation. In other species, maturation of oocytes under *in vitro* conditions has been shown to have limitations. Further, these studies have indicated that oocytes matured *in vivo* have higher developmental competence than those matured *in vitro* (Rizos et al., 2002; Dieleman et al., 2002). Therefore, the capacity of wood bison oocytes to mature under *in vitro* or *in vivo* conditions and their subsequent developmental potential, were investigated. The objective of this work was to generate information to assess the potential for IVP in bison conservation efforts. In addition, washing techniques to disinfect diseased *in vitro*-produced bison embryos were investigated to provide evidence about the utility of these techniques in the prevention of disease transmission via embryo transfer.

Four studies were conducted in the present thesis to test whether the improvement of the competence of oocytes collected from superstimulated bison will enhance the production of *in vitro*-produced wood bison embryos. The studies of the present thesis were performed using cumulus-oocyte complexes (COC) collected by transvaginal ultrasound guided follicular aspiration from live wood bison. Bison were superstimulated with two doses of follicle stimulating hormone (FSH) in hyaluronan after synchronizing the emergence of a new follicular wave by follicular ablation. The synchronization, superstimulation and COC collection procedures were

based on protocols described in previous studies in wood bison (Palomino et al., 2013; Palomino et al., 2014a) and allowed us to obtain the oocytes from the wood bison captive herd maintained at the Western College of Veterinary Medicine's Native Hoofstock Centre. Results of the four studies (Chapters 3 to 6) of this thesis will be summarized and discussed in this Chapter (Chapter 7).

The experiments described in Chapter 3 were performed to: 1) determine the optimal interval of time after human chorionic gonadotropin (hCG) treatment required for *in vivo* oocyte maturation in wood bison, 2) compare the maturational characteristics of COC after *in vitro* vs. *in vivo* maturation, and 3) compare the maturational capacity of bison oocytes collected during the anovulatory vs. ovulatory seasons. In cattle, several studies suggest differences between oocytes matured *in vivo* vs. those matured *in vitro* (Kastrop et al., 1991; Hyttel et al., 1986; Hendriksen et al., 2000). Additionally, researchers have investigated the effect of the timing of oocyte collection following superstimulation and have adjusted their protocols to optimize oocyte competence before removing them from the follicle (Krisher, 2013). Specifically, a higher number of expanded COC collected from preovulatory follicles 24 h after the luteinizing hormone (LH) surge reached the blastocyst stage after *in vitro* fertilization than those collected 2 h before the LH surge (Dielemann et al., 2002). Likewise, oocytes collected 20 to 26 h after GnRH or LH treatments had greater developmental competence than those collected from un-treated control cows (Bordignon et al., 1997; Rizos et al., 2002; Dadarwal et al., 2015). Nevertheless, basic data in bison such as the time required for an oocyte to achieve the mature stage (metaphase II) under *in vitro* and/or *in vivo* conditions have not been reported. Moreover, protocols designed to increase the competence of the oocytes had not been investigated in bison. Furthermore, knowing that wood bison is a seasonal breeding species, it was important to investigate the effect of seasonality on oocyte maturation. We performed two experiments to compare the *in vivo* and *in vitro* maturational

characteristics of wood bison COC. Experiment 1 was conducted during the anovulatory season (May–June), while Experiment 2 was conducted during the ovulatory season (October–November). The results from this study showed that 60–70% of oocytes reached the MII stage 24 h after *in vitro* maturation while only 25–27% of oocytes reached the MII stage 24 h after *in vivo* maturation. Findings indicated that nuclear maturation occurred more rapidly during *in vitro* vs. *in vivo* maturation, but nuclear maturation *in vitro* was associated with a lower degree of cumulus expansion than with *in vivo* maturation. It is important to note that the degree of cumulus expansion has been reported to be a valuable predictor of developmental competence of bovine oocytes (Furnus et al., 1998). Additionally, *in vivo* oocyte maturation was more complete at 30 vs. 24 h after hCG treatment, and season had no effect on the maturational capacity of wood bison oocytes. Therefore, implementation of these findings was important for designing subsequent studies. Implications of the differences between oocyte maturation *in vitro* vs. *in vivo* in wood bison on *in vitro* embryo development remained to be investigated. Likewise, whether *in vitro* fertilization of mature oocytes from different seasons will affect *in vitro* embryo production outcomes also require further investigation.

The experiments described in Chapter 4 were performed to investigate the developmental competence of bison oocytes matured *in vitro*. Wood bison oocytes underwent 24 h of *in vitro* maturation before fertilization. This was based on the previous study (Chapter 3), where *in vitro* oocyte maturation was maximal after 24 h. Furthermore, numerous studies have supported the notion that morphology of the COC is related to the *in vitro* developmental potential of oocytes of different species (e.g., cattle [Shioya et al., 1988; Hazeleger et al., 1995; Boni et al., 2002; Madison et al., 1992; Bakri et al. 2016]; goat [Katska-Ksiazkiewicz et al., 2007]; sheep [Kelly et al. 2007; Dadashpour Davachia et al., 2012]; buffalo [Singh et al., 2012]). Consequently, cattle oocytes are routinely selected for IVP on the basis of the appearance of the ooplasm and the characteristics of

cumulus cells surrounding the oocyte (i.e., compactness and number of cell layers) (Gordon, 2004). Researchers have shown that immature bovine oocytes with more than three layers of cumulus cells have a higher maturation rate (Kakkassery et al., 2010) and cleavage rate (Shioya et al., 1988) than oocytes with partial cumulus cell layers or denuded oocytes. Furthermore, blastocyst rates were higher for bovine oocytes surrounded by multiple layers of compact cumulus cells than for those with less compact and fewer layers of cumulus cells (Madison et al., 1992). Therefore, we hypothesized that morphologic characteristics of wood bison COC are reflective of the ability of the immature oocyte to develop to an advanced embryonic stage after *in vitro* maturation, fertilization and culture. The results of these experiments showed that oocytes with compact cumulus cell layers had the greatest competence to develop to the blastocyst stage in wood bison. Findings also demonstrate a higher blastocyst rate (54.0% vs. 25.0%, respectively) for oocytes with more than three layers of cumulus cells (i.e., good compact) vs. those with fewer layers of cumulus cells (i.e., regular compact). These results supported the hypothesis that morphologic characteristics of wood bison COC were associated with the potential of immature oocytes to develop to an advanced embryo stage after *in vitro* maturation, fertilization and culture. This finding is in accordance with previous reports in other species (e.g., cat [Wood and Wildt, 1997]; catlle [Kelly et al., 2007]; sheep [Kelly et al., 2007; Dadashpour Davachia et al., 2012]). To our knowledge, this is the first report of *in vitro* maturation and fertilization and development to the blastocyst stage of immature COC collected from live wood bison. A remarkable improvement in the blastocyst production rate (overall 28%) was achieved in comparison to previous studies in which oocytes were collected from slaughtered ovaries (i.e., no more than 16% of blastocyst production; Thundathil et al., 2007; Aurini et al., 2009; Barfield and Siedel, 2011). Differences in the competence of oocytes collected from live vs. slaughtered bison may account, in part, for the differences in *in vitro* embryo production between studies. In water buffalo, oocytes

collected by follicular aspiration from live animals had greater developmental competence compared to slaughterhouse-derived oocytes (Neglia et al., 2003).

With the purpose of increasing the developmental competence of the wood bison oocytes matured *in vitro* (Chapter 4), we also investigated the effect of FSH starvation (i.e., time interval between FSH stimulation and oocyte collection, also known as FSH withdrawal, or coasting before oocyte collection [Blondin et al., 2002]) on blastocyst production. In cattle, duration of the FSH starvation period was shown to have an effect on the proportion of medium-to-large size follicles produced and oocyte developmental competence (Blondin et al., 2012; Nivet et al., 2012). A 48-h period of FSH starvation compared to 24 and 72 h (Blondin et al., 1997) was associated with larger follicles and higher developmental competence. The effect of extending the FSH starvation period after treatment of wood bison with FSH (in a slow-release formulation, 0.5% hyaluronan) by 24 h on the number and size of the follicles at the time of collection, oocyte morphological characteristics, and blastocyst development rate was studied. In contrast to reports in cattle, under the conditions of our study, there was no effect of extending the FSH starvation period on the parameters investigated. There was no clear explanation as to why results differed from those reported in cattle. One possibility may be related to species differences between wood bison and cattle. Future studies on the effects of different FSH doses and FSH starvation periods are required to elucidate whether there is an optimal period of coasting for the acquisition of oocyte competence in wood bison.

In a previous study (Chapter 3), *in vivo* oocyte maturation was more complete at 30 h than at 24 h after treatment of superstimulated wood bison with hCG. Oocytes collected 30 h after hCG treatment were used for subsequent studies reported in this thesis (Chapter 5). Also, while more than one-third of oocytes collected at 30 h post-hCG treatment were at the MII (i.e., mature) stage and had fully expanded cumulus cell layers, another third of the oocytes were at MI stage despite

having fully expanded cumulus cells. These findings provided rationale for the hypothesis that additional maturation time was required for the expanded wood bison COC at the MI stage to reach the MII stage. Thus, the experiments described in Chapter 5 were performed to determine the effect of an additional 4 h of *in vitro* maturation on embryo development of *in vivo* matured oocytes collected 30 h after hCG treatment (Experiment 1), and to determine if extending the interval between hCG treatment and COC collection from 30 to 34 h would improve *in vitro* embryo production (Experiment 2). The results showed that developmental competence of expanded COC collected 30 h after hCG treatment from superstimulated wood bison was greater after an additional 4 h (total 34 h) of *in vitro* maturation. Competence was further increased when the interval between hCG treatment and COC collection (i.e., *in vivo* maturation time) was extended from 30 to 34 h. Collectively, these findings supported the hypothesis that additional maturation time was required for expanded wood bison COC at the MI stage to reach the MII stage. These results are in agreement with those reported in similar studies of other species (e.g., monkeys [Lanzendorf et al., 1990], humans [De Vos et al., 1999; Vanhoutte et al., 2005]). To our knowledge, this is the first report of the *in vitro* production of blastocysts from *in vivo* matured oocytes collected from live wood bison. The percentage of oocytes that reached the blastocyst stage was 54% in the present study, in comparison to 8 to 16% in previous studies in bison (Thundathil et al., 2007; Aurini et al., 2009; Barfield and Siedel, 2011). The source of oocytes (i.e., live bison vs. slaughterhouse-derived ovaries) and the *in vitro* embryo production conditions (i.e., *in vivo* maturation vs. *in vitro* maturation, different *in vitro* culture media) may explain the differences in results between studies. In this study (Chapter 5), 40 blastocysts were produced from 74 *in vivo*-matured oocytes that were fertilized immediately after collection (oocytes were collected from 14 superstimulated bison, 34 h after hCG treatment), while in the previous study (Chapter 4), 44 blastocysts were produced from 159 oocytes fertilized 24 h after *in vitro* maturation (oocytes were

collected from 32 superstimulated bison). No statistical comparison between the two studies was performed, because the studies were not conducted simultaneously. However, we can infer that inducing *in vivo* maturation of oocytes before collection from live wood bison appears to be a reasonable protocol for producing competent oocytes for the purpose of IVP in this species. Additionally, in Chapter 5, the effect of season (ovulatory vs. anovulatory) on the number of follicles ≥5 mm available for aspiration at the time of COC collection, the number of expanded COC collected after inducing *in vivo* maturation, and the *in vitro* production of wood bison embryos was examined. Results indicated that there was no seasonal effect on the developmental capacity of oocytes in the present study, as a similar percentage of COC developed into blastocysts during both the ovulatory and anovulatory seasons. Nevertheless, the absolute number of embryos produced during the anovulatory season was more than double than that of the ovulatory as a result of a nearly two-fold increase in the number of follicles aspirated and number of COC collected during the anovulatory season. A greater superstimulatory response during the anovulatory season has been reported previously in bison (Palomino, 2015), but the reason is unknown. Collectively, the results demonstrated that high quality *in vitro*-produced embryos (Grade 1) were produced during both the anovulatory and ovulatory season in wood bison. This is an important finding, as IVP could be effectively accomplished in wood bison during any time of the year. Therefore, IVP may have advantages over other reproductive technologies that have shown to be effective only during the ovulatory season in this wild species (i.e., production of *in vivo*-derived embryos [Palomino, 2015]).

Obtaining healthy offspring after transfer of an *in vitro*-produced embryo depends on existing knowledge of the reproductive physiology of each particular species, and usually little is known about the physiology of most wild animals (Comizzoli, 2015). With the knowledge generated on female bison, from earlier studies (Adams et al., 2009; McCorkell et al., 2010; McCorkell et al.,

2013a; Toosi et al., 2013; Palomino et al., 2013; Palomino et al., 2014a; 2014b; Palomino, 2015) and the present thesis (Chapter 3, 4 and 5), a pilot embryo transfer study of *in vitro*-produced wood bison embryos was conducted. Three healthy wood bison offspring were born following the transfer of ten *in vitro*-produced wood bison embryos to synchronized wood bison recipients. This is a reproductive first for this species (IVF wood bison calves, Appendix I) and is an important contribution for future conservation efforts of threatened wildlife species.

Based on the previous studies (Chapters 4 and 5), *in vitro*-produced embryos were produced for the final study (Chapter 6) using *in vivo*-matured wood bison oocytes collected 34 h post-hCG treatment. In this study, the hypothesis that the IETS standardized embryo washing procedure was an effective technique for removing *Brucella* bacteria from *in vitro*-produced wood bison embryos was tested. The primary objective was to determine the effectiveness of washing procedures with or without antibiotics for removing *Brucella abortus* from *Brucella*-infected *in vitro*-produced embryos. The process involves a 10-step transfer of embryos from one wash medium to another containing clean medium, and continued for 10 washes at 100 fold dilution. By the 10th wash, bacterial loads are expected to decrease to undetectable levels (Bielanski, 2007; Stringfellow and Givens, 2010). In earlier studies, the IETS washing procedures were shown to be effective in disinfecting *in vivo*-derived embryos exposed *in vitro* to *Brucella* in cattle (Stringfellow et al., 1986) and wood bison (Palomino et al., 2015b). However, the findings of other studies indicated that the washing procedures are less effective in removing pathogens from *in vitro*- vs. *in vivo*-derived embryos in cattle (Bielanski, 2007). Some pathogens (e.g., bovine viral diarrhea virus) persisted on *in vitro*-produced bovine embryos despite being subjected to appropriate washing procedures (Stringfellow and Givens, 2000). However, results of the current study revealed that the IETS washing procedures were effective in removing *Brucella* from *in vitro*-produced bison embryos following *in vitro* exposure to the pathogen. Although there were no differences in

embryo disinfection following the use of a wash medium with or without antibiotics, antibiotics should be added to the wash medium to ensure bacteria-free embryos. Studies in other species (e.g., human) have shown that washing procedures did not compromise survival and development of embryos (Bielanski, 2007). This is valuable information, as future studies in wood bison should determine whether the transfer of disinfected *in vitro*-produced embryos to disease-free recipients will result in *Brucella*-free calves. The last study in this thesis (Chapter 6) is fundamental in determining if the combination of the current IVP system and the IETS washing procedures can be used to produce disease-free *in vitro*-produced embryos for the reclamation of threatened wood bison in the WBNP.

In addition, in the last study (Chapter 6), the ovarian superstimulatory response in wood bison cows following implementation of two different FSH treatment protocols was compared. In previous studies in wood bison, constant and decreasing doses of FSH for the purpose of superstimulation have been compared (Palomino et al., 2013; Palomino et al., 2014b). The constant doses of FSH in saline were administered subcutaneously, while in the current study, FSH in 0.5% hyaluronan was administered intramuscularly. The effect of the administration regimen (i.e., constant-dose regimen of 200 mg or decreasing-dose regimen of 300 mg and 100 mg) of exogenous pFSH in 0.5% hyaluronan for a total dose of 400 mg NIH-FSH-P1 on the number of follicles ≥ 5 mm at the time of COC collection, the number of COC collected per bison, and the number of COC in each morphological category was examined. No effect of treatment regimen on any parameter investigated was found. In cattle, the limited number of studies conducted in this area are not sufficient to make conclusions regarding the efficacy of such regimens (Seidel and Moore Seidel, 1991). Future work should address whether there is a particular regimen that result in a greater number of follicles ≥ 5 mm for COC collection, and subsequently in a greater number

of COC collected per bison for the purpose of IVP. Further, the effect of dose of FSH on oocyte production and competence is needed in wood bison.

Overall, an efficient procedure to produce wood bison embryos *in vitro* throughout the ovulatory and anovulatory seasons was developed. The results reported in this thesis indicate that IVP is a technique that may be suitable for implementation in future conservation programs and potentially for production purposes in wood bison. Additionally, the effectiveness of embryo washing procedures for producing *Brucella*-free embryos under *in vitro* conditions was demonstrated. Washing procedures are recommended for the removal of bacteria from *in vitro*-produced wood bison embryos.

CHAPTER 8. GENERAL CONCLUSIONS

Overall, the results of this doctoral dissertation support the hypothesis that *in vitro* embryo production technologies can be utilized efficiently in wood bison by improving the competence of oocytes collected from live wood bison.

Based on these results, it can be concluded that:

- Oocyte collected from superstimulated wood bison can mature to the MII stage *in vitro* or *in vivo*.
- Reproductive season had no effect on the maturational capacity of wood bison oocytes.
- *In vivo* oocyte maturation was more complete at 30 vs. 24 h after hCG treatment.
- Immature oocytes with compact cumulus cells had higher *in vitro* blastocyst development rates than immature oocytes with expanded cumulus cells or denuded oocytes.
- Immature wood bison oocytes did not have greater *in vitro* blastocyst production rates after being exposed to an extended FSH starvation between FSH treatment and oocyte collection (FSH coasting period or FSH withdrawal).
- Developmental competence, in terms of blastocyst development, of expanded COC collected 30 h after hCG treatment (i.e., *in vivo* matured oocytes) from superstimulated wood bison was greater after an additional maturation period of 4 h, either *in vitro* or *in vivo*. The greatest improvement was achieved when the *in vivo* maturation time was increased from 30 to 34 h.
- There was no effect of reproductive season on the developmental competence of *in vivo* matured oocytes in wood bison. Similar percentages of expanded COC developed into blastocysts during both the ovulatory and anovulatory seasons.
- The IETS washing procedures were effective to remove *Brucella* bacteria from *in vitro*-derived wood bison embryos following *in vitro* exposure to the pathogen.

CHAPTER 9. FUTURE STUDIES

- Determine the effect of reproductive season on the developmental competence of bison oocytes matured *in vitro*.
- Investigate the effect of different FSH starvation periods on the developmental competence of immature oocytes collected from superstimulated bison during the ovulatory and anovulatory seasons.
- Evaluate protocols for the cryopreservation of bison oocytes and *in vitro*-produced bison embryos.
- Evaluate the pregnancy and live birth rates following transference of fresh and frozen/thawed *in vitro*-produced wood bison embryos produced from disease-free bison to recipient bison.
- Investigate whether transfer of washed *in vitro*-produced embryos exposed *in vitro* to *Brucella* to disease-free recipients will result in disease-free calves and disease-free wood bison recipients.
- Evaluate the production of *in vitro*-produced wood bison embryos from individuals or germplasm from in and around WBNP and/or from isolated wild wood bison herds.

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APPENDIX A.

World's first bison calves produced by *in vitro* fertilization (July 2016; Native Hoofstock Centre, University of Saskatchewan). A) Healthy calves running together with their surrogate mothers. B) Few-weeks old calf suckling her surrogate mother.

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