DISSERTATION

INVESTIGATION OF ASSISTED REPRODUCTIVE TECHNOLOGIES (ART) FOR CONSERVATION OF BOVIDAE

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ABSTRACT

INVESTIGATION OF ASSISTED REPRODUCTIVE TECHNOLOGIES (ART) FOR CONSERVATION OF BOVIDAE

There is an ongoing loss in global biodiversity in both wildlife and domestic species, creating a need to protect and preserve valuable genetics for the maintenance and sustainability of these populations. One of the proposed strategies to combat this loss is through the use of assisted reproductive technologies (ART), not as a replacement to natural breeding but as a strategy to augment and increase the tools we have to preserve genetic diversity. Currently, the application of ARTs is not broadly used as there are gaps in knowledge of species-specific reproductive biology and gamete biology, hindering the ability to make fast progress in implementing these techniques for conservation purposes in species and populations that are rapidly declining. ARTs used in domestic species in the Bovidae family, including cattle, have been developing over the past century, and recently are being adapted for use in non-domestic Bovidae. Arguably, one of the most critical ARTs developed for genetic rescue and conservation goals has been the successful utilization of cryopreservation techniques, which has allowed for the creation of genetic resource banks (GRBs), which are biorepositories for gametes, embryos, and tissues. Through GRBs breeding programs can achieve similar or greater levels of heterozygosity and increase the effective population size as represented by embryos and germplasm, compared to captive populations. In this work, we describe studies developing in vitro oocyte maturation (IVM) and in vitro embryo production (IVP) techniques used for the preservation of valuable bison genetics using gametes collected post-mortem, and cryopreservation techniques of bovine

oocytes and ovarian tissue for fertility preservation. Using oocytes collected post-mortem, from bison within the YNP herd (a population with endemic brucellosis) we created disease-free embryos for subsequent transfer into healthy surrogate bison. The transfer of brucellosis-free embryos resulted in the live birth of a healthy brucellosis-negative bison calf. Next, by assessing the follicle size and duration of in vitro maturation (IVM) of bison oocytes collected from abattoir ovaries, out of season, we determined that seasonality does impact oocyte competence and blastocyst production, although viable bison embryos can be created independent of seasonal effects. While cryopreservation of female gametes in bovid species remains experimental, vitrification of oocytes may provide an opportunity for infusing greater genetic diversity into future generations. By assessing mitochondrial function of bovine oocytes during vitrification, we found that germinal vesicle (GV) stage oocytes may require additional support through the vitrification process, as they demonstrated a reduced ability to handle cryo-induced oxidative stress post-vitrification. Additionally, vitrification of bovine ovarian cortical tissue coupled with techniques for vitro activation (IVA) of primordial follicles may be an alternative way to preserve female germplasm. Follicle viability in bovine ovarian cortical tissue was partially preserved after vitrification, making it feasible to biobank vitrified tissue from valuable domestic or wild Bovidae, while in vitro tissue culture and/or IVA treatments significantly reduced tissue and follicle viability was unsuccessful. Further investigation of germline preservation in Bovidae is needed before these techniques can be broadly implemented.

These ARTs are a toolbox of approaches to conserve biodiversity and valuable genetics through production, use, and preservation of tissue, gametes and embryos.

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DEDICATION

This work is dedicated to my family, whose unwavering love and support made this possible.

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CHAPTER 1: REVIEW OF LITERATURE

Introduction

Reproduction is essential for the preservation and evolution of living organisms on earth, making it a cornerstone in conservation biology. In both domestic and wildlife species, one of the core concepts in conservation is to sustain sufficient genetic diversity in a population to support genetic exchange and to maintain heterozygosity, fertility, and adaptive responsiveness to environmental fluctuations (Comizzoli and Holt 2019). For wildlife species, loss of habitat due to human activity, excessive hunting of exotic species, and climate-related environmental changes, has resulted in the unprecedented decline of many species. The IUCN Redlist of Threatened Species currently lists 18,620 species as endangered, critically endangered, or extinct in the wild. Of the 139 species in the family Bovidae, 18 are listed as endangered and 7 as critically endangered (IUCN 2020). Many species are now facing extinction before protocols can be developed that enable successful preservation of their genetics, including in the Bovidae family (Wildt 1992; Alderson 2018).

Domestic breed development has been driven by a combination of environmental, human, and economic issues. Over the past few decades, there has been a precipitous decline in allelic diversity between and within breeds due to increased calls for uniformity in individuals to streamline processes in intensive agriculture (eg. mega-farming). This may result in a loss of breed-specific genes of interest (e.g. fecundity and thermotolerance related genes) and overall genetic variance, particularly in heritage breeds (Mishra *et al.* 2009; Prentice and Anzar 2011; Renaudeau *et al.* 2012; Mara *et al.* 2013). According to the Food Agricultural Organization, genetic erosion and loss of diversity over the past 20 years has caused 17% of the 8774 categorized domestic agricultural breeds to be at risk of extinction, and 1350 domestic breeds are

considered threatened due to indiscriminate breeding, cross breeding, economic drivers, changing market demand, poor animal genetic resources (AnGr) management programs, degradation of natural resources, changes in livelihood and lifestyle opportunities for rural communities, and large-scale high-input farming (FAO 2020). Horses, sheep, and cattle are currently listed as the mammalian species with the highest number of breeds classified as at-risk (FAO 2020). AnGr managers in domestic breeds face similar challenges to preserve genetic diversity. Inadequate breed-specific knowledge and a lack of monitoring population sizes and trends are impeding the estimation of risk status. Adequate genetic diversity and environmental suitably are crucial if breeds are going to successfully meet current and future production challenges. The rapid and ongoing loss of biodiversity calls for reproductive managers of both domestic and wildlife species to consider the importance, usefulness, and potential of incorporating alternative reproductive strategies into breeding management programs.

Current Limitations of Conservation Breeding Programs and Zoos

Some argue that current conservation paradigms are not able to adequately address the rapid progression of anthropogenic habitat destruction and loss of biodiversity and call for the investigation of new methodologies that may accelerate genetic rescue (Piaggio *et al.* 2017; Bell *et al.* 2019). Genetic rescue is defined as 'a decrease in population extinction probability owing to gene flow, best measured as an increase in population growth rate' (Bell *et al.* 2019). The World Association of Zoos and Aquariums indicates many critical breeding programs are not projected to be able to maintain genetic goals to sustain biodiversity representative of once healthy wild populations (Lacy* 2013). Conservation breeding programs (CBP) manage existing populations by monitoring breeding individuals and populations in confined, managed reserves (ex situ populations) with human intervention. Healthy sustainable wild populations in natural

environments (in situ populations) are declining around the globe (Comizzoli and Holt 2019). CBPs have been used successfully to reestablish previously extinct populations in the wild back into their natural habitat (Hoffmann *et al.* 2015). For many species, including bison, conservation strategies consider monitoring and management of genetic variability to be of upmost importance as the majority of existing populations are small with limited space to expand (Cherry *et al.* 2019). However, current programs have difficulty sourcing enough individuals with founder genetics to maintain a minimum number of animals to avoid inbreeding and allelic loss (Mastromonaco and Songsasen 2020).

Zoos face significant challenges in trying to understand and manage the complex reproductive mechanisms of extensively diverse taxa (Comizzoli *et al.* 2018). Most collections in zoos represent small populations containing too few founders and with aged, sterile, acyclic females. Research is limited by inadequate funding, regulatory restrictions, limited personnel with reproduction expertise, invasiveness of technique/difficulty and cost of technology, small data sets, difficult and potentially dangerous handling or breeding behavior, and animals that are highly susceptible to handling/captivity related stress. These compounding factors result in a major gap in basic scientific knowledge of species-specific reproductive physiology and breeding behavior and knowledge of genetic management for a given species (Comizzoli 2015). Indeed, it is estimated that reproductive biology is only understood in 0.25% of the world's 40,000 vertebrate species (WILDT *et al.* 2003).

Implementing assisted reproductive technologies (ART) requires a critical understanding of the unique anatomy and ovarian functions of each species (Lueders and Allen 2020). Basic ARTs are no longer experimental technologies in some species, however ART techniques that are successful in humans and livestock do not always easily translate to wildlife (Schmidt and

Kappelhof 2019). In reality, birth rates for ARTs such as artificial insemination (AI) and embryo transfer (ET) have only resulted in live offspring in 50 to 60 wildlife species (Roth and Swanson 2018). Repeatability in basic technologies, such as AI, require well-studied captive populations, of which there are only a few, including black footed ferrets (Holt 2016), the giant panda (Martin-Wintle *et al.* 2019), and elephants (Schmidt and Kappelhof 2019) to name a few. Another hurdle of ART in threatened species is that often these techniques are only considered and put into action when a species is already in a compromised state nearing extinction; at which point, animal numbers are already low, and gametes and tissues are not expendable for research purposes. Too often, CPB and AnGr managers are faced with a situation in which they are developing ART techniques while simultaneously trying to employ them, which too often ends unsuccessfully (Lueders and Allen 2020). For example, ET, which is considered a basic technique in many domestic large animal species, has not yet been successful in rhinos, even though it is desperately needed.

The implementation of ART in conservation strategies offers opportunities to shift focus away from breeding within small, genetically isolated populations that are restricted to regional exchange of live animals, an endeavor limited by cost and animal availability. Instead, ART enables exchange between animal groups globally, creating internationally interconnected populations, called metapopulations, through exchange of cryopreserved germplasm or embryos. The end goal of both in situ and ex situ conservation is to maintain sustainably sized, genetically diverse populations that represent a healthy wild population as closely as possible. ART has been proposed, not as a replacement to natural breeding but as a strategy to augment and increase the rate of genetic rescue (Herrick 2019). The beneficial impacts of genetic rescue are promising and may have a broader reaching and longer lasting effect if ART were incorporated, but in most

species increased basic knowledge of reproductive and gamete biology are still required to realize these benefits (Roth and Swanson 2018).

Application of ART in Conservation

Broad clinical use of ART in humans makes it easy to underestimate the complexities of adapting these technologies to nondomestic species (Comizzoli *et al.* 2018; Herrick 2019). Early successes of ARTs in exotic mammals were based on knowledge gleaned from bovine studies using frozen semen in the 1950s (Comizzoli 2015). Commercial livestock industries have invested large amounts of time and resources into basic biology and research to develop ART techniques. In particular, ART implementation in wild ungulates has outpaced those in other taxa because of translatable bovine and ovine technologies, which led to key successes, including the first reindeer offspring produced from AI in 1973 (Dott and Utsi 1973). Since then, the use of AI using fresh or frozen-thawed sperm has resulted in offspring from many endangered species including black-footed ferrets, elephants, and rhinoceroses (Saragusty *et al.* 2009; Holt 2016; Pennington and Durrant 2019) Some species in the Bovidae (bison) and Cervidae (white-tail deer) families have been commercialized as a food commodity or for sport (commercial hunting farms), with captive breeding becoming more commonplace due to strong financial incentives. For example, in 2003 private for-profit hunting farms containing up to 75,000 deer in North America representing USD $111 million in animals, with that number growing. These alternative animal industries are able to provide increased material and capitol into the development of species-specific ART including AI, ET, and in vitro embryo production (IVP). Private groups are investing time and money into developing these techniques allowing information to be gathered about non-traditional species which may benefit ART efforts for conservation (Long *et al.* 2003).

However, there are issues when using traditional livestock species as reproductive models for wildlife. Arguably, many of the reproductive adaptations to the environment, climate, diet, and herd social structure are not adequately represented in domestic species, as these are traits have been purposefully bred out of domestic livestock for ease and consistency of reproductive management. Highly variable adaptations including the strategy for mate choice by females, sperm competition, monogamous verses polygamous mating, reproductive seasonality, spontaneous or induced ovulations, estrous cycle length, reproductive anatomy, gamete morphology and composition, and ovulation indicators can alter the ability for broad adaptation of ART strategies even within groups and families. Combined knowledge of all of these factors can affect the potential of ART efficiency.

The reproductive cycles of domestic bovids, such as cattle and sheep, have been well characterized over the past five decades, allowing for the development and widespread utilization of ARTs to increase reproductive efficiency. Major milestones in bovine reproductive technology were achieved in large part due to needs within the dairy industry to increase the rate of genetic gain of dairy cows through the extensive use of sires with superior genetic value (Moore and Hasler 2017). The use of AI and frozen semen in the 1950's furthered the potential of this technology when semen could be stored and transported long distances and used over multiple generations (Polge and Rowson 1952). Development of non-surgical embryo transfer and the use of frozen embryos in the 1970's furthered the growth of large-scale operations relying on ARTs (Wilmut and Rowson 1973). Improved understanding of neuroendocrine mechanisms regulating ovarian function allowed for development of superovulation protocols, increasing the impact genetically valuable females could have by increasing embryo yields per estrous cycle. As well, techniques such as ovum pick up (OPU) in the 1980's allowed for the use

of newly developing in vitro techniques such as in vitro oocyte maturation (IVM), in vitro fertilization (IVF), and in vitro embryo culture (IVC) (Hasler 2014). Used in combination, in vitro embryo production (IVP) offered an alternative to superovulation protocols and by the 1990's – 2000's bovine IVP operations were established (Looney *et al.* 1994; Hasler 2014).

The use of similar techniques in other less common Bovidae species such as bison could improve conservation efforts, however improved basic understanding of the reproductive physiology in this species is necessary for their effective application. The reproductive physiology of bison is less well known compared to bovine. The reproductive systems of both species are quite similar (estrus length, follicle size, age at estrous, gestation length, anatomy), making cattle a relevant model for some, but not all aspects of bison reproduction (Dorn 1995). Unlike cattle, bison are seasonal breeders (Cervantes *et al.* 2016). The neuroendocrine mechanisms that control ovarian function observed in cattle are not the same in bison, during the anovulatory season, as has been seen in other domestic seasonal breeders (sheep) as they change from breeding versus non-breeding season (McCorkell *et al.* 2013).

Bison are seasonally polyestrous, with multiple estrous cycles occurring during the ovulatory season unless conception is achieved. They are considered short day breeders, with the follicular and luteal activity which marks the ovulatory season beginning as the photoperiod becomes shorter in late summer/early fall (Thiéry *et al.* 2002). This allows for a favorable spring calving period when resources are more available. In mammalian seasonal breeders, photoperiod is the predominant influence that synchronizes and triggers the onset/ cessation of the breeding season (ViviD and Bentley 2018). In short day breeders like bison, decreasing length of daylight increases secretion of melatonin, a hormone secreted by the pineal gland (Dahl *et al.* 2000), that acts as a messenger for the GnRH release in seasonal breeders (Malpaux *et al.* 1996; ViviD and

Bentley 2018). The hypothalamo-pituitary-ovarian axis regulates the release of reproductive hormones (GnRH, LH and FSH), and functions similarly in the breeding season of seasonal breeders as it does in non-seasonal species (ViviD and Bentley 2018). However, during the non-breeding season, an increase in estradiol 17ß negative feedback prevents estrus and ovulation by acting as an inhibitor of GnRH and LH secretion (Legan and Winans 1981).

Early studies in bison show that similar to other seasonal breeders including sheep (Rosa and Bryant 2003) and elk (McCorkell *et al.* 2004), the pattern of continuous follicular activity continues to occur throughout the anovulatory season even though luteal activity has ceased, allowing for year-long collection of gametes for use in ARTs. However, the effects of these seasonal differences in endocrine changes on the developmental potential of gametes collected during the breeding versus non-breeding season has not been evaluated in depth in non-domestic bovids including bison and may impact the effectiveness of ARTs, particularly when gametes are collected post mortem from wild animals, when exogenous endocrine manipulation is not possible.

Obtaining sufficient amounts of reproductive material, particularly female gametes, for research is often a limiting factor (Herrick 2019). This lack of resources makes it difficult to develop species-specific techniques. Most often, post-mortem ovaries are harvested from closely related domestic models (domestic hoofstock, felines, canines, birds), or research groups will take advantage of culls of both commercial (cervids) and wild populations (bison, African oryx) to use in the development of ART(s) including IVM, IVF and IVC (Durrant *et al.* 2011; Benham *et al.* 2020). For example, heterologous studies using domestic cattle oocytes has improved fertilization fertilization rates for studies using frozen-thawed sperm from springbok, impala, and blesbok (Chatiza *et al.* 2013). Seasonal effects on oocyte competence, embryo cleavage rates,

and blastocyst rates have been studied using domestic swine and sheep models (Bertoldo *et al.* 2010; Mara *et al.* 2014). When possible, studies in non-domestic species shed greater light on important affecters of reproduction including seasonality. For example, decreased quality and fertilization success rate in semen collected during the nonbreeding season has been reported in fallow deer (*Dama dama*), tufted deer (*Elaphodus cephalophus*), and wood bison (*Bison athabascae*) (Krishnakumar *et al.* 2015; Panyaboriban *et al.* 2016; Uccheddu *et al.* 2020). Similarly, reduced cleavage rates have been reported for oocytes collected during the anovulatory season in wood bison (Krishnakumar *et al.* 2015).

While the difficulties and complexities are often underestimated, and applicability overestimated, the benefits of incorporating ART into CBP are far reaching (Comizzoli *et al.* 2018). The foundational research conducted in both domestic and non-domestic species has contributed to the collective knowledge of reproductive physiology and gamete biology and paved the way for many conservation applications. For example, frozen semen and AI can be used to infuse wild genetics into captive populations, increasing heterozygosity and genetic variance without ever having to introduce or remove individuals from wild population (Crosier *et al.* 2006; Hildebrandt *et al.* 2012). Semen sex sorting techniques that was originally developed for use in dairy cattle has now been used in the rhinoceroses, elephants, and dolphins to select for females, that better suit the goals of captive programs (O'brien *et al.* 2009). Use of IVP has also produced pathogen free gametes or embryos to mitigate disease transmission (Herrick 2019; Benham *et al.* 2020). Despite the increased application of basic ARTs in non-domestic species, pregnancy rates are consistently lower than those seen in livestock using similar techniques (Mastromonaco and Songsasen 2020). Artificial insemination using cryopreserved sperm has been moderately successful. However, small sample sizes lead to low study repeatability, with

only 20% of attempts resulting in at least one successful pregnancy (Fickel *et al.* 2007), and the combined use of IVP and embryo transfer is still considered inefficient and experimental in the vast majority of wildlife species. Often, embryo transfer is not an option due to either recipient animal availability or the resource scope of the research, thus, pregnancy outcomes using IVP embryos often remain undetermined (Mastromonaco and Songsasen 2020).

ART in Females

Understanding follicular and gamete biology is critical for utilization and preservation of female gametes for genetic conservation in threatened breeds or species. Many mammalian species have a follicle pool that is established during fetal life. In domestic cattle, a significant population of primordial follicles spontaneously undergo apoptosis during fetal development, resulting in an population of about 200,000 oocytes at birth (Santos *et al.* 2013). Primordial follicles have been isolated as early as 3 months of age in cattle (Fortune *et al.* 2000).

The ovary is divided into two main regions, the inner medulla and the outer cortex. The inner medulla is comprised of softer, highly vascular tissue that supports immature and growing follicles with hormones and gas exchange (Kerr *et al.* 2013). Alternatively, the outer ovarian cortex surrounds the medulla and contains immature and newly activated follicles and germ cells as well as supportive cells that produce hormones and growth factors necessary for early follicular development and recruitment. The outermost part of the cortex is surrounded by a dense, fibrous outer layer that provides structural support for the quiescent primordial follicle population. The primordial follicle is the most basic functional unit in the ovary and contains an immature oocyte arrested in the diplotene stage of meiosis I that is surrounded by a single layer of squamous pre-granulosa cells (Kerr *et al.* 2013). The rigidity of the outer ovarian cortex tissue

acts to maintain dormancy of the primordial follicle in part via mechanical signaling-regulated pathways (Kawashima and Kawamura 2018; Shah *et al.* 2018).

Folliculogenesis begins when a selected cohort of follicles is triggered via cellular signals to activate and transition from the primordial to primary follicle stage (McLaughlin and McIver 2008). This initial activation is gonadotropin (follicle stimulating hormone (FSH) or luteinizing hormone (LH)) independent and is initiated via non-endocrine factors (Fortune *et al.* 2010). Pre-granulosa cells will transform and proliferate into a single compact layer of granulosa cells and the zona pellucida will begin to develop around the oocyte. The oocyte will secrete oocyte growth factors such as growth and differentiation factor 9 (GDF9), bone morphogenic protein 15 (BMP15) and transforming growth factor β (TGF- β) which will stimulate granulosa cell proliferation and differentiation and responsiveness to estrogens, androgens, and insulin. (Richards and Pangas 2010). Once the follicle is surrounded by at least two complete granulosa cell layers it has reached the secondary stage, at which point the granulosa cells begin to secrete paracrine factors to initiate cellular interactions, and acquire FSH receptors (FSHr), preparing the follicle for gonadotropin dependent growth. As follicle growth continues, an antral cavity will begin to form and the existing granulosa cells will differentiate into two distinct cell populations, the cumulus and mural granulosa. The growing follicles acquire aromatase (CYP19) activity and, as a result of FSH stimulation, convert testosterone to estrogen to continue to promote follicle growth (Zeleznik 2004). If the follicle reaches an ovulatory size, the oocyte can resume meiosis I in preparation for ovulation and possible fertilization.

The Hippo signaling pathway, which has been identified in multiple species including mouse, human, and bovine is a regulator of primordial follicle activation (Kawashima and Kawamura 2018; Shah *et al.* 2018). Mechanical signaling (mechanotransduction) has been

shown to play a critical role in primordial follicle storage, dormancy, and activation, via the Hippo signaling pathway. In dormant follicles, Hippo signaling mediators have an inhibitory effect on cellular proliferation and promote cellular quiescence (Pan 2007; Kawamura *et al.* 2013; Xiang *et al.* 2015). When Hippo signaling is disrupted by mechanical stimuli, dephosphorylation and activation of key transcriptional coactivators stimulate down-stream effectors, including growth factors and apoptosis inhibitor proteins which promote granulosa cell proliferation and survival (Pan 2007; Holbourn *et al.* 2008). Another pathway associated with follicular activation is the PI3k-Akt-PTEN signaling pathway which regulates primordial follicle activation by suppressing a regulator of follicular quiescence. PTEN negative regulation of the Akt signaling pathway is considered essential for maintenance of follicle quiescence (Kawamura *et al.* 2013). Both pathways are highly conserved growth pathways and maintenance of dormancy or release from this are regulated by relief of follicle activation inhibition, the predominant state.

In vitro activation (IVA) approaches targeting these two signaling pathways have been successful in multiple species including mice (Li *et al.* 2010; Kawamura *et al.* 2013), non-human primates (Ting and Zelinski 2017), humans (Li *et al.* 2010; Kawamura *et al.* 2013), pigs (Moniruzzaman *et al.* 2010), sheep (dos Santos *et al.* 2018), and cattle (Bao *et al.* 2011). IVA can be used to amplify the impact of ART in females by increasing the potential to recover greater numbers of oocytes available for future development in vitro (Silva *et al.* 2016). Hippo signaling disruption via tissue fragmentation disengages physical forces of cortical stromal scaffolding resulting in primordial follicle activation (Fig. 1); (Kawamura *et al.* 2013; Kawashima and Kawamura 2018) . The PI3K-Akt-PTEN signaling pathway can be chemically activated using a PI3K activating peptide, 740 Y-P, and a PTEN inhibitor, bpV(pic), to activate

primordial follicles (Fig. 2); (Li *et al.* 2010). IVA via PTEN inhibition in human tissue preserved follicle viability, endocrine function, and increased the primordial to primary follicle ratio (Novella-Maestre *et al.* 2015). These methods of IVA have been used successfully to stimulate follicular activity in ovarian fragments prior to tissue transplantation in human patients with a range of fertility issues including primary ovarian insufficiency (POI), diminishing ovarian reserve (DOR), and oncofertility related issues resulting from anti-cancer treatments (prepubertal and adult), and have resulted in live births (Kawamura *et al.* 2013; Sheshpari *et al.* 2019). Developing similar strategies in other domestic and non-domestic species may increase the potential for genetic rescue from aging animals that don't respond adequately to stimulation protocols or have insufficient ovarian reserve, gonads collected postmortem, and prepubescent or non-cyclic animals.

Maintenance of meiotic arrest in immature oocytes in non-ovulatory follicles is a critical component for the maintenance of the reproductive lifespan in female mammals. In vivo, primary oocytes in dormant primordial follicles remain arrested in prophase I of the first meiotic division and contain a large nucleus enclosed in a nuclear envelope, or germinal vesicle (GV) (Fan and Sun 2019). Resumption of the first meiotic division only occurs in activated growing antral follicles, while the majority of oocytes in follicles that do not reach this stage undergo atresia—programmed cell death. Small GV oocytes do not have the same potential for meiotic resumption as GV oocytes in growing follicles. Not until the oocyte reaches ~80% of its mature size does it become capable of germinal vesicle break down (GVBD), the initial morphological sign of meiotic resumption. The initiation and arrest of meiotic events in the oocyte are predominately regulated through the activity of one kinase, cyclin dependent kinase 1 (CDK1). CDK1 requires the subunit, cyclin B, to be functional, as limited amounts of each prevent kinase

activity. Levels of CDK1 and cyclin B increase as oocyte size increases during follicular growth. GV arrest is maintained in preovulatory follicles (that now have adequate levels of CDK1 and cyclin B) by post-translational regulatory mechanisms from granulosa cells, including cyclic adenosine 3', 5'-monophosphate (cAMP), that maintain coordination of meiotic resumption with the follicular environment. cAMP promotes protein kinase A (PKA) activity to inhibit CDK1. In vivo, at the LH surge, cAMP levels within the oocyte drop causing a signaling cascade leading to resumption of meiosis I. Similarly, removal of the oocyte from the follicular environment, such as occurs during follicular aspiration causes spontaneous GVBD, resulting in meiotic resumption due to a dramatic drop in oocyte cAMP levels. Cyclin B regulation is an additional level of regulation of this activation pathway, as loss of regulation of this pathway could result in global follicle activation or early activation, which could misalign timing of oocyte maturation with ovulation (Fan and Sun 2019). To keep cyclin B levels low, constant degradation of cyclin B occurs via anaphase-promoting complex (APC) (Madgwick and Jones 2007). Phosphodiesterases, particularly PDE3, act at GVBD to hydrolyze cAMP, reduce PKA levels, allowing for CDK1 activation and resumption of meiosis. In vitro, to slow down meiotic resumption and nuclear maturation to allow cytoplasmic maturation additional time, PDE inhibitors such as Forskolin have been used to increase cAMP levels. Finally, completion of meiosis I results in a haploid secondary oocyte and the extrusion of the first polar body followed by initiation of meiosis II. The oocyte then arrests in metaphase II (MII) until fertilization. If fertilized, the oocyte will complete meiosis II and expel a second polar body (Madgwick and Jones 2007).

In vitro maturation (IVM) systems are designed to mimic this process. When immature cumulus oocyte complexes (COCs) are removed from the inhibitory environment of the antral

follicle, nuclear maturation is initiated. IVM medium contains hormones and growth factors, including FSH and epidermal growth factor 1 (EGF1), to support maturation to the MII stage (De La Torre-Sanchez *et al.* 2006). To become competent prior to fertilization, the oocyte must complete both nuclear and cytoplasmic maturation. Cytoplasmic maturation is critical for regulating lipid and carbohydrate metabolism, mitochondrial number, mitochondrial distribution and activity, intracellular ROS reduction, cumulus cell coordination and production of growth factors (Krisher 2013). Decreased embryo quality, production rates, and cryotolerance of IVP embryos derived from IVM oocytes indicates that cytoplasmic maturation may not be fully supported in the in vitro environment. Oocyte metabolism may also be affected by the in vitro environment. Throughout oocyte maturation, glucose is metabolized by cumulus cells to supply pyruvate and lactate to the oocytes, and this glucose metabolism regulates nuclear maturation and redox balance via the pentose phosphate pathway (Sudano *et al.* 2011; Krisher 2013). Increased glucose or lipid metabolism during IVM increases mitochondrial oxidative activity, possibly resulting in increased ROS production. ROS production caused by metabolic activity causes oxidative stress, resulting in cellular damage that affects oocyte quality (Silva *et al.* 2015b). IVM is still not optimized fewer in vitro matured bovine oocytes (~40%) put into an IVC system are able to develop to the blastocyst stage compared to in vivo matured oocytes (~60%), indicating lasting negative effects of a suboptimal maturation environment (Rizos *et al.* 2002).

Oocyte quality is directly linked to embryo quality (Brevini *et al.* 2005). Studies have shown that there are many influences on bovine oocyte quality that cannot be controlled including animal age—adult cattle producing higher quality oocytes than prepubertal heifers; follicle size—small follicles producing lower quality oocytes; metabolic status of the oocyte

donor—high producing dairy cows produce lower quality oocytes that non-lactating cows; nutrition—wild bovid populations in winter in negative energy balance due to low forage availability (Rizos *et al.* 2005; Vieira *et al.* 2014). However, when working with a valuable population it is critical to be able to salvage oocytes from as many genetically distinct individuals as possible, therefore, it is important to continue improving IVM protocols to obtain maximum oocyte competence and quality regardless of the conditions the donor animals are experiencing when oocytes are.

Cryobiology

Cryopreservation of cells, embryos, and tissue is an essential technology for fertility and genetic preservation of individuals and species. Cryopreservation of male gametes for reproductive preservation dates back to the eighteenth century. In 1776, an Italian scientist named Lazzaro Spallanzani froze sperm from multiple species and successfully bred a dog by artificial insemination using frozen sperm (Spallanzani 1776). Standard methods for freezing semen used in domestic species (eg. bull, ram, pig) have been adapted for use in exotic species of similar taxa, particularly bovids, with reasonable success (Herrick *et al.* 2004). Some cryoprotectants, including glycerol, can be used for a wide range of species, from wild cats and elephants to bison (Crosier *et al.* 2006; Saragusty *et al.* 2009; Vilela *et al.* 2017). Semen collection from epididymal specimens, post-mortem, can be effectively frozen for most bovid species, and this increases the ability to preserve genetic material in the event of an unexpected loss of a valuable breeding male (Vilela *et al.* 2017).

Embryo, oocyte, and tissue preservation is most successful using vitrification to maintain ultrastructure, morphology, and cellular viability (Barrett and Woodruff 2010). Typically, vitrification requires high cryoprotective agent (CPA) concentrations. Some of these CPA's

permeate the cell and others do not permeate the cell but cause cellular dehydration. In vitrification, the medium and tissue are converted to a solid glass state during ultra-rapid cooling, thus preventing intracellular ice crystals and cellular damage (Kagawa *et al.* 2009). Permeating CPA's such as dimethyl sulfoxide (DMSO), ethylene glycol (EG), and glycerol bind to free intracellular water and interact with cytoplasmic organelles such as mitochondria and the endoplasmic reticulum (Fahy *et al.* 1990). Nonpermeating CPA's including sugars such as sucrose, galactose, and trehalose increase extracellular osmolality causing dehydration of the cells (Posillico *et al.* 2010). Addition of nonpermeating CPA's or combinations of multiple permeating CPA's to vitrification media enables the concentrations of the more toxic permeating cryoprotectants to be reduced, in attempts to limit cytotoxicity exposure during vitrification and warming (Liebermann *et al.* 2002). There is a large body of literature investigating various combinations and concentrations of cryoprotectants, length of exposure, and cooling rates to improve post thaw viability of gametes, embryos and tissues for a wide variety of species.

For many species, particularly hoofstock, the lipid content of oocytes and embryos can affect cryotolerance and should be taken into consideration when determining a vitrification protocol. Studies have shown there are four classes of lipid found in both cellular droplets and cell membranes that can affect embryo cryotolerance: triacylglycerides, phospholipids, free fatty acids, and cholesterol (Sudano *et al.* 2012). The lipid droplets observed in IVP embryos are triacylglycerides and are an important energy source for oocytes and embryos (Sturmey *et al.* 2009). Phospholipids are the most abundant lipid in eukaryotic cell membranes. Cell membrane damage effecting fluidity and permeability is often observed during cryopreservation. Interestingly, phospholipid profiles differ between bovine subspecies and between IVP versus in vivo produced embryos suggesting that it is not just lipid amount but lipid composition that may affect

cryopreservation outcomes (Sudano *et al.* 2012). Bovid embryos and oocytes have much higher cytoplasmic lipid content compared to murine and human models, making them less cryotolerant.

IVP embryos are known to have higher levels of cytoplasmic lipid content than in vivo derived embryos (Barceló-Fimbres and Seidel Jr 2011). Increased lipid accumulation in embryos may be due to altered energy metabolism, in which excess glucose is created via glycolysis with inhibition of oxidative phosphorylation caused by rising concentrations of lipid synthesis precursors (Rieger 1992; Sudano *et al.* 2011). This imbalance in the oxidation-reduction (redox) reaction affects mitochondria metabolism and impairs lipid metabolism (De La Torre-Sanchez *et al.* 2006; Barcelo-Fimbres and Seidel Jr 2007). The reduced cryotolerance associated with lipid accumulation in cytoplasm is thought to be caused by addition of fetal calf serum (FCS) to in vitro culture media (Abe *et al.* 2002). Reduction of FCS in culture media has been shown to reduce intracellular lipid accumulation, apoptosis, and improve cryotolerance. In multiple studies, the use of serum-free chemically defined media with bovine serum albumin (BSA), a more defined protein source, has resulted in increased cryotolerance and higher post-thaw survival rates of bovine embryos (De La Torre-Sanchez *et al.* 2006; Sudano *et al.* 2011). However, in vivo controls still exhibited lower lipid accumulation than in vitro produced embryos (Sudano *et al.* 2011). Increased peroxidation of higher concentrations of lipid in IVP embryos may cause the observed increase in production of ROS and increased apoptosis, thus reducing embryo quality (Sudano *et al.* 2012). High lipid content due to metabolic dysfunction may be reducing initial oocyte quality, thereby producing an inferior quality, less cryotolerant embryo (Sudano *et al.* 2011). Despite these challenges over the past 30 years, ~7,800,000 bovine cryopreserved IVP derived embryos have been transferred, with recent pregnancy rates reaching ~60% using both slow freeze and vitrification methods (Ferré *et al.* 2020).

A limiting factor for ART in conservation programs is the availability of high-quality oocytes. Oocyte vitrification in cattle is still considered experimental, with reduced survival and quality of embryos resulting from vitrified oocytes compared to fresh (Zhou *et al.* 2010; Gutnisky *et al.* 2020). While vitrification has been successfully used for human oocyte preservation, differences between human and bovine oocyte characteristics make bovine oocyte freezing more challenging. Bovine oocytes have higher intracellular lipid content and lipid-rich plasma membranes, which is associated with reduced cryotolerance, making them more sensitive to cytoskeletal damage (Hwang and Hochi 2014). This is particularly important in oocytes because it effects meiotic spindle structure and microtubule configuration which affects important organelle distribution during maturation such as mitochondria needed for adequate ATP production to provide cellular energy requirements during maturation and fertilization (Gutnisky *et al.* 2020). Vitrification has been shown to affect oocyte ultrastructure, metabolic processes, particularly by affecting mitochondria, the most abundant organelle in the oocyte, proportion of active mitochondria and ROS levels, which could be responsible for reduced competence (Cree *et al.* 2015; Gutnisky *et al.* 2020). Mitochondria affect oocyte quality by providing ATP, ROS production and regulate calcium signaling to support proper chromosome segregation, sperm binding, fertilization, and ongoing embryo development (Reynier *et al.* 2001; Van Blerkom 2004; Cree *et al.* 2015). Effects on mitochondria in bovine oocytes showing large biological variability between and within individuals (Cree *et al.* 2015).

It is currently more common to freeze mature (MII) stage oocytes, however discrepancies regarding the optimal stage for bovine oocyte vitrification is still debated, with benefits and obstacles associated with freezing both immature (GV) and MII stage oocytes (Mogas 2019). In GV oocytes, damage to ultrastructure caused by decreased membrane permeability as well as

cumulus-oocyte disruption caused by damage to gap junction integrity (Magnusson *et al.* 2008). Damage occurring in MII oocytes is predominantly associated with microtubule depolymerization, abnormal meiotic spindle assembly, and chromosomal misalignment (Brambillasca *et al.* 2013).

In cattle, initial oocyte viability after vitrification-warming does not significantly differ to meiotic stage, 94% (GV) versus 93% (MII) (Zhou *et al.* 2010; Gutnisky *et al.* 2020).

However, some studies report increased developmental potential of MII over GV bovine oocytes, with blastocyst development occurring only in groups vitrified after IVM (Diez *et al.* 2005). Others reported opposite findings, showing higher developmental potential of cumulus enclosed bovine GV oocytes (cleavage: 66%; blastocyst 11%) versus in vitro matured MII oocytes (cleavage: 35%; blastocyst 5%) (Zhou *et al.* 2010).

Ongoing contradictory findings in the literature make the optimal meiotic stage for bovine oocyte vitrification unclear and suggests that this subject requires further investigation (Mogas 2019). Most studies to improve bovine oocyte vitrification protocols involve the manipulation of CPAs and CPA exposure times, cryodevices, or by altering cellular or membrane characteristics in attempt to minimize cytotoxicity while optimizing permeation of CPAs (Yamada *et al.* 2007; Magnusson *et al.* 2008; Nohalez *et al.* 2015b; Sprícigo *et al.* 2015; Monteiro *et al.* 2017; Mogas 2019). Embryo freezing is currently more successful and therefore more routinely performed, even when embryo transfer protocols have not been optimized or even tried for a species. However, preservation of oocytes is still ideal for many reasons for genetic preservation in non-domestic species.

Cryopreserving ovarian cortical tissue has the potential to preserve thousands of oocytes at a time. Oocytes from this tissue source represent a large and otherwise inaccessible reserve of

genetic material that could be activated at a later date when species-specific IVF technologies have been developed. The ability to preserve ovarian tissue and then activate follicles would provide a mechanism to collect considerably more oocytes from individuals of any age of a given species. Primordial follicles are cryotolerant due to their small size, limited cellular differentiation, metabolic quiescence, and the oocyte's lack of a zona pellucida (Khosravi *et al.* 2013).

Ovarian tissue cryopreservation is an important initial step in pursuing experimental fertility preservation methods. The most effective technique for tissue cryopreservation is vitrification of surgically removed ovarian cortical tissue fragments (Kagawa *et al.* 2009). To date, the most common strategy to restore fertility using vitrified-warmed cortical tissue is to transplant the tissue back into the ovary or under the skin of the forearm of the patient, where exogenous hormonal stimulation can trigger resumption of folliculogenesis, in vivo follicle activation, and endocrine function restoration, post transplantation (Silber *et al.* 2010; Ford *et al.* 2020). This strategy is used clinically, particularly in patients with diminished ovarian reserve (DOR) or those undergoing cancer treatments that cause fertility loss and resulted in over 130 live births (Ford *et al.* 2020). Revascularization of transplanted tissue has been greatest challenge in this technique (Ford *et al.* 2020).

In vitro follicle growth (IVFG) is another experimental technique for supporting folliculogenesis in follicles derived from excised cortical fragments. Live follicles must be isolated from tissue either mechanically or enzymatically. The best outcomes for IVFG require 3D structural support to maintain proper special development. This has been achieved through encapsulation of isolated follicles within a biological matrix made of materials such as fibrin, collagen, and alginate (Laronda *et al.* 2014). These models allow for the structural growth and hormonal fluctuations from immature follicles through to the antral stage and can mimic the

structural environment provided by the follicle in vivo (Laronda *et al.* 2014; Skory *et al.* 2015; Xiao *et al.* 2015) with whole cortical fragments or isolated follicles can be grown in vitro this way IVFG. Alternatively, utilization of a scaffolding from either decellularized or artificial ovarian matrix and seeded with isolated ovarian cells resulted in the restoration of endocrine function and lead to the onset of puberty in mice (Laronda *et al.* 2015; Guzel and Oktem 2017; Laronda *et al.* 2017b). In the future, emerging technologies such as the use of primordial germ cells (PGCs) or induced pluripotent stem cells (IPSc) from embryonic stem cells capable of differentiating into oocyte-like cells may be used to reseed these types of ovarian scaffolds (Lan *et al.* 2013; Sasaki *et al.* 2015; Morohaku 2019).

Cryopreserved cortical tissue is a tremendous source of primordial follicles. Utilization of these immature follicles to try to attain competent oocytes for use in ARTs requires follicular activation. Primordial follicle in vitro activation (IVA) techniques are still in developmental stages (Ford *et al.* 2020). Producing developmentally competent oocytes from IVA primordial follicles has not yet been achieved in the human clinical setting. It has been observed that culture of fragmented cortical tissue leads to uncontrolled spontaneous primordial follicle activation (Bertoldo *et al.* 2018). This global activation has been attributed to disruptions of the Hippo signaling pathway due to mechanical signaling and tissue fragmentation mediated by the PI3K/Akt/PTEN pathway (Grosbois and Demeestere 2018). Studies using various chemicals known to target these pathways along are ongoing, and continued advances must be made research to understand control of these mechanisms to be able to inhibit activation until the appropriate time to optimize in vitro culture conditions (Bertoldo *et al.* 2018; Grosbois and Demeestere 2018).

Our current understanding of the depletion of ovarian reserve is routed in the concept that the pool primordial follicles is finite—it cannot be regenerated or replaced. Recent discoveries

have challenged this understanding by identification of stem cells capable of differentiating into multiple cell lineages including oocyte-like cells in vitro, known as oogonial stem cells (OSCs). OSCs have been found within the mouse ovary and are suggested to be able to give rise to functional oocytes under in vitro conditions (Albertini 2020). There is some evidence of the presence of OSCs in human ovaries, however, these findings are controversial and have not been consistently repeatable(Grieve *et al.* 2015; Martin *et al.* 2019; Wagner *et al.* 2020). In cattle, there are preliminary studies asserting the use of OSCs to differentiate oocyte-like cells (OLCs) in vitro that express mRNAs for germ cell and oocyte markers (Dunlop *et al.* 2014; de Souza *et al.* 2017). Obtaining high quality oocytes is often the rate limiting step in the application of ARTs for fertility or genetic preservation. These technologies to create gametes in the laboratory may yet become the forefront of ART using cryopreserved tissue as a source for pluripotent cells.

Open carrier systems utilize carrier devices designed to allow direct contact of the biological sample with liquid nitrogen and can achieve cooling rates of ~17,000°C/min and warming rates of ~ 23,000°C/min at 38°C (Kagawa *et al.* 2009; Youm *et al.* 2017). These ultra-rapid cooling and warming rates allow almost instantaneous transitioning through the critical temperature zone (between 15°C and -5°C) causing less cellular damage due to chilling injury and osmotic fluctuations (Massip 2003). In 2009, bovine ovarian tissue was successfully vitrified using the Cryotissue method, warmed, and transplanted back to the female from which it was taken resulting in maintained stromal and follicular viability and resumed endocrine function leading to estrous cycles 2 months post-transplant (Kagawa *et al.* 2009).

For research purposes, evaluation of efficacy of cryopreservation can be accomplished by immediately assessing follicle morphology post-warming, assessing follicle growth and

activation in vitro culture, auto-, allo-, or xenotransplantation success, tissue grafting onto the chorioallantoic membrane (CAM) of chicken embryos, or recently through in vitro follicle growth (IVFG) culture systems (Khosravi *et al.* 2013; Beck *et al.* 2020). Fortunately, research of ovarian tissue cryopreservation using Bovidae species (bovine, caprine, ovine) has occurred more than in other non-murine models, as their similar ovarian size, fibrotic structures and duration of folliculogenesis make them important experimental models for humans. Cryopreserved ovarian tissue from wombats, elephants, wallabies, and lions, has been xenotransplanted into immunodeficient mice, and all cases observed morphologically normal secondary and antral follicles in grafted tissue. Tissue grafts resulted in resumption of estradiol production within 2-4 months in cows, sheep, and goats (Baird *et al.* 1999; Santos *et al.* 2009). Studies cited ischemia as the main reason for follicular loss after tissue grafting (17%) rather than effects of cryopreservation (7%). In vitro follicle activation (IVA) methods as previously described can be used with vitrified-warmed tissue to increase primordial follicle activation. Live births have been achieved in mice and humans from oocytes derived using IVA vitrified-warmed cortical pieces (Kawashima and Kawamura 2018). Unfortunately, due to physiological size limitations, xenotransplantation of tissue in immune deficient mice cannot support bovid follicle growth through to the ovulatory state, therefore there is still the need for IVM, IVF, and IVP technologies to be in place before this approach can be realized in practice.

In conclusion, cryopreservation techniques for embryos, gametes, and tissue is essential for fertility and genetic preservation of species. Vitrification is an easy cryopreservation technique that does not require technical equipment (e.g. expensive large programmable freezers) and can be conducted almost anywhere there is access to an available LN_2 supply. This is beneficial for many CBP if an animal dies unexpectedly in the field. Genetic material from a

single individual can be incorporated into the population for years to come, increasing the genetic potential of an individual that may have otherwise been lost.

Genetic Resource Banks

In the mid-eighties, an idea was circulated by zoos calling for international collaboration to act collectively as a global genetic reservoir for species that were losing biodiversity and experiencing population declines due to habitat loss with the goal of augmenting populations (Lees and Wilcken 2009). This plan proved to be difficult to execute due to required resources and space. Instead, many zoos focused their conservation efforts into other areas including education, research and resource funding for in situ conservation projects (Lees and Wilcken 2009). This paradigm has since been translated into a less space-intensive concept, the genetic resource bank (GRB), sometimes referred to as a 'frozen zoo' (Lees and Wilcken 2009). GRBs have been embraced as critical for the preservation of plants and animal genetics, for both agricultural and wildlife conservation purposes (Holt *et al.* 1996; Long *et al.* 2003).

Along with genetic rescue and augmenting the scope of CBPs globally, biorepositories of germ cells and embryos provide a level of insurance to protect current gene diversity in existing species and against further loss of biodiversity or species extinction (Comizzoli 2015). CPBs often require the movement of live animals which can be restricted to only regional animal translocation. The combined use of ART and GRBs can achieve potentially similar, if not greater, levels of heterozygosity, particularly in small populations by instead transferring embryos or germplasm, increasing the effective population size (Santos *et al.* 2010; Herrick 2019). In fact, studies estimate using frozen sperm and AI has the potential to reduce the number of animals needed in zoos or CBPs by half for some species (Holt *et al.* 1996; Wildt *et al.* 1997). GRBs increase the impact a single individual can have on a population in years or decades after

preservation, increasing the ability to infuse founder genetics into small separated captive populations continually (Roldan *et al.* 2006; Lueders and Allen 2020). The ability to preserve genetic material from animals living or dead, both wild and currently in CBPs, without the need to remove that individual from a wild population truly is "fertility preservation". It allows for the immediate rescue of gametes from animals of any age or maturity status that die unexpectedly and to preserve them until the appropriate technology is available to utilize them effectively (Wildt 1992; Vilela *et al.* 2017).

The Black-footed ferret is an example of particular note. Once considered the most endangered mammal in North America, this species was brought back from the edge of extinction through captive breeding using AI and cryopreserved sperm from 20 years earlier. In this case, a biorepository of frozen germplasm effectively reestablished a population that would otherwise have never recovered without intervention through ART and specifically GRBs. GRBs containing sperm or embryos are now currently in use for a variety of nondomestic and domestic species (Huang *et al.* 2012; Holt 2016; De Oliveira Silva *et al.* 2019). The concept of GRBs is ever expanding in the context of conservation. The San Diego Zoo's Frozen Zoo® not only contains embryos and germ cells, but now contains induced pluripotent stem cells from species including the Northern White Rhino. As genetic technologies advance, these cells may play a role in in vitro development of embryos through nuclear transfer technologies or generation of gametes from somatic cells (Tunstall *et al.* 2018; Ryder *et al.* 2020).

Conclusions

Increasing reproductive knowledge through basic science techniques is the only way to move the technologies described here out of experimental zone and into practice. The diversity among species emphasizes the importance of studying as many taxa as possible to elucidate

mechanisms and techniques that may be applicable to species in the future. Continued research of conserved pathways may be applicable to many species because studying both the differences and similarities between species is critical to applying research findings in a field where resource material is often scarce. Application of expertise from various labs and institutions for the collective benefit will have the largest scientific and conservation impact (Herrick 2019). For bison, cattle and other wild and domestic bovids, this could increase the chance of maintaining some of the world's invaluable genetic diversity.

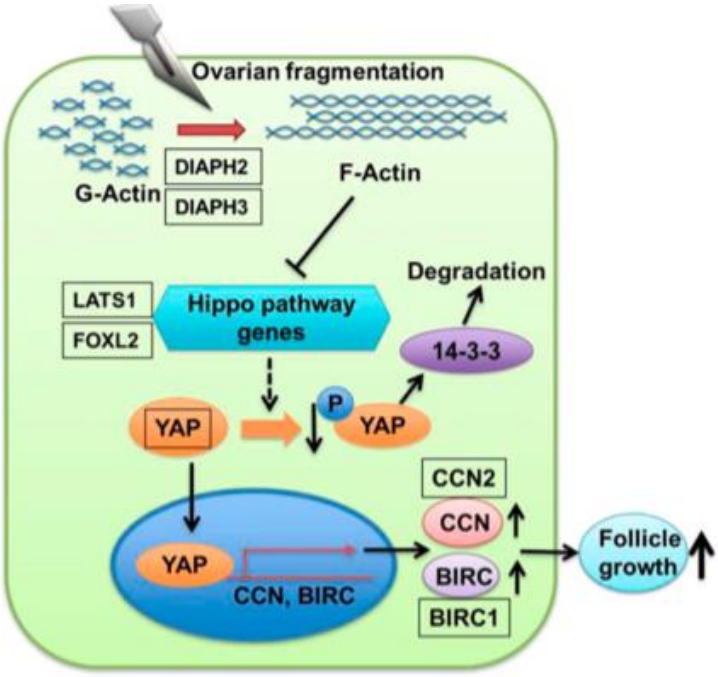

Figure 1.1. Disruption of Hippo signaling pathway via tissue fragmentation.

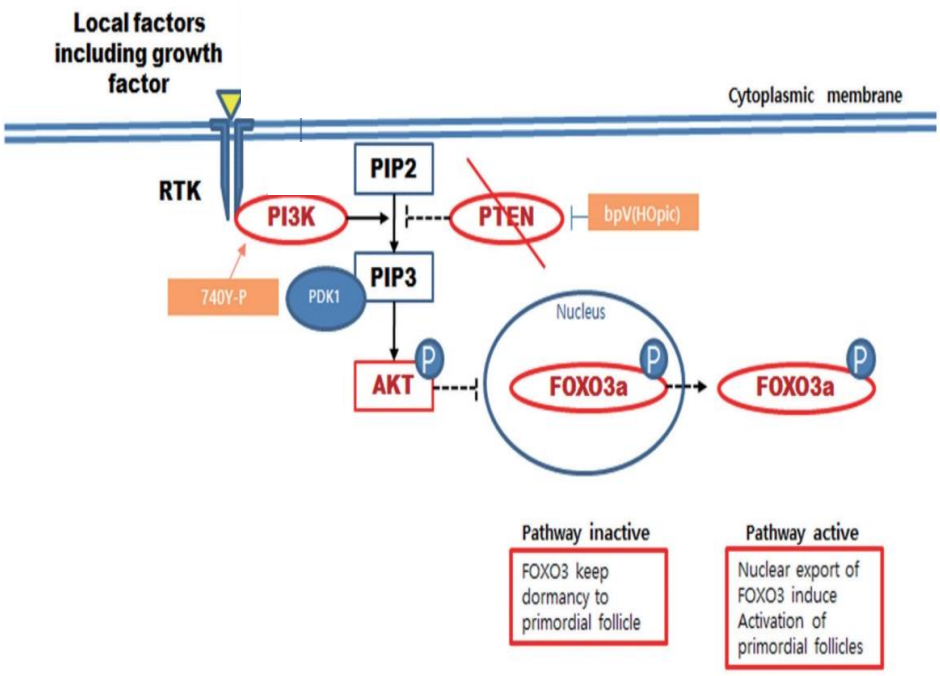

Figure 1.2. Activation of PI3K-Akt-PTEN signaling pathway via PI3K activator, 740 Y-P, and PTEN inhibitor, bpV(pic).

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CHAPTER 2: PRODUCTION OF EMBRYOS AND A LIVE OFFSPRING USING POST MORTEM REPRODUCTIVE MATERIAL FROM BISON *(Bison bison bison)* ORIGINATING IN YELLOWSTONE NATIONAL PARK, USA[1][2]

Summary

Bison from Yellowstone National Park (YNP) have an important genetic history. As one of the few wild herds of bison with no evidence of cattle DNA introgression and a large enough population to maintain genetic diversity, they are considered a conservation priority for the species. Unfortunately, there is a high prevalence of the zoonotic disease brucellosis in the herd. Part of the management strategy for controlling the disease and herd size in YNP is to remove bison from the population during the winter migration out of the park. This interagency management cull provides an opportunity to collect a large number of oocytes from a wild bison population for genetic banking and research purposes. During the winters of 2014-2018, which is the nonbreeding season for bison, oocytes were collected post mortem and used to determine the effects of donor reproductive maturity and pregnancy status on oocyte quality and *in vitro* fertilization (IVF) outcomes, and to demonstrate the feasibility of producing healthy offspring. Cumulus oocyte complexes (COCs) were placed into an *in vitro* embryo production (IVP) system, and on days 7, 7.5, and 8 of *in vitro* culture (Day 0 = day of in vitro fertilization) embryos were assessed for developmental stage and quality prior to vitrification. Embryos were then stored in liquid nitrogen until the breeding season when a subset were warmed, cultured for 6 h, evaluated for survival, and transferred to healthy bison recipients. There were no significant differences in the ability of recovered COCs to support blastocyst development based on female

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reproductive maturity or pregnancy status (juvenile 79/959 (8.2%) vs sexually mature 547/6544 (8.4%); non-pregnant 188/2302 (8.2%) vs pregnant 556/6122 (9.1%)). Following the transfer of 12 embryos to 10 recipients, one healthy female calf was born. This work demonstrates that live offspring can be generated from COCs collected from YNP bison post mortem in the non-breeding season, and that gamete recovery can be a valuable tool for conservation of valuable genetics for this species.

Introduction

North American bison (*Bison bison*) are an iconic species of cultural, conservation, and commercial importance. In the late 1800's, expansion of transcontinental railroads increased the impact of anthropogenic threats such as overhunting and competition for natural resources from domestic livestock, resulting in a drastic reduction of the bison population to fewer than 100 individuals on 5 private ranches, and a small population of wild plains bison (*Bison bison bison*) remaining in Yellowstone National Park (YNP) (Roe 1938; Meagher 1973; Coder 1975; Abe *et al.* 2002; Hedrick 2009). Today's bison population of approximately 500,000 animals descended from these foundation herds. Despite this impressive comeback, bison are still considered near threatened by the International Union for Conservation of Nature (IUCN) red list, with only 31,000 animals in conservation herds that are defined as wild (a population size large enough to prevent genetic loss and subject to the forces of natural selection) (Gogan and Dratch 2010; Aune *et al.* 2017). In addition, at the time when the 5 original foundation herds were being established, ranchers were experimenting with bison-cattle (*Bos taurus)* hybrid crosses to improve range hardiness in their cattle herds. These human-facilitated crosses, and possibly some naturally occurring ones, resulted in cattle DNA introgression in herds that became the foundation stock for many of the bison in existence today. In fact, a 2007 study reported that

evidence of cattle introgression was found in nearly half of US and Canadian public herds (6 of 14), and all except 1 of 50 private herds examined (Halbert and Derr 2007). As hybrid species are exempt from taxonomic status and are not recognized by the U.S. Endangered Species Act, as well as for their cultural significance, bison without cattle gene introgression are considered a valuable source for germplasm and are a conservation priority for the species.

The YNP bison population is considered a particularly valuable genetic resource because it has the largest population of plains bison (> 3,000 individuals) of the 4 US federal herds with no evidence of having bred with cattle, and the bison are considered wild (Halbert and Derr 2007). Unfortunately, removing bison from YNP for conservation and cultural purposes has been limited due to the high prevalence of brucellosis, a zoonotic bacterial disease that causes abortions in livestock and wildlife, and long-term illness in humans (Rhyan *et al.* 2013). This negatively impacts the ability of herd managers to introduce bison with valuable YNP genetics into existing herds or during the establishment of new herds without an extended quarantine process which can be costly and labor intensive.

Advances in reproductive technologies including cryopreservation of germplasm have made genetic resource banking an invaluable tool in conservation biology. We now have the ability to preserve many species, both exotic and domestic, using frozen reproductive cells, tissues, and embryos that have been stored for extended periods of time (Wiidt 1992; Holt *et al.* 1996; Comizzoli 2015). In addition to these techniques, *in vitro* production of embryos may provide a means to conserve valuable genetics in threatened bison populations while mitigating the transmission of brucellosis (MacPhee and Adams 2016). *In vitro*- produced embryos from potentially diseased animals can be washed free of bacterial pathogens using a protocol described by the International Embryo Technology Society (IETS) and then transferred to

disease-free recipients, thereby virtually eliminating the risk of transferring brucellosis into a healthy herd and allowing propagation of genetically valuable bison (Stringfellow 1998; Stringfellow and Givens 2010). These technologies (in vitro embryo production, embryo washing, cryopreservation and embryo transfer) were combined in this study towards the goal of establishing a biobank of bison embryos with Yellowstone genetics while mitigating the risks of brucellosis.

In vitro embryo production in bison was first reported in wood bison *(Bison bison athabascae*) using protocols based on IVP methods developed for cattle, but blastocyst production was low (<10%) (Thundathil *et al.* 2007; Aurini *et al.* 2009). Although there are few studies of *in vitro* embryo production in bison due to limited sources for abattoir-derived oocytes for research purposes, the existing research suggests that modification of cattle IVP protocols to create bison-specific methods may improve blastocyst production. For example, the addition of 5% fetal calf serum (FCS) to culture medium after the 8-cell stage of embryonic development increases blastocyst development in plains bison (Barfield 2019).

Bison are seasonal breeders, entering estrus during the late summer or early autumn in North America (Rutberg 1984). Seasonality may impact the developmental potential of abattoir-derived oocytes. Embryonic cleavage after *in vitro* fertilization (IVF) is reduced in the non-breeding season, although blastocyst development is not significantly affected for wood bison (Krishnakumar *et al.* 2015; Cervantes *et al.* 2016). However, this demonstration that bison blastocysts can be produced outside the breeding season suggests that material recovered from the annual bison culls from YNP, which takes place in winter, can potentially be used for IVP and embryo transfer to produce live offspring (Krishnakumar *et al.* 2015; Cervantes *et al.* 2016; Barfield 2019).

The study presented here is a retrospective analysis of data generated during the winters (nonbreeding season) of 2014-2018. During this time period, our laboratory, in partnership with United States Department of Agriculture (USDA)-Animal and Plant Health Inspection Service (APHIS), collected reproductive material from bison during the annual cull of YNP bison. Part of the current Interagency Bison Management Plan (IBMP) requires removal of an annually determined number of bison that migrate outside of the park to control population numbers and reduce the risk of transmission of brucellosis from bison to cattle (Interior and Agriculture. 2000; Ryan Clarke *et al.* 2014). Many of these bison go to slaughter, and until now, there has been no attempt to recover and preserve their genetics. As the YNP cull is non-selective (not based on individual animal parameters such as age, sex, disease status, or pregnancy status), we were able compare oocyte quality and IVP outcomes based on reproductive maturity and pregnancy status for a large number of animals. We then assessed the feasibility of producing live offspring from embryos generated through the IVP process. The results presented here support the use of this approach for mitigating the risk of brucellosis while preserving valuable bison genetics and led to the establishment a biobank of embryos with Yellowstone bison genetics.

Material and Methods

Animals

Reproductive material from female bison (ovaries) was recovered from animals culled during the winters of 2014-2018, specifically in the months January - March. Pregnancy status was determined by presence of a fetus in the uterus (pregnant, n=352; non pregnant, n=119), and ovaries grouped accordingly. When possible, animals were aged according to tooth eruption, and ovaries were collected from juveniles (1.5-2.5 y, n=49) and adults (3+ y, n=363). Maturity status was not recorded for animals collected in 2016, therefore oocyte and embryo outcomes resulting

from those collections are only included in data sets categorized by pregnancy status. Ovaries were harvested from 49 juvenile, 62 mature non-pregnant and 301 mature pregnant female bison in the years 2014, 2015, 2017 and 2018, and from 8 non-pregnant and 51 pregnant female bison in 2016, for a total of 471 females. Disease status of the animals was unknown. Semen from YNP bison bulls was collected either via electroejaculation or from epididymal flushes post mortem during the breeding season. All embryo transfers and semen collections were approved by the Animal Care and Use Committee at Colorado State University (CSU), IACUC protocol 17-7117A.

In vitro embryo production

Ovary collection and oocyte recovery

Ovaries were collected within 10 min of slaughter from January to March. Ovaries from individual donors were transported in separate sealed plastic bags containing ~50 mL sterile saline and kept at 25-28°C in an insulated canister. At the time of ovary collection, donor age and pregnancy status were recorded. Cumulus oocyte complexes (COCs) were vacuum aspirated from all visible follicles within 2 h of ovary collection, and COCs were collected and washed through CSU chemically defined medium for handling of oocytes (H-HCDM-M) with 10% BSA and 10 mg/mL gentamicin sulfate (De La Torre-Sanchez *et al.* 2006; Barfield 2019). Visibly degenerate COCs were excluded; no other selection of COCs was done prior to IVM. Aspirates from individual donors were kept separate from each other. Aspirations and COC processing were conducted in indoor, climate-controlled spaces which varied depending on location of bison slaughter (e.g. auxiliary rooms in slaughterhouses or rented spaces).

In vitro maturation, fertilization, and culture

Within 1 h of aspiration, COCs from individual donors were placed in 3 mL blood collection tubes without additives (Monoject™, Covidien, Mansfield, MA, USA), containing 2.5 mL CSU chemically defined medium for in vitro maturation of oocytes (IVM) pre-equilibrated at 38.5°C in 5% CO_2 and shipped by air overnight (De La Torre-Sanchez *et al.* 2006). The same equilibration conditions were used for all media used during embryo culture. IVM medium was supplemented as follows: 15 ng/mL NIDDK-oFSH-20, 1 µg/mL USDA-LH-B-5, 1 µg/mL estradiol 17ß, 50 ng/µL epidermal growth factor, and 0.1 mM cysteamine (De La Torre-Sanchez *et al.* 2006). On occasion, higher numbers of bison were sent to slaughter than anticipated. During these times, COCs from multiple donors were pooled for in vitro maturation if there were not enough materials in the field to allow for COCs from individual donors to be matured separately. If pooled, COCs from donors of similar maturity group and/or pregnancy status were combined when possible. While oocytes from all females were counted, recorded, and fertilized, females whose oocytes were pooled with unlike females or had IVP rounds with missing data points were not included in the analysis. A maximum of 50 COCs were matured in each tube for 23 h during transport in a portable incubator (MicroQ Technologies, Scottsdale, AZ, USA). If the incubator arrived prior to the completion of the 23 h maturation period, tubes were uncapped and transferred to a large box equilibration incubator in the laboratory. Otherwise, oocytes were directly transferred from the IVM tubes, along with no more than 20 µL IVM to a 4-well culture dish containing 430 µL of equilibrated CSU chemically defined medium for in vitro fertilization (FCDM)/well (De La Torre-Sanchez *et al.* 2006). All of the oocytes from one maturation tube were put into the same fertilization well (50 COCs/well) and held in the equilibration incubator until sperm was added.

Semen from YNP bison bulls was frozen in 20% egg yolk tris + 8% glycerol at ~60 million cells/mL (Vilela *et al.* 2017). Motile sperm were isolated by separating thawed semen through a 45/90 Percoll® gradient (Parrish *et al.* 1995). Post thaw progressive motility after separation ranged from 60 to 70% prior to use in IVF. Sperm were added to COCs at a final concentration of $0.5x10^6$ sperm/mL, and co-incubated in a humidified atmosphere of 5% CO_2 in air at 38.5°C for 18 h. During the 5 y period, semen from singles sires (n=10 males) or pooled semen from 2 bulls (n=6 pools) were used for IVF, for approximately 35% and 65% of fertilizations, respectively. Semen from all bulls had been used successfully in our laboratory to generate bison IVP embryos.

Following IVF, presumptive zygotes were mechanically stripped of cumulus cells by gentle micropipetting using a STRIPPER® and 150 µm and 135 µm stripper tips (Origio, CooperGenomics, Denmark). Denuded zygotes were washed through a series of CSU chemically defined medium for handling of early embryos (HCDM-1) drops until wash medium was free of loose cumulus cells and remaining spermatozoa (De La Torre-Sanchez *et al.* 2006). Cleaned zygotes were placed into wells containing 500 µL CSU chemically defined medium for in vitro culture of early embryos (CDM-1) and cultured for 56 h in a humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38.5°C (De La Torre-Sanchez *et al.* 2006). The number of embryos per well ranged from 1 to 50. Embryo cleavage was recorded after 56 h of culture. At this time, embryos containing ≥ 4 blastomeres were placed in 500 µL CSU chemically defined medium for in vitro culture of late embryos (CDM-2) + 5% FCS/well and cultured for an additional 120 h in a humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38.5°C (De La Torre-Sanchez *et al.* 2006). The maximum number of embryos per well was 30. Embryos were assessed for blastocyst development on day 7(96 h), 7.5 (108 h), and 8 (120 h) post insemination. Blastocysts were

evaluated for stage and graded morphologically in accordance with IETS guidelines (Stringfellow and Givens 2010). All spent CDM-2 media and degenerate embryos from the IVP process were cultured for presence of *Brucella abortus*.

Embryo vitrification and warming

Grade 1 and 2 early blastocysts, blastocysts, and expanded blastocysts were vitrified using a two-step equilibration process. Vitrification solutions were prepared using a base medium of H1-Mod medium (H1-Mod; H-CDM-1 + 20% FCS). Embryos were placed in an equilibration solution (V1) of 1.5 M ethylene glycol in H1-Mod for 5 min, followed by ≤ 30 s in a vitrification solution (V2) of 7 M ethylene glycol, 0.6 M galactose, and 18% ficoll (w/v) in H1-Mod (Campos-Chillon *et al.* 2009). Embryos were placed onto Cryotop® devices (Kitazato, Japan) in <1 µL V2 and plunged into liquid nitrogen (LN_2) (Campos-Chillon *et al.* 2009).

Prior to transfer into bison cows, embryos were warmed using a 4-step dilution with the following solutions:1 M galactose in H1-Mod (W1), 0.5 M galactose in H1-Mod (W2), 0.25 M galactose in H1-Mod (W3), and finally H1-Mod. First, embryos were plunged directly into 1 mL of 38.5°C W1 for 3 min. Embryos were then moved in succession into 1 mL 38.5°C W2, W3, and H1-Mod for 3 min each. After warming, embryos were incubated in CDM2+5% FCS for ≤ 6 h prior to transfer to confirm viability and blastocoel re-expansion. All embryos that were not warmed remain in cryo-storage at Colorado State University and are effectively a biobank of Yellowstone bison embryos.

Embryo washing

As the disease status of YNP bison donors was unknown, appropriate embryo washing techniques per the IETS were used to prevent transmission of pathogens from donor to recipient

(Stringfellow 1998; Stringfellow and Givens 2010). After warming and incubation prior to embryo transfer, all embryos were passed through 10 sequential washes. Embryos were held in the first wash which contained 0.25% trypsin in Ham's F-12 medium for 60 s. All subsequent washes were done in 1 mL of HCDM-2. Embryos were moved between washes in 1-2 µL of fluid or less resulting in a 1:1000 dilution of the embryo for each wash. This is a more stringent wash than the required minimum dilution of 1:100 according to IETS standards to ensure that bacteria potentially present on the zona pellucida (ZP) or in the surrounding fluid are eliminated. The ZP is considered an efficient barrier to microorganisms, so only embryos with an intact ZP were washed and transferred (Stringfellow 1998; Stringfellow and Givens 2010).

Recipient synchronization and embryo transfer

Ten bison cow recipients housed on the Foothills Campus of Colorado State University were synchronized for embryo transfer during the breeding season of 2016. While embryos were produced over multiple years, embryo transfers were only conducted in August of 2016 because of the limited availability of bison cows in a university research herd which was demonstrated to be brucellosis-free through repeated testing over a minimum of 2 years and were proven mothers. Each female received an intravaginal progesterone releasing device sixteen and a half days prior to embryo transfer (EAZI-BREED™CIDR®, Zoetis,USA), and were simultaneously administered 100 µg gonadotropin-releasing hormone (GnRH) intramuscular (im) (Cystorelin®, Merial Ltd., Duluth, GA). The CIDRs were removed 7 d later at which time animals received 25 mg prostaglandin F-2-alpha (PG) im (Lutalyse®, Zoetis,USA). Nine and a half days after CIDR removal and PG injection, 1 (n=5 recipients) or 2 (n=5 recipients) warmed embryos were transferred to recipients with a palpable corpus luteum. The highest quality embryos as evaluated after thawing and a short culture period (as indicated above) were transferred as single embryos

while lower quality embryos were paired for transfer to maximize potential pregnancy rates. Only grade 1 and 2 embryos were transferred as evaluated in accordance with IETS guidelines (Stringfellow and Givens 2010). While other synchronization methods used with bison have been previously described, they require daily or frequent animal handling and examination (Landry *et al.* 2016). As all of our recipients are eventually released back onto the landscape where they may have contact with the public, we try to handle them as little as possible. The protocol used in this study only required the animals being handled twice prior to embryo transfer. Recipient cows were housed separately from males, and had no exposure to males prior to or after embryo transfer and were not released from the facility until the completion of this project. Recipients were not evaluated for pregnancy status via palpation or ultrasound following embryo transfer to minimize stress and handling. Rather, delivery dates were estimated and visual cues (development of the udder, swelling of vulva) were used to indicate pregnancy and impending parturition.

Statistical analyses

COC recovery and donor parameters (maturity and pregnancy status) were compared by unpaired Student's t-test. Embryo cleavage and blastocyst production percentages was compared to donor parameters by 2-tailed Chi-square test. COC recovery rates are expressed as a mean ± SEM and cleavage and blastocyst production percentages as a proportion. P-values of <0.05 were considered significant. All statistics were performed using GraphPad Prism® 7.0d (GraphPad Software, La Jolla, CA, USA).

Results

Effects of donor maturity on oocyte recovery and embryo production

During the five-year sampling period, a total of 959 COCs from 49 juvenile heifers and 6544 COCs from 363 sexually mature cows were collected and used for in vitro embryo

production. The mean number of COCs collected per animal was similar in juvenile and mature females (P>0.05; Table 1). For oocytes that were used in the IVP analysis (as described), the proportion of COCs from juvenile bison that cleaved after IVF was lower than that for mature bison; however, there were no differences in the proportions of blastocysts per oocyte or blastocysts per cleaved embryo based on donor maturity (Table 1).

Effects of donor pregnancy status on oocyte recovery and embryo production

During the five year sampling period, a total of 2302 COCs from 119 non pregnant females and 6122 COCs from 352 pregnant females were collected and used for in vitro embryo production. The mean number of COCs collected per animal was higher in non-pregnant than in pregnant females (P<0.05; Table 2). While the proportion of COCs from pregnant bison that cleaved after IVF was greater than that for non-pregnant bison (P<0.05), there were no significant differences in proportions of blastocyst development per oocyte or per cleaved embryo based on donor pregnancy status (P<0.05; Table 2).

Live offspring produced from IVP embryos

All embryos warmed for embryo transfer were grade 1 or 2 and a blastocyst or expanded blastocyst at the time of vitrification. Following warming and a short culture grades ranged from 1 to 3 (5 grade 1, 8 grade 2, and 2 grade 3). In May of 2017, a healthy female calf was born to a recipient cow that had received two grade 2 blastocysts, as evaluated post warming and culture, via embryo transfer in August of 2016 (Figure 1). At the time of vitrification these two embryos were classified as a grade 1 blastocyst and a grade 2 blastocyst. Genetic testing was performed by Texas A&M University to confirm that the heifer calf born was genetically unrelated to the recipient cow, and indeed the result of embryo transfer. The genetic dam of the calf was a

pregnant mature bison cow that was harvested in the winter of 2016. Both the recipient cow and calf tested negative for brucellosis 6 months post calving. In addition, all spent media and degenerate embryos were culture negative for *Brucella abortus*. Through this research, a biobank for YNP bison was established containing more than 800 vitrified embryos that remain in cryo-storage at Colorado State University.

Discussion

To our knowledge, this is the first study to report the birth of a live calf after the transfer of IVP embryos derived from reproductive material collected post mortem during the non-breeding season in plains bison. We describe the success of our IVP system over a 5-year period. Oocytes derived from both juvenile and mature females, as well as open and pregnant females, can be used successfully for blastocyst production. Pregnancy status had a small negative impact on average COC recovered per female, possibly because the large corpus luteum (CL) present on the ovary in pregnant animals decreased surface area on the ovary for developing follicles to occupy. Neither pregnancy nor maturity status impacted the overall number of blastocysts produced per recovered COC. As is routinely conducted in cattle, these results demonstrate that COCs recovered from post mortem ovaries can be effectively used in an in vitro embryo production system, and the resulting blastocysts vitrified for future embryo transfer. Based on these results, and considering the conservation and cultural value of the Yellowstone bison population, it is important that reproductive material continue to be collected from these genetically valuable females during the annual prescribed winter cull.

Studies in cattle have similarly shown that there is no impact of pregnancy status on IVP outcomes when oocytes are collected from either transvaginal aspiration or from slaughterhouse ovaries (Behboodi *et al.* 1992). These studies similarly reported a reduction in oocyte yield from

pregnant females due to restricted follicular growth on the ovary supporting the CL (Behboodi *et al.* 1992). In cattle, the acquisition of oocyte competence is achieved by 11 months of age, when oocytes derived from calves exhibit embryo cleavage and blastocyst development similar to that of mature cows (Presicce *et al.* 1997). The nulliparous heifers used as donors and classified as juveniles in our study were between the ages of 1.5-2.5 y, and no longer prepubescent. Although higher oocyte yields can be achieved from younger donors, lower developmental competence nullifies this increase and final blastocyst production does not differ between heifers and mature cows (Rizos *et al.* 2005; Landry *et al.* 2016). Similarly, while we did see reduced embryonic cleavage in embryos from juvenile animals, blastocyst development per recovered oocyte did not differ between juveniles and mature cows in this study.

Potential seasonal effect on oocyte quality are an important consideration in this study because samples were collected during the winter cull. Many atretic COCs were discarded during collection. Throughout the anovulatory season, small antral follicles can persist on the ovary for a long period of time, possibly increasing the number of recovered oocytes that are atretic or with poor developmental potential (Rizos *et al.* 2005). A seasonal effect on *in vitro* fertilization success has been described in wood bison, inferring reduced oocyte competence during the non-breeding season (Krishnakumar *et al.* 2015). In contrast to the current study, these oocytes were collected from live, super-stimulated, non-pregnant bison. Approximately 80% of the YNP bison collected post mortem in the nonbreeding season were pregnant and the oocytes had not been exposed to FSH, unlike the wood bison study (Cervantes *et al.* 2016). While there may be a seasonal component to the overall lower blastocyst development reported here, viable blastocysts were generated and cryopreserved each year of collection. Overall cleavage and blastocyst production rates (respectively) per year were as follows: 2014-61%, 8.4%; 2015-51%, 10%;

2016-55%: 12%; 2017-50%, 7%, 2018-59%, 10%. Because of high variability in numbers of animals, collection conditions, technicians conducting the work, and other factors from year to year, data were not analyzed according to year. Low blastocyst development could be due in part to the difficulty in managing unpredictable field conditions during the time of ovary and oocyte collection, particularly adverse weather conditions during the winter in YNP. It is also worth noting that these bison are wild and subject to the stresses of low forage availability in the winter; indeed, this is the reason for their migration out of YNP (Treanor *et al.* 2015). As a result, carcasses evaluated from bison harvested from YNP in winter have been found to be in negative energy and protein balance, with juvenile animals more strongly impacted than adults (Treanor *et al.* 2015).

While the production of a live calf from an IVF-produced embryo is a promising success and proof of concept, continued work in this area is needed to improve pregnancy rates. Species-specific techniques, particularly in nondomestic species, take substantial time and resources to develop, often with a low probability of producing live offspring from newly developed protocols (Herrick 2019). The success of assisted reproductive technology (ART) in these species has relied heavily on work done in domestic species of similar taxa (Sontakke 2018). In this case, research with domestic cattle has provided a tremendous body of work from which to draw technical information. While cattle protocols have provided a strong foundation for bison-specific protocols, the low blastocyst development and pregnancy rates described here suggest that further research is needed to improve these techniques for use in bison. In particular, bison embryos have higher cytoplasmic lipid content than cattle embryos, reducing cryotolerance (Abe *et al.* 2002; Sudano *et al.* 2011; Meneghel *et al.* 2017; Barfield 2019). Developing cryopreservation and embryo warming methods that are more effective with lipid rich embryos

and increase post thaw embryo viability is critical to improve IVP embryo pregnancy outcomes. It is also possible that culturing embryos post warming had an impact on pregnancy rates. In these experiments culturing was done post-thaw to confirm viability of embryos so that only the best embryos were transferred to recipients. The recipient herd size available for this study limited the number of embryo transfers that could be performed. Many more transfers will be needed to evaluate the potential pregnancy rates from embryos generated in this manner; however, the transfers performed here provide proof of principle that this technology can result in live offspring.

As a result of this research, we established a biobank of over 800 embryos for Yellowstone bison. Biobanks or genetic resource banks are an important component of modern conservation strategies. A large number of genetically diverse individuals can be preserved as gametes or embryos, or even tissue, in a small amount of space. This material can be easily transported without the stress of moving animals, and the potential effective population size could be much larger than what exists in current conservation herds (Herrick 2019). Translocating animals is stressful and potentially dangerous for bison, can increase risk of disease transmission, and disrupt a herd's social order (Dickens *et al.* 2010). These technologies are not intended to replace natural breeding for sustainable bison populations (in situ conservation), but instead the value lies in being able to circumvent disease, and preserve and/or recover valuable genetics that can be introduced over time into captive conservation herds (ex situ conservation) to maintain genetic diversity and fitness (Cherry *et al.* 2019; Comizzoli and Holt 2019). For bison from Yellowstone National park, the ability to preserve genetics while mitigating the risks of brucellosis transmission is particularly valuable because of their high conservation and cultural value.

In conclusion, during a 5-year period we were able to harvest and preserve large quantities of material from the genetically valuable Yellowstone bison population without affecting the wild population. Here we provide a method that can be followed when valuable bison die or go to slaughter and which may be useful for other valuable ungulates. We have demonstrated that there is value in collecting oocytes from females across age categories and pregnancy status and those oocytes, collected in the non-breeding season, can result in viable embryos that may lead to live offspring. This work has allowed us to establish a biobank of embryos for Yellowstone bison that continues to grow annually. Continued research is needed to increase the efficiency of the ART techniques described here to realize their full potential in conserving bison.

Table 2.1. Development of in vitro produced (IVP) plains bison embryos produced from cumulus oocyte complexes (COC) collected post mortem from juvenile (1.5-2.5 y) or sexually mature (≥ 3 y) females. Embryo cleavage and blastocyst development per oocyte and per cleaved embryo was collective from Day 8. Number of COC collected presented as mean ± SEM; Day 0 = day of in vitro fertilization.[ab] Within rows, values with different superscripts are different (P<0.05, Chi-square test).

End point	Juvenile	Mature
Number of bison	49	363
Number of COC collected/donor	25.9 ± 2	23.6 ± 1
Cleaved embryos (%)	472/959 (49.2%)[a]	3621/6544 (55.3%)[b]
Blastocysts per COC (%)	79/959 (8.2%)	547/6544 (8.4%)
Blastocysts per cleaved embryo (%)	79/472 (16.7%)	547/3621 (15.1%)

Table 2.2. Development of in vitro produced (IVP) plains bison embryos produced from cumulus oocyte complexes (COC) collected post mortem from either non-pregnant or pregnant females. Embryo cleavage and blastocyst development per oocyte and per cleaved embryo was collective from Day 8. Number of COC collected presented as mean ± SEM; Day 0 = day of in vitro fertilization.[ab] Within rows, values with different superscripts are different (P<0.05, Chi-square test).

End point	Non Pregnant	Pregnant
Number of bison	119	352
Number of COC collected/animal	26.9 ± 2[a]	22.4 ± 1[b]
Cleaved embryos (%)	1135/2302 (49.3%) [a]	3477/6122 (56.8%) [b]
Blastocysts per COC (%)	188/2302 (8.2%)	556/6122 (9.1%)
Blastocysts per cleaved embryo (%)	188/1135 (16.6 %)	556/3477 (16.0%)

Figure 2.1. A healthy female calf (IVF1) was born in May 2017 to a recipient bison cow that received two grade 2 in vitro produced blastocysts at the time of transfer. The genetic dam of the calf was a pregnant mature YNP bison cow that was harvested in winter of 2016. Both the recipient cow and calf tested negative for brucellosis 6 months post calving.

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CHAPTER 3: EFFECT OF REPRODUCTIVE SEASONALITY ON IN VITRO OOCYTE MATURATION AND EMBRYO PRODUCTION IN PLAINS BISON (Bison bison bison)[3]

Summary

The effect of season on in vitro oocyte maturation (IVM) outcomes in plains bison were assessed in relation to follicle size and length of IVM. In experiment 1, nuclear maturation was assessed for oocytes aspirated from small (< 2 mm), medium (2-5 mm), or large (> 5 mm) follicles. In experiment 2, nuclear maturation and embryo development were assessed in oocytes matured in vitro for either 24 h (control) or 29-34 h (extended). The proportion of oocytes reaching the MII stage of nuclear maturation increased with increasing follicular diameter ($P < 0.05$) and differed between seasons ($P<0.05$). Additionally, the proportion of oocytes reaching the MII stage of nuclear maturation increased with extended in vitro maturation time ($P< 0.05$) and was lower in Fall than all other seasons ($P<0.05$). The proportion of blastocyst development was higher using the control maturation time than the extended maturation time ($P< 0.05$). Oocyte cleavage rates were highest in Summer ($P<0.05$) and the mean proportion of blastocyst development differed between Winter and Summer ($P<0.05$), but did not differ when comparing between the other seasons. These studies indicate that nuclear maturation and embryo production can be achieved from oocytes harvested throughout the year, however higher blastocyst rates are achieved when using oocytes harvested in Summer.

Introduction

North American bison (Bison bison) are valued for conservation, cultural, and commercial reasons. Having once roamed the continent in the tens of millions, by the beginning of the

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twentieth century bison populations drastically declined to fewer than 100 animals on private ranches and in national parks (Roe 1938; Meagher 1973; Coder 1975; Hedrick 2009). Today's bison population of approximately 500,000 animals are descended from these small protected populations. Despite this population growth, bison are still considered near threatened by the International Union for Conservation of Nature (IUCN), and only 31,000 animals are in conservation herds that are defined as wild (i.e., a population that is subject to the forces of natural selection and large enough to prevent loss of genetic diversity) (Gogan and Dratch 2010; Aune *et al.* 2017). International interest in preserving genetic diversity from both plains (Bison bison bison) and wood bison (Bison bison athabascae) using assisted reproductive technologies (ART) such as in vitro embryo production (IVP) and germplasm cryopreservation has been growing over the past decades. Of particular interest is the genetic preservation of bison that have valuable genetic lineages and using ART as a means of preventing transmission of diseases such as brucellosis (Cervantes *et al.* 2017a; Vilela *et al.* 2017; Benham *et al.* 2021).

Genetic resource banks (GRB), particularly germplasm biorepositories, are one mechanism of preserving biodiversity. The combination of GRBs and ARTs allows for the maintenance of germplasm and embryos which can be used to improve heterozygosity in herds across the continent and abroad (Comizzoli 2015). However, successful GRBs rely on efficient ART protocols that must be developed on a species by species basis (Herrick 2019). Bison are considered both wildlife and livestock and the ability to access slaughterhouse ovaries or have access to bison in research herds is rare, which has impeded the broad development of ARTs relative to other domestic species. Fortunately, there has been extensive research in ARTs in domestic species of similar taxa, particularly in cattle, which has provided information from which to draw for bison (Barfield 2019; Herrick 2019).

Even when there are relevant domestic model species available, the ability to employ ART for wild animals can be challenging. One such challenge is dealing with issues of reproductive seasonality. Bison are seasonal breeders, entering estrus during the late summer or early autumn in North America (Rutberg 1984). Seasonality is known to effect gamete quality, gamete recovery, and in vitro fertilization success in species such as sheep, water buffalo, and a variety of wildlife species (Koonjaenak *et al.* 2007; Sontakke 2018; Zhu *et al.* 2018), making the timing of access to valuable reproductive material an important factor for potential success. Studies in wood bison indicate that while no seasonal effect is observed in the capacity of oocytes collected from live animals to mature, embryonic cleavage after in vitro fertilization (IVF) is reduced in the non-breeding season for oocytes collected post mortem, although blastocyst development rates were not affected (Krishnakumar *et al.* 2015; Cervantes *et al.* 2016). In some cases, reproductive material from wildlife can only be accessed post mortem, thus reproducing these studies using abattoir-derived plains bison oocytes is important for elucidating seasonal differences between oocytes collected from live donors and those harvested post mortem.

In vitro embryo production followed by cryopreservation is a primary method for preserving genetic diversity for valuable or endangered species. In vitro embryo production in bison was first described in wood bison (Bison bison athabascae) using abattoir-derived oocytes and resulted in blastocyst production rates of <10% (Thundathil *et al.* 2007). There have been only a few studies dealing with in vitro embryo production in bison, and recently oocytes sourced from live hormone-stimulated bison have produced blastocyst rates (20-40%) (Cervantes *et al.* 2017a). While these results are promising, using oocytes aspirated transvaginally from live hormone-stimulated animals may not be possible from genetically valuable bison. In most cases,

reproductive material is only be possible post mortem, particularly for wild bison herds (Benham *et al.* 2021). Thus, abattoir-derived oocytes are an excellent model for developing IVP protocols that can be implemented in situations where access to genetically valuable bison is unpredictable and/or limited.

The in vitro maturation of bison oocytes is essential for producing IVP bison embryos. These oocytes must complete both a nuclear and a cytoplasmic maturation to acquire developmental competence and fertilization capacity (Zhu *et al.* 2018). An oocyte is categorized as "mature" following the extrusion of the first polar body and when the chromosomes align on the metaphase plate for the second meiotic division (MII) – an indication of nuclear maturation. Studies, in wood bison, indicate that higher proportions of in vitro matured oocytes complete nuclear maturation (60-70%) than in vivo matured oocytes (25-27%) over a 24 h maturation period (Cervantes *et al.* 2016). This is not unique to bison, as cattle oocytes matured in vitro also have higher maturation rates but lower blastocyst rates than their in vivo counterparts (Rizos *et al.* 2002). Only a small percentage of in vitro matured oocytes that complete nuclear maturation reach the blastocyst stage after IVF, which may indicate incomplete cytoplasmic maturation, due to suboptimal IVM conditions, and suggests that an extended IVM period may permit more complete oocyte maturation and improved oocyte competence (Krisher 2013). Extending oocyte maturation time increased blastocyst rates for oocytes collected from hormone-stimulated wood bison (Cervantes *et al.* 2017a), but increasing the maturation time of abattoir derived bison oocytes has not been evaluated. In addition, in vivo studies using wood bison, showed a correlation between follicle size in the ovary at the time of collection and oocyte developmental competence, with better oocyte competence associated with follicles >5 mm. One of the advantages of using abattoir derived ovaries is the ability to collect greater numbers of oocytes

from all visible antral follicles, compared to the smaller number of preovulatory follicles that can be collected during in vivo oocyte aspiration. In cattle, abattoir derived oocytes collected from <3 mm follicles were still capable of reaching the blastocyst stage after fertilization (Shabankareh *et al.* 2014). While oocyte maturation rates from small diameter follicles may be lower, the effects of follicular diameter on the ability of oocytes to mature adequately, have not been assessed using abattoir-derived bison oocytes.

In this study we attempt to fill in knowledge gaps regarding the seasonal effects of bison oocyte competence after IVM and their capacity to produce in vitro embryos when bison oocytes are collected post mortem. Oocytes were collected each month over a year and a half, to evaluate if reproductive seasonality affects oocyte meiotic competence of oocytes collected from follicles of different diameters (experiment 1) and whether longer in vitro maturation time benefited bison oocyte meiotic competence and IVP outcomes (experiment 2).

Materials and Methods

Experiment 1

Ovaries were transported to the laboratory in sterile saline at 25-28°C within 2 h of collection. Ovaries were collected within 10 minutes of slaughter at a local abattoir from November 2017- March 2019. Because ovaries were unavailable certain months, the collection period spanned multiple years to ensure material was collected during each calendar month. If suffiecient oocytes were collected in one day, replicates of both experiments were conducted. Cumulus oocyte complexes (COCs) were collected separately from <2 mm, 2-5 mm, or >5 mm diameter follicles using an 18-gauge needle attached to a vacuum pump set to 50mm Hg, (Pioneer Pro Pump, Life Global Group, LLC, Guliford, CT, USA). Aspiration tubing was rinsed with CSU medium for handling oocytes (H-CDM-M) with 10% BSA and gentamicin (10

mg/mL) prior to and after each aspiration, providing a holding medium for COCs during aspiration, as well as a rinse for the aspiration line to ensure complete COC recovery. All media used for COC and embryo handling and culture were chemically-defined (CDM) and prepared as described by Barfield (2019). Recovered COCs with a minimum of two layers of intact cumulus cells were washed multiple times through H-CDM-M to remove debris and COCs placed in 4 well dishes containing 1 mL CSU medium for in vitro maturation of oocytes (IVM) pre-equilibrated at 38.5°C in 5% CO_2 and air for ≥ 5 h. Oocytes were matured in IVM medium supplemented with: 15 ng/mL NIDDK-oFSH-20, 1 μg/mL USDA-LH-B-5, 1 μg/mL estradiol 17β, 50 ng/μL epidermal growth factor, 0.1 mM cysteamine (De La Torre-Sanchez *et al.* 2006). COCs obtained from different size follicles were placed in separate IVM wells.

After 23 h maturation, oocytes were mechanically stripped of cumulus cells by gentle micropipetting using a STRIPPER® pipette with a 150 μm tip (Origio, CooperGenomics, Denmark) in 0.01% hyaluronidase in CSU medium for handling of early embryos (HCDM-1). Denuded oocytes were then washed in HCDM-1 drops until the wash medium was free of loose cumulus cells (approximately 6 times). Denuded oocytes were mounted on poly-L lysine coated slides and covered with a glass coverslip. Two lines of a petroleum jelly- paraffin wax material was applied close to the outer edges of the side prior to mounting the coverslip to prevent the oocytes from being squished (Mullen and Critser 2004). Oocytes were then fixed with a 1:3 (acetic acid: 70% ethanol) solution for 48 h. After fixation, oocytes were stained with 1.5 μL/mL Hoechst 33342 working stock (10mg/mL) (Invitrogen, Thermo Fisher, H3570) for 5 minutes prior to imaging. Oocytes were categorized as GV, GVBD (this category included all oocytes in metaphase I, anaphase I, and telophase I), or MII using fluorescence microscopy at 40X magnification (Nikon Eclipse E800) (Marei *et al.* 2009) (Fig. 1).

Experiment 2

Ovaries were collected and transported to the laboratory as described in experiment 1. COCs were aspirated from 2-5 mm follicles, and washed in HCDM-M, as described in experiment 1, before being placed in 1 mL IVM medium for 24 h (control IVM) or 29-34 h (extended IVM) in a 38.5°C humidified incubator with 5% CO^2 in air. Early experiments with 34 h maturation resulted in drastically reduced blastocyst production, thus 29 h was used for the majority of months (see supplemental data).

After maturation, approximately 10 oocytes from each well were collected for nuclear staining and evaluated for meiotic stage as previously described, and the remainder of COCs were moved to a 4-well culture dish containing 430 µL of equilibrated CSU medium for in vitro fertilization (FCDM)/well. Approximately, 50 mature COCs were transferred into FCDM wells in ≤ 20 µL IVM medium.

Sperm from slaughtered bison, was collected by flushing post mortem epididymides and frozen in 20% egg yolk tris + 8% glycerol at ~60 million cells/mL (Vilela *et al.* 2017). Sperm from the same bull were used for all IVF procedures. Motile sperm for fertilization were isolated by washing thawed sperm through a 45/90 Percoll® gradient (Parrish *et al.* 1995). Sperm were resuspended in HCDM-1 and post thaw sperm motility ranged from 60 to 90% as assessed by light microscopy at 200X magnification, and quantified using a hemocytometer, prior to preparation for use in IVF. Sperm were added to COCs to a final concentration of 0.5×10^6 sperm/mL and co-incubated for 18 h at 38.5°C and 5% CO_2 in air.

After 18 h of co-incubation, presumptive zygotes were mechanically stripped of cumulus cells by gentle micropipetting as described above except without the hyaluronidase. Denuded zygotes were then placed into wells containing 500 µL of equilibrated CSU medium for in vitro culture of early embryos (CDM-1) per well and cultured for 56 h at 38.5°C in an atmosphere of

5% CO_2, 5% O_2, 90% N_2. The percentages of oocytes that cleaved was determined after 56 h of culture.

Embryos (up to 30/well) containing ≥ 4 blastomeres (Fig. 2) at 56 h were placed in 500 µL of equilibrated CSU medium for in vitro culture of late embryos (CDM-2) + 5% FCS per well, and cultured for an additional 96-120 h at 38.5°C in an atmosphere of 5% CO_2, 5% O_2, 90% N_2 (Barfield 2019). On days 7 and 8 post-fertilization (Day 0 = day of fertilization) embryos were assessed to determine stage of development (Fig. 2). The stage was determined in accordance with International Embryo Technologies Society (IETS) guidelines (Stringfellow and Givens 2010).

Statistical Analysis

The frequencies of oocytes at each stage of nuclear maturation, cleavage rates, and blastocyst rates were compared by 2-tailed Chi-square test or Fisher's exact test for both experiments. Treatment differences were considered significant at $P<0.05$. Logistic regression was used to determine marginal mean effects, with follicle developmental status or maturation time as response. Predictors include follicle size (3 levels) season (4 levels) and the interaction. Pairwise comparisons were considered in experiment 1. All statistics were performed using GraphPad Prism® 8.0d (GraphPad Software, La Jolla, CA, USA) and JMP®, Version 13.0 (SAS Institute Inc., Cary, NC, USA, 1989-2019).

Results

Experiment 1

Oocytes collected from follicles of each size group exhibited some nuclear maturation (Table 1). The proportion of oocytes that reached the MII nuclear stage was higher as follicle size increased irrespective of season: small, <2 mm (41.5%); medium, 2-5 mm (57.9%); and

large, >5 mm (68.4%) (Table 1). Across seasons, the mean effect on the proportion of oocytes reaching MII differed between small and medium follicles, small and large follicles, and medium and large follicles (Fig 3). Season had a significant effect on the proportion of oocytes reaching MII, irrespective of follicular diameter: with oocytes collected in the Fall achieving lower percentages of MII oocytes (51.5%) than oocytes collected in Winter and Spring (~59%). No differences were observed in the proportion of oocytes reaching MII between Winter and Spring, and the proportion of oocytes reaching MII did not differ between Summer (53.3%) and any other season. A significant interaction was observed between follicle size and season. Data listed by month can be found in Supplemental Table 1.

Experiment 2

The time for which oocytes maturated affected the proportion of oocytes reaching MII. Longer IVM times increased the percentages of oocytes achieving the MII stage, irrespective of season (Table 2; Figure 4). In addition, season affected the percentage of oocytes achieving MII with only 48% reaching MII in the Fall compared to >59% reaching MII in the other seasons, irrespective of in vitro maturation length. No interactions were observed between IVM time and season. Data listed by month and IVM duration can be found in Supplemental Table 2.

IVM time did not affect mean oocyte cleavage rates, irrespective of season (Table 3; Figure 5); However, IVM time did affect the percentage of embryos reaching the blastocyst stage at both Day 7 and Day 8, with higher percentages of embryos reaching the stage when oocytes were matured for only 24 h compared to longer maturation times (Table 3; Figs. 6 and 7).

Season affected oocyte cleavage rates irrespective of IVM time, with cleavage rates for all seasons: Winter (44%), Spring (62%), Summer (71%), and Fall (54%) being different from each other. Season also affected the overall D7 blastocyst rate irrespective of IVM length with

embryos cultured in Summer (9%) having higher blastocyst rates than those cultured in the Winter (6%), Spring (5%), and Fall (6%). No difference was observed in D7 blastocyst rates for embryos cultured in the Winter, Spring or Fall. The D8 blastocyst rate in Summer (11%) was higher than in the Winter (7%), but did not differ from Spring (7%) or Fall (8%). No difference was observed in D8 blastocyst rates between the Winter, Spring or Fall. No interaction was observed between IVM time and season. Data listed by month and IVM duration can be found in Supplemental Table 3.

Discussion

Reproductive seasonality can significantly impact the effectiveness of ART in wildlife species. This year-round study investigated the effects of follicular diameter and IVM duration on oocyte meiotic competence and in vitro embryo production from oocytes collected post-mortem. Understanding the potential of oocytes collected across all seasons, including transitional times between ovulatory and anovulatory seasons, is valuable information for researchers working with species for which access to genetically valuable reproductive material is unpredictable.

Development of species-specific reproductive technologies for bison has been limited, due to a scarcity of reproductive material. Abattoir-derived ovaries from bison are an important resource of bison gametes for research purposes and provide a model for material collected from wild bison post mortem. They have the potential to produce greater numbers of oocytes and embryos than when aspirating ovaries in live bison and can be collected throughout the year, allowing the continuous development of research strategies. In this study, we have shown that abattoir-derived oocytes can be matured and fertilized; and resulting embryos can develop to the blastocyst stage, when they are collected from follicles of various sizes and collected throughout

the year. However, more embryos are produced on a per oocyte basis during the summer months. These results may help set expectations for IVP outcomes under conditions in which gametes are recovered post- mortem, which is particularly relevant when using ARTs in conservation strategies with valuable bison in wild populations.

In Experiment 1, the proportion of oocytes that reached the MII maturation stage was higher when they came from larger follicles, with the highest percentage of oocytes achieving MII, when they came from follicles > 5mm (68%). Follicle size has been linked to oocyte competence in a variety of species including dogs (Songsasen and Wildt 2005), cattle (Lonergan *et al.* 1994), and goats (Crozet *et al.* 1995). For each of these species, there is a size of follicle above which the oocyte is able to progress to metaphase II of meiosis (i.e. dogs (<0.5 mm), cattle (>3 mm), goats (>2 mm) (Lonergan *et al.* 1994; Crozet *et al.* 1995; Songsasen and Wildt 2005). This threshold has not been determined for abattoir bison follicles. A study using abattoir-derived bovine ovaries reported that oocytes collected from follicles >4 mm in diameter achieved higher maturation rates (87.8 ± 3.0%) and blastocyst rates (57.3 ± 3.0%) than oocytes collected from follicles <4 mm (72.2 ± 5.8% and 34.0 ± 4.0%, respectively) (Barros *et al.* 2019). Another study using live hormone-stimulated wood bison reported that follicles < 5mm were not aspirated, due to the reduced competence of oocytes coming from these smaller follicles (Cervantes *et al.* 2017b). However, in this study, while higher percentages of oocytes reached MII from large follicles, oocytes derived from both medium and small follicles were still able to reach MII at rates that would merit collection when collecting oocytes from genetically valuable animals, 58% and 42%, respectively. There are usually high numbers of small follicles on ovaries throughout the year, particularly during the anovulatory season, which are not currently collected when performing follicular aspirations in live bison (Cano 2015; Cervantes *et al.* 2016;

Cervantes *et al.* 2017a; Cervantes *et al.* 2017b), but can be easily accessed post mortem. Utilization of oocytes collected from follicles of all sizes increases the overall pool of oocytes that can be put in to an IVP system, thus increasing embryo production potential.

In the present study, higher percentages of oocytes reached MII during the Winter and Spring compared to Fall. Similar results were seen using oocytes collected from hormone-stimulated live bison after in vitro maturation, with a higher percentage of oocytes achieving MII observed during the anovulatory season (73%; May-June) vs ovulatory season (62%; Oct-Nov) (Cervantes *et al.* 2016). The reason for higher percentages of oocytes achieving MII in the non-breeding season in both our study and the wood bison study remains unclear, but it does infer that in vitro methods can be successfully used to mature bison oocytes during the anovulatory season. Interestingly, in our study an interaction was observed between effects of season and follicular diameter on the proportion of oocytes that reached MII. A similar proportion of oocytes from small follicles (<2 mm) and medium (2-5 mm) follicles reached MII in the Spring.

Previous studies in hormone-stimulated wood bison report that nuclear maturation and cumulus cell expansion occurred faster in vitro than in vivo, with 60% of in vitro matured oocytes reaching MII at 24 h post hCG treatment compared to 25% in vivo in COCs collected from follicles >5 mm, potentially indicating that nuclear maturation in vitro is happening too quickly to allow for completion of cytoplasmic maturation (Cervantes *et al.* 2016). Further studies showed that an additional 4 h of in vitro maturation following 30 h in vivo maturation increased the proportion of oocytes reaching MII phase, indicating that bison oocytes may require a longer in vitro maturation period to achieve MII although this was not examined for abattoir derived oocytes that had not been exposed to exogenous hormones (Cervantes *et al.* 2017a). In Experiment 2 of this study, results consistently showed that extending the in vitro maturation time improved the

percentage of oocytes that were able to achieve MII from (54%; 24 h) to (64%; 29-34 h) across all seasons but this did not result in higher in developmental competence, as measured by blastocyst production rates. There was a seasonal effect on the percentage of oocytes that were able to achieve MII irrespective of length of maturation, with the percentage of oocytes that were able to achieve MII in Winter being lower than oocytes collected the other seasons, indicating a reduced competence in oocytes as the ovulatory season comes to an end. Similar results from previous studies using in vivo matured oocytes also showed an increase of expanded COC collected during the anovulatory season (7.0±0.9 COC/bison; April-May) compared to ovulatory season (3.6 ±0.7 COC/bison; Sep-Nov), where fully expanded cumulus cells were used as an indicator of maturational competence (Cervantes *et al.* 2017a). Despite these differences observed in cumulus cell expansion, embryo cleavage and blastocyst production rates were similar between seasons indicating no seasonal effect on developmental capacity (Cervantes *et al.* 2017a).

Optimal in vitro maturation times for oocytes vary across species. Cattle oocytes are typically matured in vitro for 23-24 hours prior to fertilization and this has been used as the model for bison in vitro embryo production, and is supported by other reports for bison oocytes (Krishnakumar *et al.* 2015; Barfield 2019). The in vivo maturation of oocytes from live wood bison for 34 h post hCG trigger showed higher cleavage rates (74%) and blastocyst rates (54%) when compared to oocytes matured in vivo for 30 h post hCG, plus an additional 4h in vitro maturation (57% and 37%, respectively); (Cervantes *et al.* 2017a). Similar cleavage rates are currently reported using in vitro systems, however blastocyst/oocyte rates using oocytes derived from live bison far exceed what has been reported using abattoir derived ovaries, 54% versus 8-16%, respectively (Thundathil *et al.* 2007; Aurini *et al.* 2009; Barfield and Seidel 2011; Cervantes *et al.* 2017a; Benham *et al.* 2021). In Experiment 2, embryo cleavage rates were not

affected by in vitro maturation time, and cleavage rates for both maturation times were similar to those for oocytes from hormone-stimulated wood bison, matured for 34 h (58%) (Cervantes *et al.* 2017a; Cervantes *et al.* 2017b). The blastocyst rates at Day 7 and Day 8 were both higher when oocytes were in vitro matured for only 24 h compared to longer in vitro maturation times. Increasing the in vitro maturation to 34 h had detrimental effects on blastocyst development in this study, with blastocyst rates at Day 8 as low as 2%. Therefore, the extended maturation treatment time was reduced to 29 h after the first three months (November 2017, January 2018, February 2018) of this experiment. Blastocyst rates after 29 h maturation were either similar or lower to those achieved after 24 h maturation, indicating no benefit of increased maturation time (Supplemental Table 3). While longer in vitro maturation time improved oocyte competence and embryo production rates using oocytes derived from live animals (Cervantes *et al.* 2017a), these improvements were not observed using oocytes collected from slaughter house ovaries.

In wood bison, a seasonal effect on in vitro fertilization success has been described, inferring reduced oocyte competence during the non-breeding season (Krishnakumar *et al.* 2015). Throughout the anovulatory season, small antral follicles can persist on the ovary for a long period of time, possibly increasing the number of recovered oocytes that are atretic or have poor developmental potential, thereby reducing embryo production (Rizos *et al.* 2005). In the present study, seasonal effects were observed on embryo cleavage and blastocyst production rates. Cleavage rates were highest in Summer (71%), similar to previous results in wood bison using slaughterhouse derived oocytes (84%; July-Sep vs 74%; Jan-Mar) (Krishnakumar *et al.* 2015). As well, Day 7 blastocyst rates were highest in Summer, while Day 8 blastocyst rates did not differ between Spring, Summer, or Fall. While studies of other seasonally breeding species including sheep and cats have shown periodic embryo yield reductions in non-breeding season

(Spindler and Wildt 1999; Mara *et al.* 2014), this is the first description of seasonal differences in blastocyst production across all calendar months in bison.

Oocyte competence is a key factor determining the yield and quality of blastocysts following IVF, while post fertilization environment does not have as great an effect on these parameters (Rizos *et al.* 2002). Indeed, studies using in vivo derived wood bison reported high blastocyst rates (50%) from excellent or good compact COC, indicating that embryo quality is integrally affected by oocyte quality (Cervantes *et al.* 2017b). The data presented in this study shows that similar embryonic cleavage rates can be achieved using oocytes from slaughterhouse sourced ovaries compared to live hormone-stimulated animals (Cervantes *et al.* 2017a). Promisingly, when oocyte maturation occurs under optimal conditions (in vivo matured), bison IVP outcomes can be comparable to those seen in cattle. However, despite similar rates of fertilization and cell cleavage, reduced blastocyst rates, as presented here, indicate that embryonic developmental potential is still compromised using in vitro matured oocytes. Together, these results emphasize the importance of directing our research toward improving IVM protocols that promote complete (nuclear and cytoplasmic) oocyte maturation to produce competent oocytes that can develop into blastocysts. This is a crucial step towards generating large numbers of high-quality embryos for the establishment of GRBs.

Modifications to IVM and IVP protocols using bison oocytes sourced from abattoir-derived ovaries is essential to developing strategies to create a larger resource of female gametes available for research purposes throughout the year. Improving oocyte competence following in vitro maturation is a critical step in producing high quality embryos from reproductive material from valuable animals for conservation purposes, thereby strengthening the foundation on which many of our more advanced assisted reproductive techniques rely.

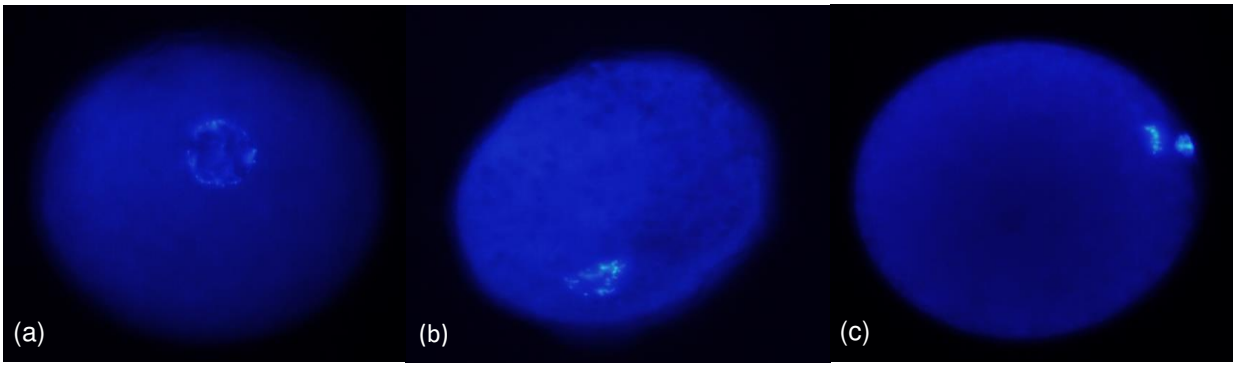

Figure 3.1. Bison oocytes stained with Hoechst 33342 to determine nuclear maturation stage (a) germinal vesicle (GV); (b) germinal vesicle break down (GVBD); (c) metaphase II (MII) with extruded polar body

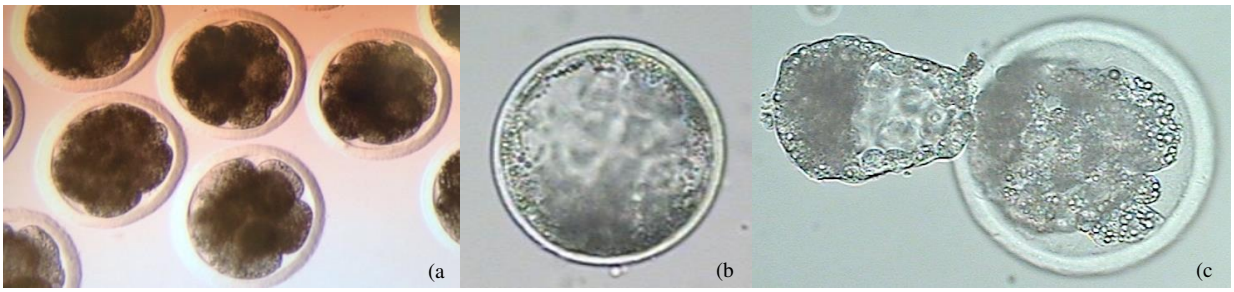

Figure 3.2. Representative images of plains bison in vitro produced embryos on (a) Day 3, 8-cell embryos; (b) Day 7, expanded blastocyst; (c) Day 8, hatching blastocyst (Day 0 = day of fertilization).

Table 3.1. Meiotic competence of plains bison oocytes produced from cumulus oocyte complexes (COC) derived from different sized diameter follicles over 4 seasons. Seasons were divided to represent the winter (January-March), spring (April-June), summer (July-September), and fall (October-December) seasons. Oocytes from <2 mm, 2-5 mm, or >5 mm follicles were matured in vitro for 23 h at 38.5°C in 5% CO_2 in air. Nuclear status was determined using fluorescence microscopy and oocytes were classified as still having a germinal vesicle (GV), having undergone germinal vesicle breakdown (GVBD), or having completed meiosis II (MII) (Experiment 1). [abc] Within rows, [xy] within columns, values with different superscripts significantly differ ($P < 0.05$).

End point	Follicular Diameter			Marginal mean effect on MII (%)
	<2 mm	2-5 mm	>5 mm	
Winter (January-March)				
COC submitted to IVM (n)	165	176	93	
GV	31/165 (18.8%)	20/176 (11.4%)	8/93 (8.6%)	
GVBD	59/165 (35.8%)	44/176 (25.0%)	22/93 (23.7%)	
MII	75/165 (45.5%)[a]	112/176 (63.6%)[b]	63/93 (67.7%)[b]	58.9 %[x]
Spring (April-June)				
COC submitted to IVM (n)	187	301	85	
GV	30/187 (16.0%)	42/301 (14.0%)	12/85 (14.1%)	
GVBD	55/187 (29.4%)	87/301 (28.9%)	15/85 (17.6%)	
MII	102/187 (54.5%)[a]	172/301 (57.1%)[ab]	58/85 (68.2%)[b]	59.9%[x]
Summer (July-September)				
COC submitted to IVM (n)	175	298	107	
GV	45/175 (25.7%)	57/298 (19.1%)	9/107 (8.4%)	
GVBD	68/175 (38.9%)	87/298 (29.2%)	20/107 (18.7%)	
MII	62/175 (35.4%)[a]	154/298 (51.7%)[b]	78/107 (72.9%)[c]	53.3%[xy]
Fall (October-December)				
COC submitted	235	271	54	

End point	Follicular Diameter			Marginal mean effect on MII (%)
	<2 mm	2-5 mm	>5 mm	
to IVM (n)				
GV	53/235 (22.6%)	25/271 (9.2%)	7/54 (13.0%)	
GVBD	110/235 (46.8%)	86/271 (31.7%)	12/54 (22.2%)	
MII 7	2/235 (30.6%)[a]	160/271 (59.0%)[b]	35/54 (64.8%)[b]	51.5%[y]
Marginal mean effect on MII (%)	41.5%[a]	57.9%[b]	68.4%[c]	

Table 3.2. Meiotic competence of plains bison oocytes produced from cumulus oocyte complexes (COC) collected from 2-5mm follicles and matured in vitro for different lengths of time over 4 seasons. Seasons were divided to represent the winter (January-March), spring (April-June), summer (July-September), and fall (October-December) seasons. Oocytes were matured in vitro for 24h, 29 h, or 34 h at 38.5°C in 5% CO_2 in air. Nuclear status was determined using fluorescence microscopy and oocytes were classified as still having a germinal vesicle (GV), having undergone germinal vesicle breakdown (GVBD), or having completed meiosis II (MII) (Experiment 2). [ab] Within rows, [xy] within columns, values with different superscripts significantly differ ($P < 0.05$).

End point[1]	**Length of time of in vitro maturation**		**Marginal mean effect on MII (%)**
	24 h	**29 or 34 h**	
Winter (January-March)			
COC submitted to IVM (n)	117	110	
GV	13/117 (11.1%)	11/110 (10.0%)	
GVBD	31/117 (26.5%)	20/110 (18.2%)	
MII	73/117 (62.4%)	79/110 (71.8%)	67.1%[x]
Spring (April-June)			
COC submitted to IVM (n)	156	143	
GV	20/156 (12.8%)	13/143 (9.1%)	
GVBD	47/156 (30.1%)	34/143 (23.8%)	
MII	89/156 (57.1%)	96/143 (67.1%)	62.1%[x]
Summer (July-September)			
COC submitted to IVM (n)	140	138	
GV	18/140 (12.9%)	11/138 (8.0%)	
GVBD	46/140 (32.9%)	40/138 (29.0%)	
MII	76/140 (54.3%)	87/138 (63.0%)	58.7%[x]

End point[1]	Length of time of in vitro maturation		Marginal mean effect on MII (%)
	24 h	29 or 34 h	
Fall (October-December)			
COC submitted to IVM (n)	113	114	
GV	21/113 (18.6%)	14/114 (12.3%)	
GVBD	44/113 (38.9%)	40/114 (35.1%)	
MII	48/113 (42.5%)	60/114 (52.6%)	47.6%[y]
Marginal mean effect on MII (%)	54.1%[a]	63.6%[b]	

Table 3.3. In vitro production of plains bison embryos produced from cumulus oocyte complexes (COC) collected from 2-5 mm follicles and matured in vitro for different lengths of time over 4 seasons. Seasons were divided to represent the winter (January-March), spring (April-June), summer (July-September), and fall (October-December) seasons. Oocytes were matured for 24h, 29 h or 34 h, fertilized, and cultured in vitro (Day 0 = day of in vitro fertilization). Blastocyst rates are presented as blastocysts/oocyte (Experiment 2). [ab] Within rows, [wxyz] within columns, values with different superscripts significantly differ ($P < 0.05$).

End point	Length of time of in vitro maturation		Marginal mean effect on end point (%)
	24 h	29 or 34 h	
Winter (January-March)			
COC submitted to IVF (n)	581	615	
Cleaved oocytes	256/581 (44.1%)	275/615 (44.7%)	44.4%^{w}
Blastocysts on Day 7	46/581 (7.9%)^{a}	25/615 (4.1%)^{b}	6.0%^{x}
Blastocysts on Day 8	56/581 (9.6%)^{a}	26/615 (4.2%)^{b}	6.9%^{x}
Spring (April-June)			
COC submitted to IVF (n)	668	752	
Cleaved oocytes	421/668 (63.0%)	463/752 (61.6%)	62.3%^{x}
Blastocysts on Day 7	42/668 (6.3%)^{a}	27/752 (3.6%)^{b}	5.0%^{x}
Blastocysts on Day 8	59/668 (8.8%)^{a}	44/752 (5.9%)^{b}	7.4%^{xy}
Summer (July-September)			
COC submitted to IVF (n)	462	436	
Cleaved oocytes	313/462 (67.8%)	320/436 (73.4%)	70.6%^{y}
Blastocysts on Day 7	49/462 (10.6%)	34/436 (7.8%)	9.2%^{y}
Blastocysts on Day 8	58/462 (12.6%)	39/436 (8.9%)	10.8%^{y}
Fall (October-December)			
COC submitted to IVF (n)	520	524	
Cleaved oocytes	281/520 (54.0%)	285/524 (54.4%)	54.2%^{z}
Blastocysts on Day 7	43/520 (8.3%)^{a}	17/524 (3.2%)^{b}	5.8%^{x}
Blastocysts on Day 8	54/520 (10.4%)^{a}	26/524 (5.0%)^{b}	7.7%^{xy}
Marginal mean effect on:			

End point	Length of time of in vitro maturation		Marginal mean effect on end point (%)
	24 h	29 or 34 h	
Cleaved oocytes (%)	57.2%	58.5%	
Blastocysts on Day 7 (%)	8.3%[a]	4.7%[b]	
Blastocysts on Day 8 (%)	10.4%[a]	6.0%[b]	

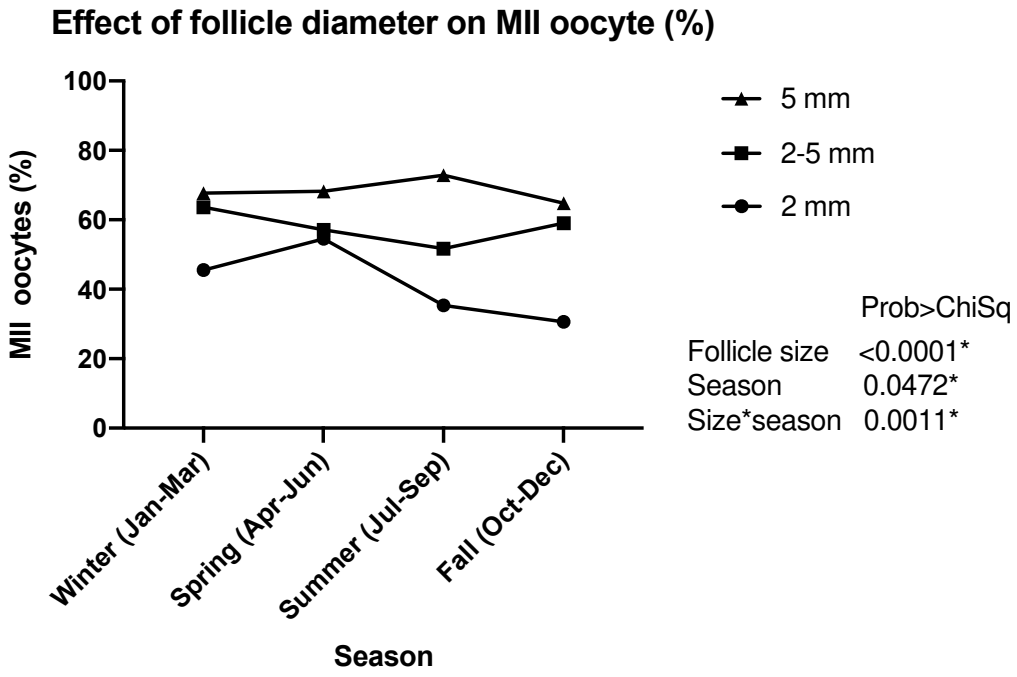

Figure 3.3. Experiment 1: Effects of season and follicle size on MII% in IVM plains bison oocytes.

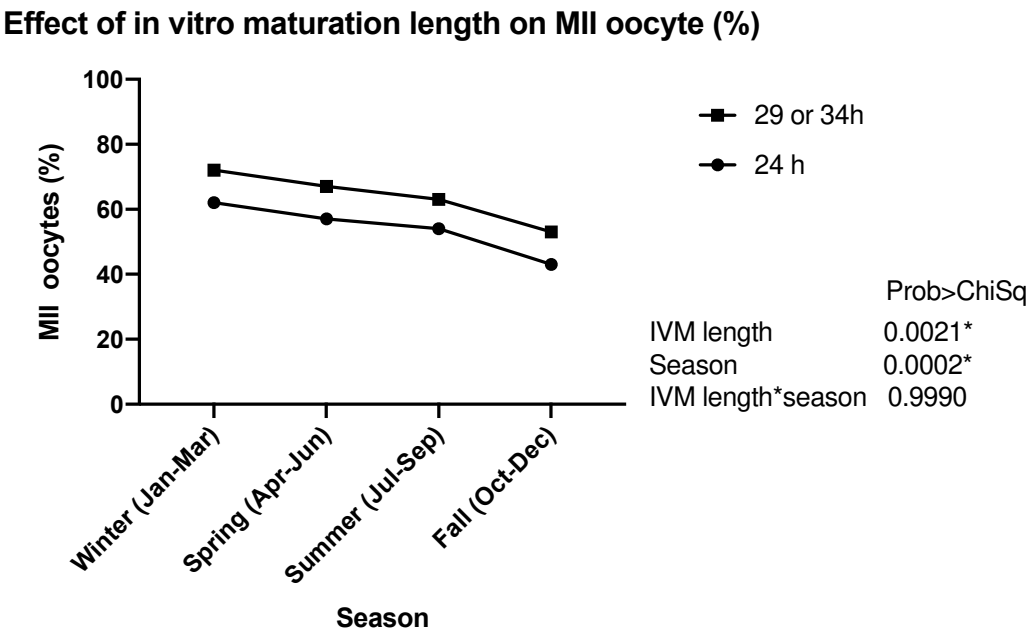

Figure 3.4. Experiment 2: Effects of season and IVM duration on MII% in plains bison oocytes.

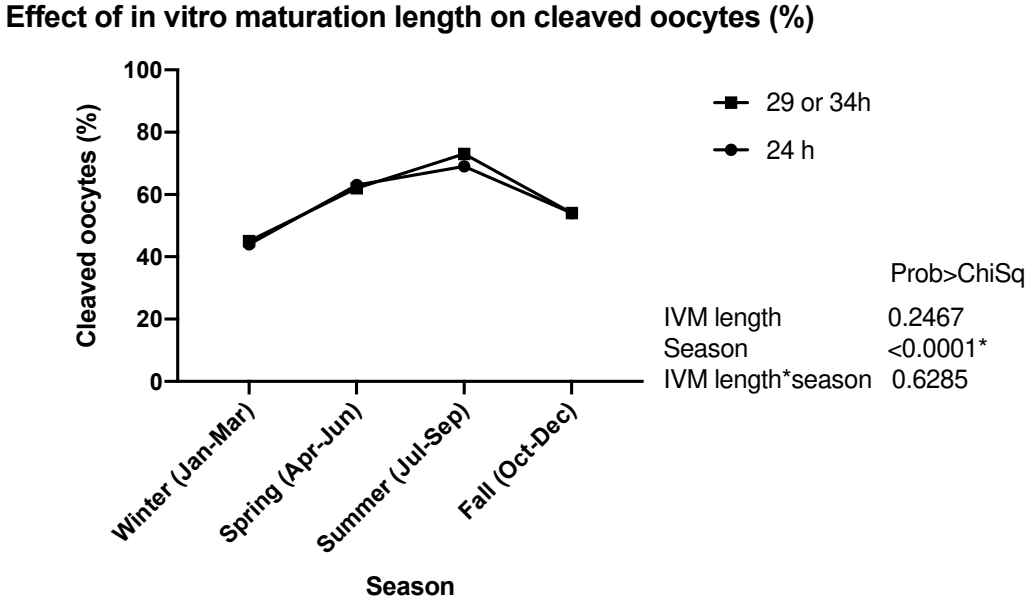

Figure 3.5. Experiment 2: Effects of season and IVM duration on mean oocyte cleavage rates in plains bison.

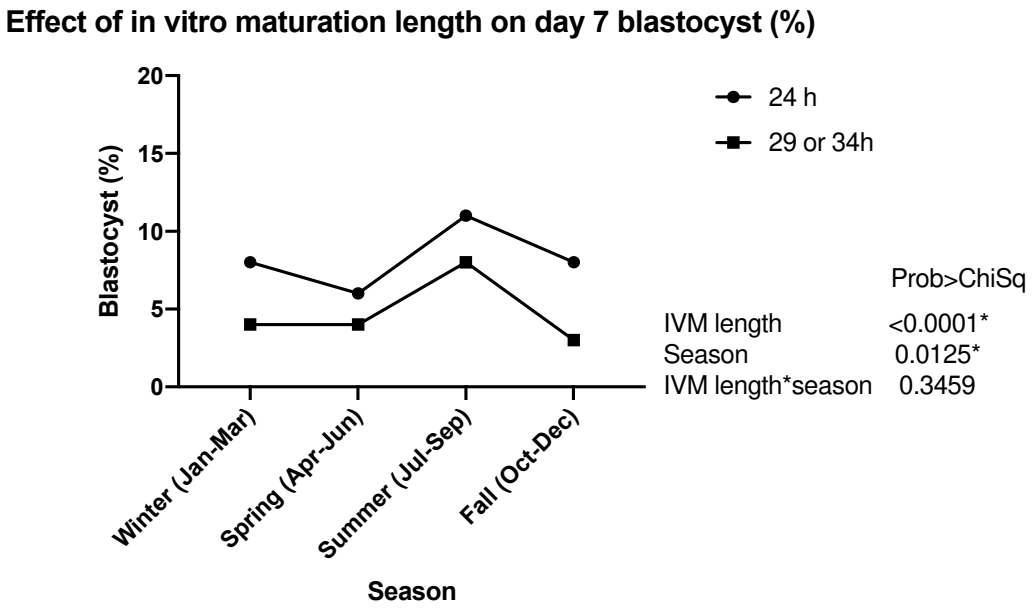

Figure 3.6. Experiment 2: Effects of season and IVM duration on Day 7 mean blastocyst rates in plains bison.

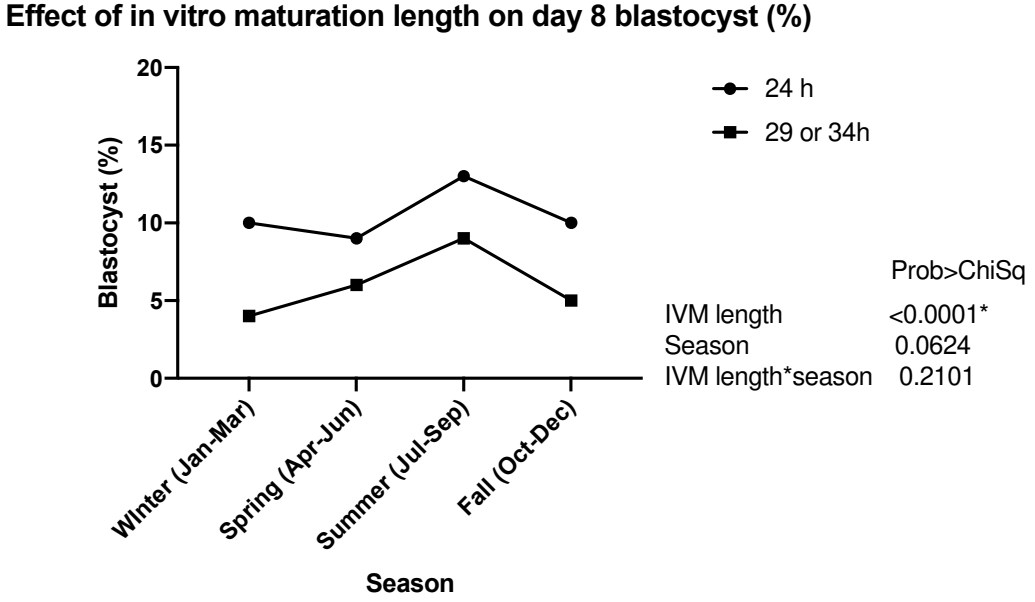

Figure 3.7. Experiment 2: Effects of season and IVM duration on Day 8 mean blastocyst rates in plains bison.

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CHAPTER 4: EFFECTS OF VITRIFICATION ON BOVINE GV OOCYTE AND MII EGG MITOCHONDRAIL NUMBER AND FUNCTION[4]

Summary

Bovine oocytes currently do not survive vitrification well, this is partially due to the high lipid content in the ooplasm, but also owing to cell cytoskeletal structure damage and poor mitochondrial function during the vitrification and warming process. The objective of this work was to determine how vitrification affects bovine oocyte mitochondrial DNA (mtDNA) copy number, activity and gene expression. Bovine oocytes were collected from slaughterhouse ovaries and split into four treatment groups in which oocytes were assayed immediately; 1) fresh germinal vesicle (GV) oocytes (control); 2) vitrified immediately as GV oocytes; 3) in vitro matured to the MII stage; and 4) in vitro matured to the MII stage and then vitrified. Non-vitrified oocytes were analyzed when reaching the designated stage of development and all vitrified oocytes were processed for analysis immediately after warming. mtDNA copy number in single oocytes was determined by real-time quantitative PCR (RT-qPCR), using an absolute quantification assay (n=20 oocytes/group). Mitochondrial activity was determined by staining oocytes with MitoTracker® Orange CMTMRos and imaging the oocytes using confocal microscopy (n=20 oocytes/group). The expression of genes related to oxidative stress response and lipid metabolism (*GCLC*, *GPX1*, *NRF2*, *KEAP1*, *SOD2*, *PARAα*, *SREBP1*, and *CPT2*) was determined by RT-qPCR in three biological replicates (10 pooled oocytes per replicate). mtDNA copy number were similar for fresh and vitrified GV and MII oocytes (P>0.05). Active mitochondria were distributed in the cortical region of the oocytes in all treatment groups.

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[4] This chapter is written and formatted for submission to *Reproduction, Fertility and Development*

Cortical mitochondrial activity was lower in MII compared to GV oocytes ($p<0.05$) but was higher for vitrified GV oocytes compared to fresh GV oocytes ($P<0.05$).

Expression of *GCLC* was lower for vitrified GV oocytes than for fresh GV oocytes but was similar for fresh and vitrified MII oocytes. In summary, vitrification of bovine oocytes did not significantly affect mitochondrial DNA copy number but did increase mitochondrial activity in GV stage oocytes compared to vitrified GV oocytes. Reduced *GCLC* expression in vitrified GV oocytes may indicate a compromised antioxidative stress response. While alterations caused by vitrification were more evident in immature oocytes, future studies will investigate if adding antioxidants during vitrification could benefit both immature and mature oocytes.

Introduction

Oocytes are viable for a finite time between ovulation or removal from the follicle and fertilization or activation (Rho *et al.* 2002). The recent surge in the use of ovum pickup (OPU) in the commercial cattle industry makes oocyte preservation critical in overcoming the associated logistical problems of oocyte transportation to IVF clinics and the capacity of the clinics to fertilize these oocytes in a timely manner (Moore and Hasler 2017). Currently, oocytes must be used within a small time window of oocyte viability and vitrification of oocytes would provide more flexibility in time-sensitive logistics for getting oocytes to labs and using them. In addition, preserving bovine oocytes from slaughterhouse ovaries would allow greater utilization of ART procedures, including in vitro embryo production (IVP), cloning, and transgenic animal production (Brevini *et al.* 2005; Yamada *et al.* 2007). Preservation of bovine oocytes, particularly immature oocytes, would not only allow greater utilization of ART techniques but increase the ability of commercial veterinarians to collect genetically valuable oocytes on farm and preserve them without the need for an onsite IVF laboratory.

Vitrification uses ultra-rapid cooling and warming rates with small volumes of solutions containing high concentrations of cryoprotectants. These vitrification solutions and a rapid cooling rate cause the solution to form glass-like physical state within the cell, rather than intracellular ice, that results in cell injury/death (Campos-Chillon *et al.* 2009; Sprícigo *et al.* 2014; Gutnisky *et al.* 2020); vitrification is used successfully to cryopreserve embryos and oocytes in many species (Zhou *et al.* 2010). Oocytes are large single cells with low surface area to volume ratio, that have high cytoplasmic lipid content, and highly permeable membranes, all which make them more susceptible to cryodamage than embryos (Moawad *et al.* 2014). Currently, there is no standardized vitrification-warming procedure for in vitro produced (IVP) bovine embryos, survival rates for vitrified bovine oocytes are inconsistent (Mogas 2019). Developing vitrification protocols for bovine oocytes has been ongoing for the past 25 years (Fuku *et al.* 1995), however it is still considered an experimental technology (Gutnisky *et al.* 2020) and improving bovine oocyte vitrification outcomes is imperative for commercial application of this technique.

Oocyte quality is a critical factor affecting both fertilization capacity and the subsequent embryonic development to the blastocyst stage in an in vitro embryo production system, however the comprehensive assessment of oocyte quality parameters proves challenging as many processes are involved in generating a competent oocyte (Brevini *et al.* 2005). The effect that vitrification has on an oocyte's quality remain unclear. Components of cytoplasmic maturation including spindle formation, mRNA and protein synthesis, and redistribution of organelles play a crucial role in maintaining oocyte developmental potential and require sufficient levels of adenosine triphosphate (ATP) to occur efficiently (Magnusson *et al.* 2008; Machatkova *et al.* 2012). In particular, mitochondria are involved in ATP production and are dynamic during

oocyte maturation, preparing the oocyte for fertilization and embryo development. ATP is produced through oxidation of metabolites in the ooplasm (glycolysis) or by oxidative phosphorylation in the mitochondria (Chen *et al.* 2012). Parameters of mitochondrial function including oxidative activity and membrane potential, distribution, and total copy number are increasingly viewed as markers of oocyte quality.

Previous studies have shown that vitrification negatively impacts mitochondrial function in oocytes from multiple species (bovine, porcine, mice, human), which may contribute to increased effects of reactive oxygen species (ROS) damage in vitrified oocytes, however no consensus has been reached regarding whether or not there is an ideal meiotic stage for which effects of vitrification may be minimized (Brevini *et al.* 2005; Zhao *et al.* 2011a; Zhao *et al.* 2011b; Chen *et al.* 2012; Moawad *et al.* 2014; Nohalez *et al.* 2015a). ROS's that from within the oocyte as by-products of energy metabolism pathways, including mitochondrial phosphorylation, can result in cellular damage caused by oxidative stress (Dai *et al.* 2015). Oxidative stress is suggested as a reason for reduced oocyte competence after vitrification and functioning mitochondria are critical to a cell's ability to combat oxidative stress (Gutnisky *et al.* 2020). Indeed, high levels of ROS and genes related to oxidative stress and lipid metabolism have been seen in oocytes with compromised mitochondrial function, indicating a correlation between mitochondrial function and oocyte quality (Zhao *et al.* 2011a; Zhao *et al.* 2011b; Dai *et al.* 2015; Gutnisky *et al.* 2020).

While the optimal meiotic stage for vitrifying bovine oocytes remains unclear and results inconsistent, oocyte maturation status may impact viability and developmental capacity after cryopreservation (Nohalez *et al.* 2015a). During oocyte maturation, the distribution pattern of mitochondria and mitochondrial activity change (Rho *et al.* 2002; Brevini *et al.* 2005;

Machatkova *et al.* 2012). Improper mitochondrial distribution can occur due to a variety of stressors (IVM conditions, cryopreservation, advanced donor age) can result in impaired oocyte competence and may indicate mitochondrial dysfunction that will result in te compromised metabolism, often seen in poor quality oocytes (Brevini *et al.* 2005; Pasquariello *et al.* 2019; Gutnisky *et al.* 2020). Chilling damage can occur in both immature and mature oocytes, due to specific properties associated with each type of oocyte, including oocyte-cumulus cell interaction, membrane permeability, and meiotic spindle formation (Sprícigo *et al.* 2014).

It is currently more common to freeze mature (MII) stage oocytes, however, discrepancies regarding the optimal stage for bovine oocyte vitrification still abound, with benefits and liabilities associated with freezing both immature (GV) and MII stage oocytes (Mogas 2019). In GV oocytes, damage can occur to the oocyte's ultrastructure due to decreased membrane permeability and cumulus-oocyte disruption can occur due to gap junction damage (Magnusson *et al.* 2008). Damage occurring in MII oocytes is predominantly associated with microtubule depolymerization, abnormal meiotic spindle assembly, and chromosomal misalignment (Brambillasca *et al.* 2013).

The objectives of this study were to determine if vitrification effects the absolute mitochondrial DNA copy number, activity, distribution, and gene expression of oxidative stress response and lipid metabolism when bovine oocytes were vitrified at the GV or MII stage.

Materials and Methods

Oocyte recovery and in vitro maturation

Ovaries were collected at a local slaughterhouse and were transported to the laboratory in sterile saline at 25-28°C within 60 min of collection. Cumulus oocyte complexes (COCs), were aspirated from follicles 2-5 mm in diameter within 2 h of ovary collection, using an 18-gauge

needle and a vacuum pump set to 50mm Hg (Pioneer Pro Pump, Life Global Group, LLC, Guliford, CT, USA). All media used for COC and embryo handling and culture were prepared as described by De La Torre-Sanchez et al. 2006 (De La Torre-Sanchez *et al.* 2006). Aspiration lines were rinsed with CSU chemically defined medium for handling oocytes (H-CDM-M) supplemented with 10% BSA (Sigma Aldrich, USA) and gentamicin (10 mg/mL) prior to and after aspiration. This rinse provided a holding medium for COCs in the collection tube during aspiration, as well as ensured complete COC recovery (Stringfellow 1998; De La Torre-Sanchez *et al.* 2006). After aspiration, COCs with a minimum of two layers of intact cumulus cells were isolated and washed through H-CDM-M twice to remove all debris. COCs were then placed into dishes containing 1 mL CSU chemically defined medium for in vitro maturation of oocytes (IVM) pre-equilibrated at 38.5°C in 5% CO_2 and air for ≥ 5 h prior to addition of COCs. The IVM medium was supplemented with: 15 ng/mL NIDDK-oFSH-20, 1 μg/mL USDA-LH-B-5, 1 μg/mL estradiol 17β, 50 ng/μL epidermal growth factor, 0.1 mM cysteamine (De La Torre-Sanchez *et al.* 2006). To obtain MII oocytes, COCs were matured for 23h.

Oocyte vitrification and warming

Vitrification and warming were done using a base medium of H1-Mod medium (H1-Mod; H-CDM-1 + 20% FCS). GV and MII oocytes with intact cumulus cells were washed three times in a first equilibration solution V1; H1-Mod containing 7.5 % ethylene glycol and 7.5% dimethylsufoxide (DMSO) for a total of 9 min, followed by 45-60 s in a second vitrification solution V2; H1-Mod containing 15% ethylene glycol, 15% DMSO, and 0.5 M sucrose (Sprícigo *et al.* 2014). Oocytes were placed onto Cryotop® devices (Kitazato, Japan) in <1 μL V2 and plunged into liquid nitrogen and kept in cryostorage for at least one week (Sprícigo *et al.* 2014). Oocytes were warmed by placing the vitrified oocytes into 1 mL W1 (H1-Mod containing 1M

sucrose) at 38.5°C for 1 min. The oocytes were then moved in succession into 1 mL W2 (H1-Mod containing 0.5M sucrose) at 38.5°C for 3 min, and then H1-Mod for 3 min. The oocytes were then mechanically stripped of cumulus cells by gentle micropipetting using a STRIPPER® equipped with a 150 μm stripper tip (Origio, CooperGenomics, Denmark) in 0.01% hyaluronidase (Sigma Aldrich, USA) in HCDM-1. Denuded oocytes were washed 6 times through a series of CSU chemically defined medium for handling of early embryos (HCDM-1) until the wash medium was clean of cumulus cells. Denuded oocytes were held in H1-Mod until processed for either RT-qPCR or fluorescence staining. The meiotic stage was determined in GV oocytes by nuclear staining and in MII oocytes by visualization of a polar body.

Absolute quantitation of mitochondrial DNA content by real-time PCR

The mtDNA content of single oocytes (n=20 oocytes/treatment group) was quantified using real time PCR (RT-qPCR) as described by Pasquariello et al. (Pasquariello *et al.* 2019). Briefly, denuded oocytes were washed in Dulbecco's Phosphate-Buffered Saline containing no calcium or magnesium (DPBS, GIBCO/ThermoFisher Scientific, USA), but with 0.01% PVP (Polyvinylpyrrolidone; Sigma Aldrich, USA), and the oocytes were stored in 3 μL of this solution at −80°C. DNA was extracted using X-Tract™ DNA Extraction Buffer (Lucigen Corporation, USA). Briefly, 7 μL of extraction buffer were added to each tube containing an oocyte followed by lysing the zona pellucida using a vortex mixer. The tubes were then incubated at 65°C for 15 min, and 98°C for 2 min. After which, 40 μL nuclease free water (Thermo Fisher Scientific, USA) were added to each tube and the samples stored at -20°C until analysis. For analysis, each of the DNA samples were diluted 1:10 in RNase free water and analyzed by RT-qPCR using an absolute quantification assay. To do this, quantification standards were prepared. A 969 nucleotide (nt) fragment of the bovine DNA mitochondrial

region (Supplemental Table 2) was amplified by PCR using the Long Range PCR kit (Qiagen, USA) and then purified by using the QIAquick PCR purification kit (Qiagen, USA). The PCR fragment was cloned using the Qiagen PCR cloning kit (Qiagen, USA). Plasmid DNA was purified from bacteria using QIAprep Miniprep kit (Qiagen, Germantown, MD), and the concentration determined using a Nanodrop ND1000 Spectrophotometer (ThermoFisher Scientific, USA). A standard curve was generated using seven, tenfold serial dilutions (10^7 to 10 copies), and standard curve correlation coefficients were consistently greater than 0.99. Real-time quantitative PCR was run in triplicate for each standard dilution and sample using SYBR green PCR master mix (Applied Biosystems, Foster City, CA), a Quantstudio 5 real time machine (Applied Biosystems, USA), and the following amplification program: 50° C for 2 min, 95° C for 2 min, 95° C for 15 sec, followed by 40 cycles of 60° C for 30 sec and 72°C for 1 min. A melting curve was run for each experiment to assess specificity of the primers (primer sequences are detailed in Supplementary table 2). Standard curves were created for each run and sample copy number was generated from the equation of Ct value against copy number for the corresponding standard curve. Oocyte DNA samples were compared on the same RT-qPCR plate in order to produce comparable results for oocytes either fresh or vitrified at the GV or MII stage.

Analysis of mitochondrial activity and distribution

Oocyte mitochondrial distribution and activity were determined using MitoTracker® Orange CMTMRos (Molecular Probes, Eugene, OR, USA). This probe is sequestered into actively respiring mitochondria based on the oxidative activity of the mitochondria, and is retained in the mitochondria after fixation (Brevini *et al.* 2005; Moawad *et al.* 2014). Fresh or vitrified oocytes were denuded of cumulus cells by adding the oocytes to H-CDM1 + 0.1% (w/v)

hyaluronidase (Sigma Aldrich, H3506) and gently micropipetting them using a STRIPPER® equipped with a 150 μm stripper tip (Origio, CooperGenomics, Denmark). Oocytes were then placed into 1 mL PBS + 0.01% PVA with MitoTracker® Orange CMTMRos (250 nM) for 30 min at 38.5°C in 5% CO_2 in an atmosphere of air. Following incubation, to confirm meiotic stage the DNA was stained with 1.5 μg/ml Hoechst 33342 (Life Technologies, Eugene, OR, USA) for 15 min at 38.5°C. After staining, the oocytes were rinsed in PBS + 0.01% PVA and fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. The oocytes were then washed three times in PBS and the oocytes mounted on a slide using a 9 mm x 0.12 mm imaging spacer (Grace Bio-Labs Secure Seal™, Sigma Aldrich) attached to the center of a glass slide. A total of 5-6 fixed oocytes were placed into the center of the spacer in ≤ 5 μL PBS and a cover slip was placed on top and sealed. Slides were stored at 4°C until evaluation (Fluoview, Olympus, Center Valley, PA). Imaging was done using an Olympus Fluoview FV10-ASW 4.1 microscope (Olympus) at 60x magnification using excitation/emission at 554/576 nm (rhod-2/red fluorescence) for oxidized MitoTracker® Orange CMTMRos and 350/461 nm (DAPI/blue fluorescence) for Hoechst 33342.

Mitochondrial activity was determined as described previously (Brevini *et al.* 2005; Mitchell *et al.* 2009; Silva *et al.* 2015a; Pasquariello *et al.* 2019). The equatorial plane of each oocyte was scanned, and the fluorescence intensity was measured using ImageJ (Schneider *et al.* 2012). The mitochondrial activity was determined by measuring the pixel intensity of red channels in four areas (quarters) of the oocytes (using equal sized squares) within three different regions (i.e. cortical, intermediate, and nuclear regions) (Fig. 4.1). The mean value of red fluorescence intensity in the four areas was calculated, and results were expressed in arbitrary units.

Oocyte gene expression

Expression of the following genes: glutamate-cysteine ligase (GCL)-catalytic subunit (*GCLC*), glutathione peroxidase 1 (*GPX1*), nuclear respiratory factor 2 (*NRF2*), kelch-like ECH-associated protein 1 (*KEAP1*), superoxide dismutase-2 (*SOD2*), sterol regulatory element binding protein transcription factor 1 (*SREBP1*), peroxisome proliferator-activated receptor alpha (*PPARAα*), carnitine palmitoyl transferase-2 (*CPT2*) (Table 4.1). Data were normalized using expression of the housekeeping gene 18S ribosomal RNA (*18S*). A total of three biological replicates (pools of 10 eggs) for each group (fresh GV, vitrified GV, fresh MII, and vitrified MII) were used for quantitative real-time PCR. RNA extraction was performed using the PicoPure RNA Isolation Kit (ThermoFisher Scientific) with on-column DNase treatment (Qiagen). Complementary DNA (cDNA) was synthesized using the iScript™ cDNA Synthesis Kit (BiORAD, Hercules, CA), following the manufacturer's protocol. The cDNA samples were diluted 1:2 using RNase-free water and stored at -20°C until qPCR was conducted. Each PCR reaction was performed in triplicate using 7.5 µL Power SYBR™ Green PCR Master Mix (Applied Biosystems), 1.5 µL 10 µM primer mix, and 4 µL diluted cDNA sample. The qPCR program was as follows: 50°C for 2 min for first cycle, 95°C for 10 min for second cycle followed by 40 cycles of amplification step at 95°C for 15 s and 60°C for 1 min. A melting curve was analyzed for each experiment to assess the specificity of primer amplification. Normalized data were analyzed and plotted using the comparative C_T method ($2^{\wedge-\Delta C_T}$) (Schmittgen and Livak 2008).

Statistical analysis

All statistics were performed using GraphPad Prism® 8.0d (GraphPad Software, La Jolla, CA, USA). Student *t*-test (2 treatments) and one-way ANOVA (multiple comparisons) were

conducted depending on the number of experimental groups. Analysis of gene expression data was performed using one-way ANOVA with Tukey's multiple range test. Results were considered statistically significant when $P < 0.05$. Unless otherwise stated, results are presented as mean ± SEM.

Results

Absolute mitochondrial DNA (mtDNA) copy number

Absolute mitochondrial copy number were similar for fresh (968,076 ± 54,904 copies) and vitrified immature oocytes (777,920 ± 68,213 copies), and for fresh (863,154 ± 64,876 mtDNA copies) and vitrified mature oocytes (999,686 ± 63,764 copies) ($P>0.05$). No differences in mtDNA were observed between fresh or vitrified oocytes based on maturation stage (Fig. 4.2).

Mitochondrial oxidative activity and distribution

The majority of active mitochondria were distributed in the cortical region of the oocyte for all groups (Fig. 4.3b). Mitochondrial oxidative activity was lower in the cortical region of matured eggs compared to GV oocytes ($p<0.05$); (Fig. 4.3a). Mitochondrial activity was higher in the cortical region of vitrified GV oocytes compared fresh GV oocytes ($p<0.05$); (Fig. 4.3d). Mitochondrial activity was similar in the cortical regions for both fresh and vitrified mature oocytes (Figure 4.3c).

Expression of oxidative stress response and lipid metabolism genes

Expression of *GCLC* was lower for vitrified compared to fresh GV oocytes ($P<0.05$); (Fig.4.4), however its expression in both fresh and vitrified MII oocytes was similar ($P>0.05$). *GCLC* expression was lower in vitrified GV oocytes compared to vitrified MII oocytes ($P<0.05$). Expression of *GPX1* was similar between fresh and vitrified GV and MII oocytes but was lower

in fresh GV oocytes than vitrified MII oocytes (P<0.05). *KEAP1* expression was similar between fresh and vitrified GV oocytes and fresh and vitrified MII oocytes. *KEAP1* expression was higher in vitrified MII oocytes than GV oocytes (P<0.05). Expression of *CPT2* was lower in vitrified GV oocytes (P<0.05) compared to fresh GV oocytes but was similar in vitrified versus fresh MII oocytes. Expression of *NFR2*, *PARAα*, *SOD2*, and *SREBP1* was similar for all groups.

Discussion

We used three mitochondrial function parameters to assess the effects that vitrification can have oocyte quality. The first, mtDNA copy number, has been associated with oocyte competence in mice, and vitrification of MII oocytes reduced the total mtDNA copy number (Reynier *et al.* 2001; Amoushahi *et al.* 2017). In our study, vitrification did not affect the total mtDNA copy number of vitrified GV or MII oocytes compared to non-vitrified control oocytes, nor were there differences in mtDNA copy number between GV and MII oocytes.

Previous studies have presented conflicting data regarding whether meiotic stage affects mtDNA copy number. mtDNA copy number was shown to be higher in MII porcine oocytes after IVM compared to GV stage oocytes (GV: 167,365 versus MII: 275,132) (Mao *et al.* 2012; Lee *et al.* 2014), and similar results have been reported for bovine oocytes (GV: 368,118 versus MII: 807,794) (Iwata *et al.* 2011). However, similar to our findings, the mtDNA copy number was similar for GV and MII stage, ovine oocyte (Cotterill *et al.* 2013), mouse oocytes (Pasquariello *et al.* 2019), and human oocytes (Barritt *et al.* 2002; Pasquariello *et al.* 2019). A study evaluating immature ovine oocytes throughout the process of folliculogenesis showed that the majority of mtDNA replication occurs during initial stages of oocyte growth, with a significant increase in mtDNA occurring between the secondary follicles to early antral stage follicles, at which time mtDNA production plateaued but the absolute mtDNA copy number did

not differ between GV and MII stage oocytes (Cotterill *et al.* 2013). It is postulated that this increase in mtDNA copy number is needed to facilitate the production of sufficient ATP to sustain cytoplasmic and cytoskeletal reorganization in oocytes prior to nuclear maturation and fertilization. mtDNA copy number increases throughout folliculogenesis, with a 6-fold increase from secondary follicles to preantral, prior to oocyte maturation, suggesting the majority of mtDNA replication occurs during folliculogenesis, with limited mitochondrial production post maturation (Cotterill *et al.* 2013).

Vitrification can negatively impact mitochondrial structure and function due to ultrastructural and biomolecular alterations (Zhou *et al.* 2010; Mogas 2019; Daddangadi *et al.* 2020), however few studies have been conducted to determine if vitrification affects absolute mitochondrial copy number in mammalian oocytes. Vitrification of MII mouse oocytes showed less mtDNA copy number after 1 h incubation post warming (Amoushahi *et al.* 2017), but similar investigations using bovine oocytes have not been done. Our results indicate that mtDNA copy number was similar for both fresh and vitrified oocytes immediately after warming. However, quantification after culturing of vitrified-warmed oocytes, in future studies, may show similar quantitative differences as seen in the mouse study (Amoushahi *et al.* 2017).

High mitochondrial content is associated with increased oocyte quality and competence. A developmental threshold of 100,000 mitochondria has been proposed for human and mouse oocytes (Wai *et al.* 2010). However, several conditions associated with reduced mtDNA content have been shown to reduce oocyte quality. Oocytes from individuals of advanced maternal age (AMA) have lower mtDNA content than oocytes from young individuals (Pasquariello *et al.* 2019), and oocytes from patients with primary ovarian insufficiency (POI) contain threefold less mtDNA than oocytes from control individuals (May-Panloup *et al.* 2005). In addition, inherited

mtDNA depletion syndromes are associated with female infertility (Luoma *et al.* 2004; Pagnamenta *et al.* 2006). Furthermore, when mtDNA copy numbers were reduced, in porcine oocytes, using 2'3 dideoxycytidine (ddC), blastocyst production was reduced, compared to controls; supporting the principle that oocytes with few mitochondria affects oocyte and embryo developmental potential (Lee *et al.* 2014). Tests in mice showed that embryos with low mtDNA copy number, determined by single blastomere biopsy at 8-cell stage, exhibited reduced post-implantation development (11%) vs embryos with high copy numbers (60%) (Wai *et al.* 2010). Wai et. Al. (2010) concluded that oocyte mtDNA content below the lower end of the biologically normal distribution (50,000 copies), seen in wild type oocytes were unlikely to progress normally through post-implantation development, suggesting that development is only compromised if levels fall below a critical threshold.

In our study, mtDNA copy number for individual oocytes showed large variation between biological replicates (range GV fresh: 13,232 – 1,273,116; GV vitrified: 759,108 – 1,332,084; MII fresh: 433,856 – 1,331,027; MII vitrified: 713,993 – 1,423,727). Similar biological variation in mtDNA copy number between oocytes has also been reported in human and ovine oocytes (Cotterill *et al.* 2013; Pasquariello *et al.* 2019). Biological variability in the mtDNA content of mouse oocytes also showed tremendous variability (Wai *et al.* 2010). It has been postulated that mtDNA variations may be reflective of oocyte quality and achieving an optimum range of mitochondrial activity and/or ATP production, that may be species-specific, is necessary for fertilization and embryogenesis. Therefore, oocytes with mtDNA levels below or above this range, may have reduced developmental potential (Reynier *et al.* 2001; Barritt *et al.* 2002; Santos *et al.* 2006; Zeng *et al.* 2007; Cotterill *et al.* 2013; Pasquariello *et al.* 2019). This inherent variability makes it difficult to establish thresholds, particularly with so much observed

variation between species (Wai *et al.* 2010). Although we did not see significant differences in the absolute mtDNA copy number between treatment groups, the observed variation between individual oocytes indicates that inherent oocyte quality may also be affected by copy number. Interpretation of these data is problematic as it requires the sample to be destroyed to obtain the data. To highlight the degree of variability, human studies have shown range of 240,000-1,550,000, within oocytes from the same individual (Barritt *et al.* 2002; Cotterill *et al.* 2013). Bovine oocytes were recently shown to have 300,000 copies mtDNA, similarly reported in humans (Cree *et al.* 2015).

Oocytes, at different maturation stages of exhibit different physiologies, including differences in oocyte-cumulus cell interactions, plasma membrane permeability, presence or absence of nuclear membranes, differences in cytoskeletal structure and chromosome configuration (Mogas 2019), all of which can be affected during vitrification. Therefore, it seems reasonable to assume there would be specific problems associated with vitrification depending on meiotic stage. Vitrifying an oocyte at the GV stage avoids damage to meiotic spindles and chromosomes, as the spindles are not yet organized, and chromosomes are still within the nucleus. However, mature oocytes may have greater cryotolerance since they have already completed meiosis I and can avoid problems associated with polar body extrusion and organelle redistribution (Sprícigo *et al.* 2014). Results from the present study showed that vitrification did not alter absolute mtDNA copy number, but did change mitochondrial activity and oxidative stress related gene expression of immature GV oocytes.

Vitrification of MII stage oocytes may produce issues with male pronuclei formation and reduces intracellular glutathione levels (Somfai *et al.* 2007; Liang *et al.* 2012; Egerszegi *et al.* 2013), but other studies have reported higher blastocyst rates, for oocytes vitrified at the MII

stage (Magnusson *et al.* 2008; Moawad *et al.* 2014; Nohalez *et al.* 2015a). Studies also report that, even though oocyte viability is high, for vitrified GV oocytes matured in vitro, fertilization rates and developmental capacity is decreased resulting in low blastocyst rates (Magnusson *et al.* 2008; Nohalez *et al.* 2015a). This may be due in part to the GV oocyte cytoplasmic membrane being less permeable to CPAs compared to mature oocytes, making them more susceptible to cryodamage, such as gap junction disruption causing issues with cumulus cell-oocyte communication (Diez *et al.* 2005; Zhou *et al.* 2010; Brambillasca *et al.* 2013; Sprícigo *et al.* 2014). The plasma membrane of mature oocytes contain higher concentrations of saturated and polyunsaturated fatty acids, making them more fluid and allowing for greater resistance to chilling injury at low temperatures (Brambillasca *et al.* 2013). As well as meiotic stage, in vitro culture conditions may affect cryo-sensitivity in in vitro matured oocytes. Plasma membranes of in vitro matured oocytes have fewer aquaporins than in vivo matured oocytes, which may cause decreased membrane permeability of penetrating CPAs (Brambillasca *et al.* 2013). The presence of cumulus cells on GV oocytes further inhibits CPA permeability, but denuding the oocytes of cumulus cells at this meiotic stage limits oocyte developmental capacity after warming (Nohalez *et al.* 2015a). While the optimal meiotic stage for oocyte vitrification remains unknown, our results show that GV oocytes are more susceptible to mitochondrial alterations after vitrification.

In the present study, mitochondrial activity increased after vitrification of GV oocytes but was similar for fresh and vitrified MII oocytes. Recent studies similarly showed that vitrified bovine and ovine oocytes exhibited higher mitochondrial activity immediately after warming, suggesting this may be due to higher energy requirements for reorganization of organelles, cytoplasm, and redox potential after oocyte vitrification/warming (Gutnisky *et al.* 2020). Conversely, porcine oocytes exhibited a reduction in mitochondrial membrane potential (MMP),

ATP quantification, and evidence of ultra-structural damage post vitrification (Dai *et al.* 2015). Human MII oocytes also exhibited reduced MMP and lower intracellular ATP after vitrification and warming, but those recovered to levels observed in fresh oocytes by 4 h of incubation, inferring that given time, oocytes can restore mitochondrial activity balance (Chen *et al.* 2012). Our results suggest that vitrification alters mitochondrial oxidative activity in immature oocytes, indicating that GV oocytes may be more susceptible to cryodamage than mature MII eggs. It is unclear why oxidative activity increased in response to vitrification, as higher mitochondrial activity is associated with increased oocyte quality. It is possible that this increase is a temporary compensatory mechanism employed by the oocyte to increase ATP levels to overcome vitrification induced stress (Cotterill *et al.* 2013; Moawad *et al.* 2014; Pasquariello *et al.* 2019; Gutnisky *et al.* 2020).

We observed higher mitochondrial activity in both fresh and vitrified GV oocytes compared to MII oocytes. These results were unexpected, as previous studies using human oocytes, showed increased mitochondrial oxidative activity in MII oocytes after in vitro maturation and suggested reduced developmental potential when this rise was not observed (Van Blerkom and Davis 2007). Similar to our study, a recent study in mice showed decreased membrane potential and activity in MII vs GV oocytes after vitrification, indicating alterations in mitochondrial function in MII oocytes but not GV oocytes (Daddangadi *et al.* 2020). Reductions in intracellular calcium levels and thus cortical granule redistribution were also greater in vitrified MII oocytes versus GV oocytes, with both being reduced from control oocytes (Daddangadi *et al.* 2020). This may indicate that in vitrified MII oocytes, the integrity of zona receptors was more compromised due to chilling injuries than immature oocytes (Daddangadi *et al.* 2020). However, conflicting results from additional studies in murine (Blerkom *et al.* 2003), ovine (Cotterill *et al.* 2013), pig (Brevini *et al.* 2005), and

bovine (Stojkovic *et al.* 2001) oocytes showed no differences in mitochondrial activity between GV and MII oocytes. There was no observed increase in mitochondrial potential or oxidative activity, however ATP levels were increased during in vitro maturation (Stojkovic *et al.* 2001; Brevini *et al.* 2005). Interestingly, the increase in ATP production observed in in vitro matured MII oocytes was not observed in in vivo matured MII oocytes, possibly indicating that higher energy requirements may be generated to overcome inadequate in vitro conditions (Van Blerkom 2004; Brevini *et al.* 2005). Contradicting findings in this study and by others using bovine oocytes emphasizes the incongruity of information that remains regarding normal mitochondrial function due to meiotic stage (Tarazona *et al.* 2006).

Mitochondrial distribution is thought to affect meiotic spindle assembly, chromosomal segregation, and redistribution of active mitochondria during maturation and has been associated with the increased production of ATP production during the MI to MII transition required for these molecular changes (Van Blerkom 2004; Yu *et al.* 2010). ATP is a product of mitochondrial respiratory oxidative phosphorylation and provides energy for spindle assembly and subsequent embryonic development (Stojkovic *et al.* 2001). Some studies have shown that mitochondrial redistribution from cortical regions to subcortical and perinuclear regions at MII result in increased ATP levels prior to spindle assembly and fertilization (Stojkovic *et al.* 2001; Cotterill *et al.* 2013). Other studies have alternatively suggested that peripherally (cortical) concentration mitochondria may be required for correct segregation of sister chromatids at fertilization (Dalton and Carroll 2013). The relocation of mitochondria to different areas within the ooplasm during cytoplasmic maturation is orchestrated by microtubule networks within the cytoskeleton (Brevini *et al.* 2007). Therefore, damage to this network can alter the ability for mitochondrial redistribution and the subsequent metabolism within the oocyte (Magnusson *et al.* 2008).

A cortical distribution pattern of active mitochondria was observed in all categories of oocyte in this study. Similarly, previous studies have reported cortical distribution of active mitochondria in fresh and vitrified bovine (Zhao *et al.* 2011b) and human (Chen *et al.* 2012) oocytes and in both GV and MII mouse oocytes (Pasquariello *et al.* 2019). Others have observed a more central distribution of mitochondria throughout the MII oocyte (Rho *et al.* 2002; Machatkova *et al.* 2012). The significance of centrally dispersed mitochondria throughout the intermediate and nuclear regions of the ooplasm after maturation is associated with rising ATP levels and meiotic spindle assembly (Yu *et al.* 2010). One study showed that abnormal mitochondrial distribution was higher in vitrified oocytes than in fresh GV and MII oocytes, and that irreversible damage to the cytoskeleton was incurred during vitrification (Dai *et al.* 2015). Dehydration of oocytes during vitrification may induce microtubule network damage, altering proper mitochondrial redistribution during oocyte maturation (Rho *et al.* 2002). Mitochondrial distribution did not change after oocyte maturation or vitrification, therefore in this case, effects of vitrification on microtubule integrity and organelle distribution are not clear. Oocyte quality may affect mitochondrial distribution as well. Studies looking at oocytes from aged mice versus young mice showed that oocytes originating from aged groups had reduced cortical distribution (15%) compared to those from young mice (50%), as well as reduced metabolism (Pasquariello *et al.* 2019). Interestingly, the addition of antioxidants increased cortical distribution in oocytes from aged mice and increased activity to rates comparable to that of young oocytes, indicating that the observed perinuclear distribution may be influenced by negative effects of increased ROS in oocytes from older donors (Pasquariello *et al.* 2019).

ROS are highly reactive molecules that can damage intracellular molecules and structures such as proteins, lipids, DNA, and membranes leading to compromised mitochondrial function

and membrane lipid peroxidation which can affect an oocyte's developmental capacity, gene expression and metabolism (Balaban *et al.* 2005; Powers and Jackson 2008; Amin *et al.* 2014). In the present study, reduced expression of certain genes associated with oxidative stress was only observed in immature GV oocytes. Oxidative stress can be induced through exposure to suboptimal conditions in the in vitro environment resulting from medium composition, pH, oxygen tension and from within the oocyte, as by-products of metabolic pathways including mitochondrial phosphorylation, leading to the accumulation of ROS (Amin *et al.* 2014).

When under oxidative stress, oocytes can activate responses to deal with those challenges such as the *NRF2* oxidative-stress-response pathway (Amin *et al.* 2014; Pasquariello *et al.* 2019). *NRF2* is an important transcription factor regulating many antioxidant related genes in mammalian cells and is involved in lipid metabolism and lipid accumulation (Tanaka *et al.* 2008; Amin *et al.* 2014). In the present study, *GCLC* (an antioxidant defense mechanism gene associated with *NRF2*) was reduced in vitrified GV oocytes compared to fresh GV oocytes which may indicate a compromised antioxidative stress response in GV oocytes, that is not occurring in MII eggs. Previous studies have shown that protection against oxidative stress, during early embryonic development, is driven by the maternal pool of antioxidant enzymes stored in the oocyte during oogenesis (Harvey *et al.* 1995), consistent with the model that *NRF2* activity and antioxidant concentration was not significantly triggered by oxidative stress before the 8-cell stage (Amin *et al.* 2014). Similar to our study, mouse oocyte quality was not impacted by expression of *NRF2* related genes, possibly indicating that stored oxidative stress enzymes are being used, rather than the *NRF2* pathway being activated (Amin *et al.* 2014; Pasquariello *et al.* 2019).

In the present study, expression of *CPT2,* a gene involved in lipid metabolism, was lower in GV vitrified oocytes than non-vitrified GV oocytes indicating possible reduction in lipid

oxidation pathways. Intracellular lipid accumulation is associated with reduced oocyte cryotolerance (Campos-Chillon *et al.* 2009; Barceló-Fimbres and Seidel Jr 2011; Gutnisky *et al.* 2020) which may be due to the inability of the oocytes to properly metabolize lipids as accumulating ROS may disrupt mitochondrial activity (Barcelo-Fimbres and Seidel Jr 2007). ROS induced by cryopreservation increases lipid peroxidation leading to mitochondrial dysfunction and compromised cellular activity (Dai *et al.* 2015). While vitrification only altered the expression of some genes in the *NRF2* pathway, it is possible that other antioxidants present within the oocyte are also being utilized (Harvey *et al.* 1995). Reduced expression of *GCLC* and *CPT2* in GV oocytes further indicates cryopreservation may have a greater impact on immature oocytes than mature oocytes.

Abnormal mitochondrial function is associated with low quality oocytes and embryos and can be used as a parameter to evaluate oocyte competence (Machatkova *et al.* 2012). It is not clear why results in this study showed increased mitochondrial activity in GV oocytes post vitrification. Similar patterns of mitochondrial distribution were observed in fresh and vitrified oocytes, suggesting that vitrification is not impacting normal mitochondrial redistribution orchestrated by microtubule networks within the cytoskeleton.

In summary, mitochondrial integrity and function in oocytes was maintained through the vitrification-warming process, but alterations to metabolic processes were observed. Our results showed that vitrification caused an increase in mitochondrial activity, similar to other studies, but contradictory to some. These discrepancies indicate that more research is necessary to understand effects of vitrification on bovine oocyte quality.

Table 4.1. a. Gene symbol, gene description, primer sequences, melting temperature (Tm) , amplicon (pair of bases) and accession number for every gene quantified by real-time quantitative PCR. b**.** Primer sequences used for the absolute quantification assay of the mitochondrial DNA.

Supplemental Table 1. Gene symbol, gene description, primer sequences, melting temperature (Tm), amplicon (pair of bases) and accession number for every gene quantified by real-time quantitative PCR

Gene symbol	Gene description	Sequence (5' → 3')		Tm (°C)	Amplicon size (bp)	Accession number
GCLC	glutamate-cysteine ligase (GCL)-catalytic subunit	forward	CAACCCTTTGGAGACCAGAA	60	113	NM_001083674.1
		reverse	CCGTTTCCTCATGTTGTCCT			
GPX1	glutathione peroxidase	forward	CCACTGGCAGGAACTTTGAT	60	137	AF080228
		reverse	TTCCTCTTCAGGGATGGTTG			
NRF2	nuclear respiratory factor 2	forward	TCCAACCTTTGTCGTCATCA	55	174	AB162435
		reverse	TTGCCCGTAGCTCATCTCTT			
KEAP1	kelch-like ECH-associated protein 1	forawrd	TCACCAGGGAAGGATCTACG	55	199	NM_001101142.1
		reverse	AGCGGCTCAACAGGTACAGT			
SOD2	super oxide dismutase-2	forward	GGATCCCCTGCAAGGAACAA	60	110	NM_201527.2
		reverse	TGGCCTTCAGATAATCGGGC			
SREBP1	sterol regulatory element binding protein transcription factor1	forward	ACCGCTCTTCCATCAATGAC	56	190	NM_001113302.1
		reverse	TTCAGCGATTTGCTTTTGTG			
PPARAα	peroxisome proliferator-activated receptor alpha	forward	CCTACGGGAATGGCTTCATA	54	219	NM_001034036.1
		reverse	GCACAATACCCTCCTGCATT			
CPT2	camitine palmitoyl transferase-2	forward	CACAACATCCTGTCCACCAG	54	209	NM_001045889.1
		reverse	CCTTCCAAGGCATCAAACAT			

Supplemental Table 2. Primer sequences used for the absolute quantification assay of the mitochondrial DNA.

Genome	Fragment length (bp)	sequence (5->3)	Primer verse	Assay
Bovine mtDNA	969	CTAAGAGGAGCTGGCATCAAG	Forward	Insert cloned in the plasmid to make standard curve
		GGTTGTTTAGTCGAGAGGGTATC	Reverse	
	120	GGGCTACATTCTCTACACCAAG	Forward	Absolute RT-qPCR

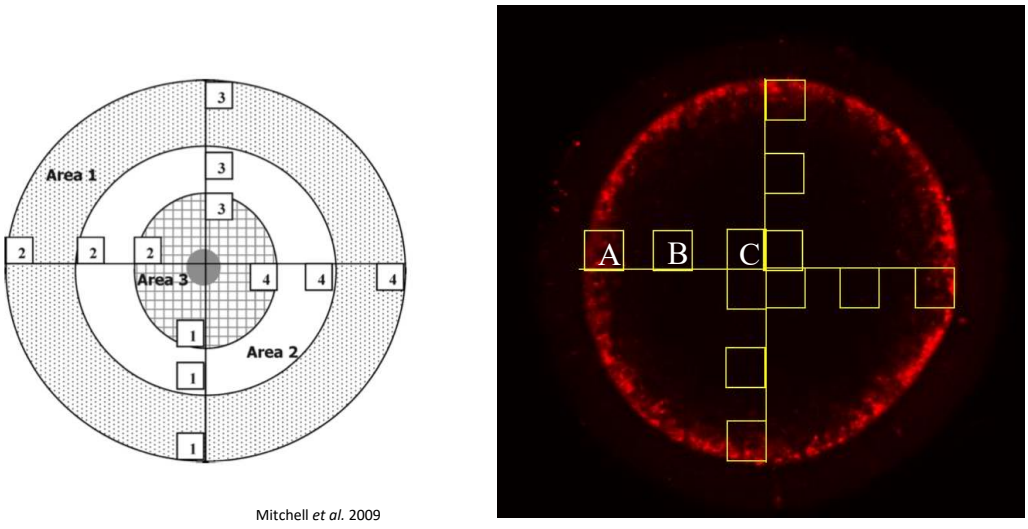

Figure 4.1. Representative diagram and images of mitochondrial activity determined by measuring pixel intensity in 4 regions (1-4) of three areas (A) cortical, (B) intermediate, and (C) nuclear.

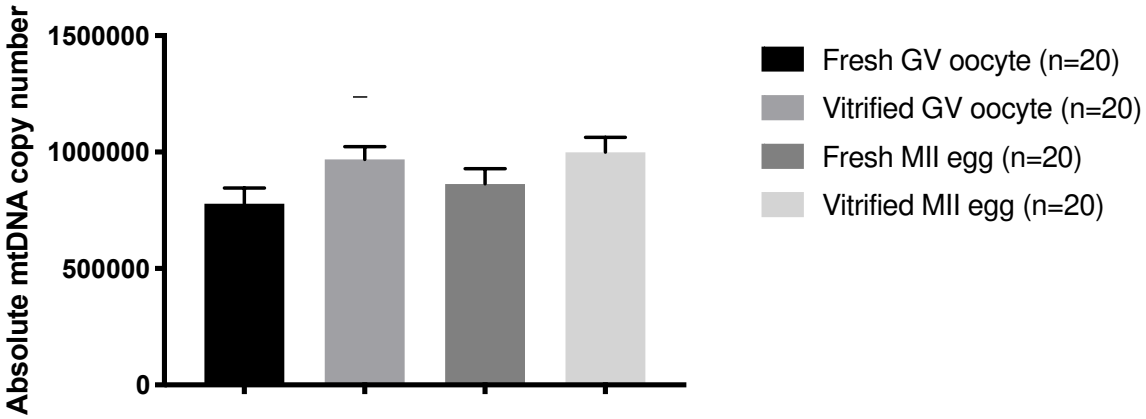

Figure 4.2. Quantification of mitochondrial DNA (mtDNA) copy number in fresh and vitrified bovine GV oocytes and in vitro matured MII eggs.

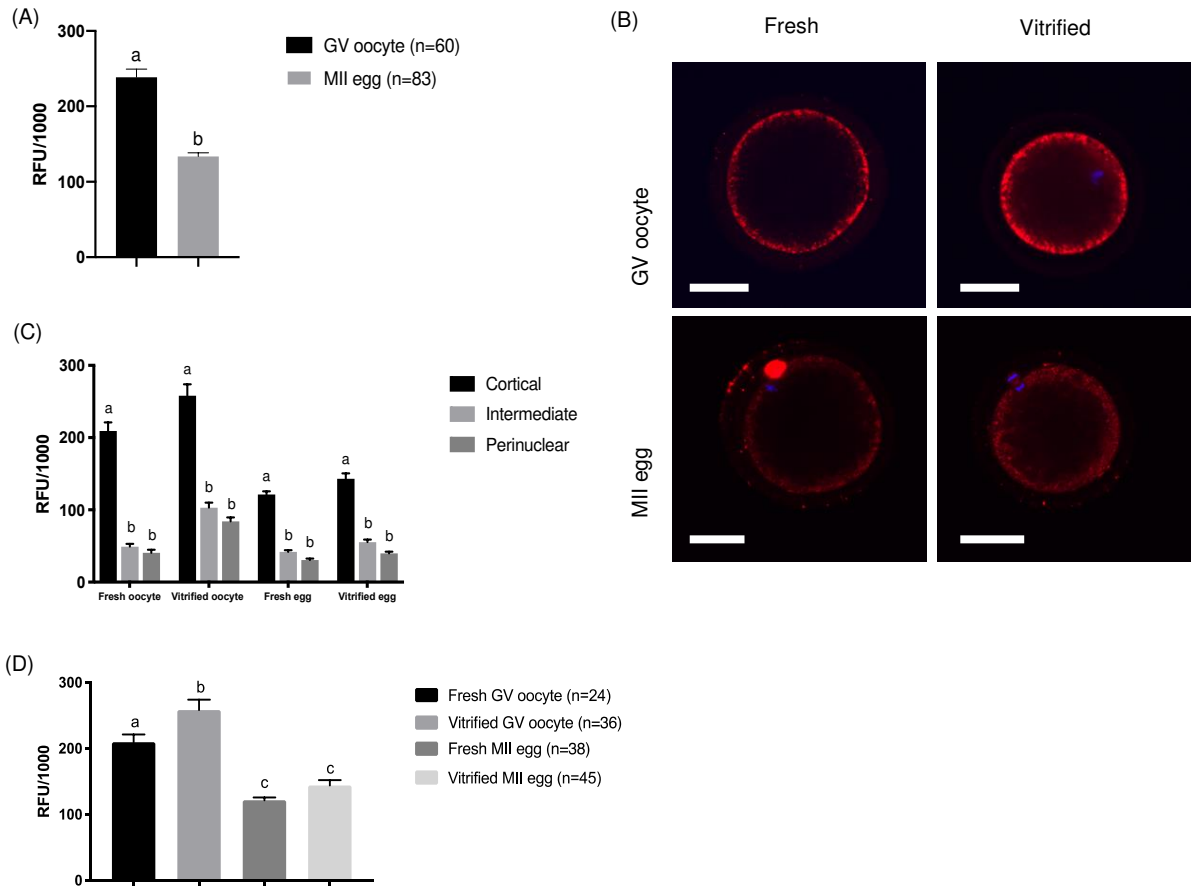

Figure 4.3. Distribution of active mitochondria in fresh and vitrified bovine GV oocytes and in vitro matured MII eggs. (A) Quantification of mitochondrial in GV oocytes and MII eggs, (B) Representative images of cortical distribution of active mitochondria in fresh and vitrification GV oocytes and MII eggs. Scale bar is equivalent to 50 µm. (C) Quantification of active mitochondria in three regions of fresh and vitrified GV oocytes and MII eggs ($P < 0.05$). (D) Quantification of active mitochondria in the cortical region of fresh and vitrified GV and MII eggs.

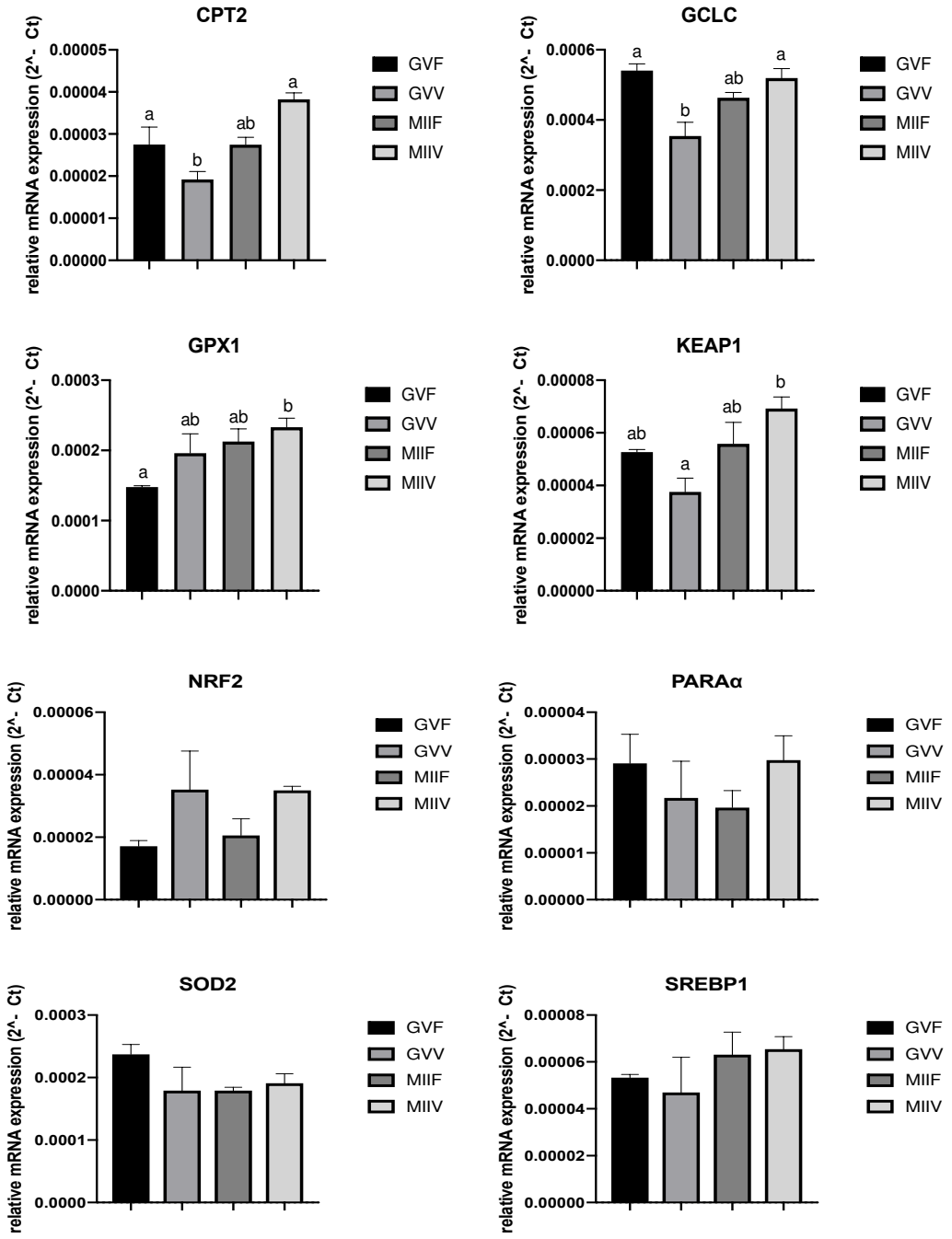

Figure 4.4. Expression of oxidative stress and lipid metabolism related genes in fresh and vitrified bovine GV oocytes and in vitro matured MII eggs. Different superscripts indicate statistical differences (P< 0.05).

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CHAPTER 5: IN VITRO ACTIVATION OF PRIMORDIAL FOLLICLES VIA HIPPO SIGNALING DISRUPTION AND PI3K-AKT-PTEN PATHWAY STIMULATION IN VITRIFIED BOVINE OVARIAN CORTICAL TISSUE

Summary

Two experiments were conducted investigating 1) the effects of vitrification and *in vitro* culture of vitrified-warmed bovine ovarian tissue on cell viability; and 2) markers of folliculogenesis and cell proliferation after *in vitro* activation (IVA) of primordial follicles via disruption of Hippo and PI3K-Akt pathway signaling. Adult and fetal bovine ovarian cortical tissue were analyzed by histological evaluation, live/dead staining, and expression genes associated with follicular and cellular growth (*AHR, BMP15, GDF9, and MKI67)*. Gene expression was similar for vitrified-warmed, vitrified-warmed-cultured, and fresh cortical tissue, indicating follicular viability was maintained in both cryopreserved and cultured cortical tissue. Histological analysis revealed morphologically normal follicles in tissues from both treatment groups, although cryodamage to tissue was observed as evident by stromal and follicular degeneration. When vitrified-warmed fetal calf ovarian tissue was subjected to short-term (48 h) IVA, expression of all genes except *CYP19* (aromatase) were similar for all IVA treatments. *CYP19* was lower in both cryopreserved treatment groups compared to fresh controls. Phosphorylation of pathway associated factors, YAP and Akt, are indicative of pathway activation or disruption and were analyzed to determine effects of IVA treatments. Total Akt, YAP, p-YAP levels were reduced in both treatment groups compared to fresh control while p-Akt levels remained similar, indicating cellular degradation in vitrified cultured tissue. Together, these data suggest that while tissue viability is maintained after vitrification, it may negatively impact follicles, resulting in poor IVA outcomes.

Introduction

Mammalian ovaries contain a finite number of germ cells and follicles that are established during fetal development. In cattle, primordial germ cells (PGCs) migrate to the genital ridge and differentiate into oogonia around 40 days postcoitum (dpc) (Wrobel and Süß 1998). Oogonia mitotically divide and proliferate, forming germ cell nests connected through cytokinesis (Pepling 2006; Kenngott *et al.* 2013). Bovine oogonia enter meiosis at 70-80 dpc and are converted into oocytes arrested in MI, shortly followed by the formation of primordial follicles, which can be isolated from ovaries of fetal calves by 140 dpc (Erickson 1966). In cattle, up to 80-90% of initial oogonia undergo apoptosis between days 130-170 resulting in an initial population of 200,000 immature oocytes in primordial follicles at birth (Erickson 1966; Santos *et al.* 2013). Multiple cellular pathways are responsible for the spontaneous and irreversible activation of a cohort of primordial follicles to begin growth, resulting in either ovulation or atresia. Folliculogenesis will continue until the follicular pool is eventually depleted resulting in reproductive quiescence (Shah *et al.* 2018). Primordial follicles are the most abundant follicular population in the ovary and comprised of a quiescent diplotene oocyte arrested in prophase of meiosis I surrounded by a single layer of flattened pre-granulosa cells (Hirshfield 1994). Regulating the activation of primordial is crucial for maintaining ovarian function and female fertility (McLaughlin and McIver 2008; Reddy *et al.* 2010).

In humans, cortical tissue can be preserved for cancer patients, as an option to preserve fertility after chemo/radiation therapy. It can also be used as a tool for banking of genetically valuable tissue from threatened species. The limited supply of competent oocytes is a significant impediment to increasing the success of assisted reproductive technologies (ART) in domestic and wildlife species (Telfer *et al.* 2020). Cattle are an ideal domestic model to develop techniques, such as in vitro follicle activation (IVA) to preserve tissue, for nondomestic hoof

stock as they are physiologically more similar than murine models, and bovine ovaries can be easily obtained from slaughterhouses for research (Santos *et al.* 2010). In cattle, this technology can be used to increase genetic gain, particularly if gametes are obtained from young prepubertal females (Martin *et al.* 2019).

The ability to preserve ovarian tissue and then activate follicles would provide a mechanism to collect reproductive material from females without concern about the impacts of factors such as seasonality, animal maturity, or mortality on oocyte competence or embryo production. Harvesting large numbers of primordial follicles at one time, for use *in vitro,* may be a way to preserve large amounts of genetic material than could occur in vivo. If prepubertal ovarian tissue containing primarily primordial follicles can be preserved and later stimulated to mature in vitro, it could represent a very large and otherwise inaccessible reserve of genetic material and potentially increase the genetic impact a single individual can have in a population.

The preferred method to preserve ovarian tissue is vitrification (Shahsavari *et al.* 2020a). Vitrification requires the use of cryoprotectant agents (CPA) at high concentrations, which both dehydrate the cell (nonpermeating CPA) and concentrate within the cell (permeating CPA) where they induce liquids to form a glass when cooled, preventing intracellular ice crystals and related cellar damage (Kagawa *et al.* 2009). Studies have shown that mouse oocytes derived from vitrified whole ovaries can be matured and fertilized in vitro resulting in the birth of live pups (Hasegawa *et al.* 2004; Hasegawa *et al.* 2006; Hasegawa and Koyama 2007).

However, the size of human and bovine ovaries, prevents the vitrification of the entire ovary. Instead, only the outer ovarian cortex, containing the majority of the primordial follicles is removed for cryopreservation (Barrett and Woodruff 2010). Primordial follicles have greater cryotolerance than more mature follicles, due to their small size, oocyte dormancy, reduced

differentiation and lack of zona pellucida (Khosravi *et al.* 2013). By cutting human and bovine cortical tissue into strips, the cryoprotectants could penetrate the stromal tissue adequately for the tissues to survive vitrification and retain follicular viability (Kagawa *et al.* 2009). For conservation and the establishment of genetic resource banks (GRBs), preservation of ovarian tissue may help increase the amount of available genetic material from small populations for use in ART, as it can be collected from juvenile as well as reproductively mature females (Santos *et al.* 2010). The vitrification of prepubertal ovaries requires additional considerations as this tissue, once thawed, contains immature follicles that need to be isolated, activated, and cultured to maturity in vitro, fertilized, and the resulting embryo cultured in vitro prior to embryo transfer.

Follicular and oocyte growth, differentiation, and activation are driven by complex molecular pathways orchestrated by a variety of hormones and growth factors (Silva *et al.* 2016). One important pathway for mouse, human, and bovine follicle activation is the Hippo signaling pathway (Shah *et al.* 2018). Through mechanical signaling, Hippo signaling regulates cell proliferation and apoptosis, and is responsible for maintaining optimal organ size via growth inhibition (Pan 2007; Kawamura *et al.* 2013). This signaling occurs when physical stimuli are relayed into biochemical signals impacting gene expression and cellular activity.

The ovary is a dynamic organ that is constantly undergoing physical remodeling during follicular and luteal phases. It is believed that mechanical signaling may play a critical role in the dynamic characteristics of the ovarian follicle including primordial follicle storage and dormancy, activation, and maturation in part via the Hippo signaling pathway (Grosbois and Demeestere 2018; Shah *et al.* 2018). In humans and mice, the Hippo pathway inhibits follicular growth and maintains follicular dormancy, as a cellular response to the rigid external environment provided by the cortical stroma. In a dormant state, Hippo pathway mediators

phosphorylate and inactivate the yes-associated protein (YAP) and the PDZ-binding motif (TAZ), keeping these proteins sequestered to the cytoplasm which maintains follicular quiescence (Kawamura *et al.* 2013; Xiang *et al.* 2015). Mechanical stress causes non-phosphorylated cytoplasmic YAP/TAZ to translocate to the nucleus, which induces the transcription growth factors and apoptosis inhibitor genes to promote cellular growth, in this case, folliculogenesis (Dupont *et al.* 2011; Dupont 2016).

A second pathway associated with follicular activation is the PI3k-Akt-PTEN signaling pathway (Li *et al.* 2010; Moniruzzaman *et al.* 2010; Reddy *et al.* 2010; Maidarti *et al.* 2019; Maidarti 2020). This pathway maintains cellular proliferation and survival in tissues throughout the body, including the ovary (Liu *et al.* 2007; Moniruzzaman *et al.* 2010; Bao *et al.* 2011; Kawamura *et al.* 2013; Shah *et al.* 2018). It regulates primordial follicle activation via two primary pathway associated factors, phosphatidylinositol 3-kinase (PI3K) and the phosphatase and tensin homolog deleted from chromosome 10 (*PTEN*) gene, with the PTEN protein negatively regulating PI3K. Once PI3K is activated via upstream pathway mediators it phosphorylates phosphatidylinositol-4, 5-biphosphate (PIP2) into phosphatidylinositol-3,4,5-triphosphate (PIP3), which phophorylates Akt (protein kinase B). Activated phosho-Akt (p-Akt) is then translocated into the nucleus where it suppresses the forkhead box 03 (FOXO3a) transcription factor, a regulator of follicular quiescence. p-Akt in the nucleus suppresses the forkhead box 03 (FOXO3a) transcription factor through phosphorylation, resulting in its nuclear export into the cytoplasm. Thus, cytoplasmic phosphor-FOXO3a is seen as a hallmark of follicle activation. PTEN negative regulation of the Akt signaling pathway is considered essential for maintenance of follicle quiescence. PTEN acts as an Akt inhibitor by dephosphorylating activated PIP3 to PIP2 which prevents activation through Akt activity suppression, leading to

nuclear export of FOXO3a into the cytoplasm. Indeed, *Foxo*$^{-/-}$ mice showed global primordial follicular activation, indicating its crucial role in follicle quiescence (Castrillon *et al.* 2003).

IVA approaches targeting the Hippo and PI3k-Akt-PTEN signaling pathways have been investigated in mice, non-human primates, humans, pigs, sheep, and cattle (Li *et al.* 2010; Moniruzzaman *et al.* 2010; Bao *et al.* 2011; Kawamura *et al.* 2013; Ting and Zelinski 2017; dos Santos *et al.* 2018). Hippo signaling disruption via tissue fragmentation is used to disengage structural forces of cortical stromal scaffolding resulting in primordial follicle activation (Kawamura *et al.* 2013; Kawashima and Kawamura 2018). This fragmentation alone has been shown to cause spontaneous follicle activation and growth in bovine cortical fragments, and studies in mice show that ovarian fragmentation causes a decrease in phosphorylated YAP levels and increased nuclear localization of YAP, causing upregulation of growth factors and apoptosis inhibitors (Wandji *et al.* 1996; Braw-Tal and Yossefi 1997; Kawamura *et al.* 2013). The PI3K-Akt-PTEN signaling pathway has been chemically activated using a PI3K activating peptide, 740 Y-P, and a PTEN inhibitor, bpV(pic), to stimulate primordial follicle activation in vitro. Neonatal mouse ovaries treated with the combination of PTEN inhibitor and an Akt activating peptide increased the nuclear export of Foxo3 in primordial oocytes, indicating that follicle activation had commenced (Li *et al.* 2010). Treatment of fresh and frozen human cortical pieces with bpV(pic) resulted in increased initiation of follicular growth, increased estradiol production, and production of competent oocytes after ovarian tissue transplantation (McLaughlin *et al.* 2014; Novella-Maestre *et al.* 2015). Together, these results suggest that IVA can be used to successfully promote primordial follicle growth using multiple regulatory pathways.

Objectives of this study were to determine the effects of vitrification on fetal and adult bovine ovarian cortical tissue viability and morphology, and to investigate the efficacy of IVA

via Hippo signaling disruption and chemical activation of the Akt-PTEN signaling pathway to induce primordial follicle activation.

Materials and Methods

Collection of fetal and adult bovine ovaries

Near-term female bovine fetuses (angus crosses and Holstein) were obtained at a local slaughterhouse (Double J Meat Packing Inc., Pierce, CO). Fetal age was estimated by crown-rump length (Kan and Cruess 1987). Whole fetal calf reproductive tracts were collected and transported to the laboratory in 50 mL conical tubes containing H-HCDM-M with 10% BSA and gentamicin (10 mg/mL) kept at 25-28°C. Adult bovine ovaries were obtained at local slaughterhouse (JBS USA Food Co., Greeley, CO) and ovaries transported to the laboratory in sterile saline kept at 25-28°C.

Ovarian cortical tissue processing

All visible antral follicles were aspirated prior to cortical tissue processing and adult ovaries with a corpus luteum (CL) were excluded. The ovarian cortexes of both fetal and adult ovaries were dissected from the medullary tissue to a thickness of 0.5-1.0 mm using either beveled dissection scissors or a Thomas Stadie-Riggs Tissue Slicer®. Cortical pieces were held in H-HCDM-M in 100 mm glass petri dishes and cut into approximately 2.0 x 4.0 x 1.0 mm^3 strips using a sterile scalpel.

Ovarian tissue vitrification-warming

Ovarian cortical pieces were vitrified following the Cryotissue method with minor modifications as described by Kagawa *et al.* (Kagawa *et al.* 2009). Briefly, cortical strips were equilibrated for 25 min in equilibration solution (15%): 7.5% ethylene glycol (EG) and 7.5%

dimethyl sulphoxide (DMSO), with 0.1% hydroxypropyl cellulose (HPC) in oocyte maturation medium based vitrification medium stock solution (OVM; supplemented with 0.5mg/mL hyasis hyaluronan (HAIM-5; Lifecore Biomedical, Chaska, MN) and 10 μg/mL gentamicin), followed by 30 min in vitrification solution (30%): 15% EG and 15% DMSO, with 0.15% HPC, 4% ficoll, and 0.6M trehalose in OVM stock solution. Cortical strips were then placed onto the metal prongs of a tissue Cryo Device® (Ova Cryo Kit Type M; Kitazato BioPharma, Fuji, Shizouka, Japan) and plunged into liquid nitrogen (LN_2); (Fig. 5.1). While submerged in LN_2, the Cryo Device® was inserted into a protective outer cryovial and held in a liquid nitrogen storage tank.

Cortical strips were warmed by removing the Cryo Device® protective cover under LN_2 and the prongs of the Cryo Device® holding the strip rapidly plunged into a sterile microcentrifuge tube containing 1 mL 37°C thawing solution (TS): OVM with 1M trehalose, 20% (v/v) synthetic serum substitute (SSS; Irvine Scientific, Santa Ana, CA) for 1 min until the cortical strip detached from the Cryo Device® and sank to the bottom of the tube. After 1 min, the tissue was poured into a 35 mm dish and the strip placed into a new dish containing 1 mL ambient temperature dilution solution (DS): OVM with 0.5M trehalose, 20% (v/v) SSS for 3 min. Finally, the strip was placed into 1 mL ambient temperature wash solution (WS): OVM with 20% (v/v) SSS for at least 5 min. Only the tissue that was translucent pink to peach color at the time of vitrification and warming was used for further experiments (Fig. 5.1). Tissue that was opaque and white, indicative of tissue cryodamage, was discarded (Laronda *et al.* 2017a).

Experiment 1: Effects of vitrification on fetal calf and mature cow ovarian cortical tissue

In vitro tissue culture

Vitrified-warmed cortical tissue from mature cows was further cut into approximately 1 x 1 x 1 mm^3 pieces, and 8-10 pieces were placed onto Millicell® cell culture inserts (Millipore

Sigma) in Nunc™ cell-culture treated 24-well dish (ThermoFisher Scientific) containing 400 µL basic medium [DMEM/12 (Life Technologies Corp.) with 25% human serum albumin (HSA; ThermoFisher), 0.01% insulin-transferrin-selenium (ITS) X100 (Life Technologies Corp.), 0.03 IU/mL ovine follicle stimulating hormone (oFSH; Natl. Hormone & Pituityary Prgm. UCLA, Torrance, CA), 0.001% L-ascorbic acid (Sigma) and 0.01% Gibco® antibiotic-antimycotic 100X (ThermoFisher)] for 24 h at 37°C in 5% CO_2 in humidified air. The culture medium was replaced after 24 h and the tissues cultured for an additional 24 h. After 48 h, the cortical pieces were removed from the inserts, rinsed with sterile PBS + 0.01% PVA, and fixed for histological evaluation or snap frozen, for use in gene expression analysis.

Histological evaluation of tissue morphology

Adult cow ovarian cortical tissue pieces were fixed in 4% paraformaldehyde (PFA) for 30 min before being placed into a tissue cassette. Liquified Richard Allen-Scientific™ HistoGel™ (Thermo Fisher Scientific, Waltham, MA) was dispensed over the tissue piece(s) until covered. Cassettes were placed into a 10% neutral buffered formalin solution for 24 h, and the blocks were then embedded in paraffin wax. The tissues were sliced into 5µm thick sections as described by Braw-Tal et al., and mounted onto poly-L-lysine coated slides (P0425; Sigma Aldrich, St. Louis, MO) and allowed to dry overnight. The samples were deparaffinized, rehydrated, and stained with hematoxylin (Harris) and eosin or VectaShield®Antifade with DAPI (Vector Laboratories, Burlingame, CA) (Braw-Tal and Yossefi 1997).

Enzymatic follicle isolation and LIVE/DEAD imaging

Vitrified cortical pieces from three fetal calves were warmed and 2.0 x 4.0 x 1.0 mm^3 pieces were dissected using a sterile scalpel and placed into a 15 mL conical tube containing 1 mL of 1

mg/mL collagenase type 1 (SCR103; Millipore, Temecula, CA) in 1X PBS (Aerts *et al.* 2008). Tubes were incubated in a 37°C water bath for 45 min during which pieces were mechanically agitated by pipetting every 15 min. Enzymatic digestion was stopped by adding an equal volume of 4-8°C PBS + 10% fetal calf serum (FCS) to the suspension. The suspension was then centrifuged at 300g for 10 min at 8°C, the supernatant was removed, and the remaining tissue pellet was resuspended in 7-10 mL PBS + 10% FCS. Primordial (oocytes with one layer of flat pre-granulosa cells present), primary (one layer cuboidal granulosa cells) and secondary (two layers cuboidal granulosa cells) follicles were dissected from the cortical tissue under a stereo microscope (Nikon SMZ1270) using 22 gauge needles (McLaughlin *et al.* 2014). Isolated follicles were collected using a STRIPPER® and 125 µm stripper tips (Origio, CooperGenomics, Denmark) and transferred into a non light-permiating dish containing equal volumes PBS + 10% FCS and a stock solution of LIVE/DEAD® Cell Imaging Kit prepared according to manufacture instructions (R37601, Thermo Fisher Scientific, Waltham, MA) for 25 min. The intracellular esterase activity of live cells converts the non-fluorescent cell-permeant calcein AM to the fluorescent calcein (green), which is retained within live cells. The second fluorophore of the LIVE/DEAD® Cell Imaging Kit, ethidium homodimer-I, is cell-impermeant and therefore only enters dead or dying cells with damaged membranes and fluoresces red upon binding to DNA. After incubation, the follicles were transferred into 1X PBS + 0.01% PVP before being placed onto poly-L-lysine coated slide in a 10 µL drop. Drops were covered with a glass coverslip and imaged using fluorescence microscopy (Nikon Eclipse E800) with FITC (ex/em 488 nm/515 nm) and TRITC (ex/em 570nm/602 nm) filters.

Gene expression in vitrified tissue

The gene expression of the following cellular proliferation and folliculogenesis associated genes were determined (Table 1): aryl hydrocarbon receptor (*AHR*), bone

morphogenic protein 15 (*BMP15*), growth differentiation factor 9 (*GDF9*), and marker of proliferation Ki-67 (*MKI67*). Data were normalized using expression of the housekeeping genes 18S ribosomal RNA (*18S*) and glyceraldehyde 3-phosphate dehydrogenase *(GAPDH)*. A total of three biological replicates (cortical tissue from 3 adult cows) for each treatment group (fresh, vitrified-warmed, vitrified-warmed-cultured) were used for quantitative real-time PCR snap-freezing in liquid nitrogen in 10 µL PBS + 0.01% PVA. Tissue homogenization was performed using an Argos Technologies® Pellet Mixer (Cole-Parmer, Vernon Hills, IL) to grind the dense, tough stromal tissue in a BioMasher II® Tissue Grinder (Kimble Chase, DWK Life Sciences, Vineland, NJ) tube followed by RNA extraction using RNeasy®Micro Kit (Qiagen) with on-column DNase treatment (Qiagen). Complementary DNA (cDNA) was synthesized using qScript™ cDNA Synthesis Kit (QuantaBio) following the manufacturer's protocol. The cDNA samples were diluted 1:5 using RNase-free water and stored at -20°C until qPCR was run. Each PCR reaction was performed in triplicate using 7.5 µL Power SYBR™ Green PCR Master Mix (Applied Biosystems), 1.5 µL 10 µM primer mix, and 3.5 µL 1:5 diluted cDNA sample. The qPCR program was as follows: 50°C for 2 min for first cycle, 95°C for 10 min for second cycle followed by 40 cycles of amplification step at 95°C for 15 s and 58°C for 1 min. A melting curve was analyzed for each experiment to assess the specificity of primer amplification. Normalized data were analyzed and plotted using the comparative C_T method ($2^{\wedge-\Delta C_T}$); (Schmittgen and Livak 2008).

Experiment 2

In vitro activation

Vitrified fetal calf cortical tissue was further dissected into approximately 1 x 1 x 1 mm^3 pieces, and the pieces were randomly divided into two groups for: Hippo disruption

(fragmentation only) or chemical activation (AKT activator 740Y-P and PTEN inhibitor bpV(pic)). A total of 8-10 fragments were placed onto Millicell® cell culture inserts (Millipore Sigma) in Nunc™ cell-culture treated 24-well dish (ThermoFisher Scientific) containing either 400 µL basic medium only (Hippo disruption group) or in in vitro activation (IVA) medium [basic medium with 150 µg/mL 740 Y-P (Tocris, Bristol, UK) and 30µM bpV(pic) (Enzo Life Science, Farmingdale, NY)] for 24 h at 37°C in 5% CO_2 in humidified air. Inserts were washed three times by placing them into wells containing 700 µL basic medium. After washing, the inserts were placed into new wells containing either 400 µL fresh basic medium or IVA medium (now supplemented with only 150 µg/mL 740 Y-P) and cultured an additional 24 h. After the 48 h culture period, cortical pieces were removed from the inserts, rinsed with sterile PBS + 0.01% PVA, and prepared for tissue fixation or snap frozen for use in gene expression analysis or protein quantification. For Experiment 2, IVA treatments for Hippo disruption by tissue fragmentation or by chemical activation via 740 Y-P and bpV(pic) will be referred to as HD or 740 Y-P+bpV(pic), respectively.

Immunohistochemistry

Fetal calf ovarian cortical tissues were fixed and embedded in paraffin as previously described. Paraffin sections were deparaffinized and rehydrated to evaluate the expression of FOXO3a. Antigen retrieval was performed in heat using 10mM sodium citrate buffer (pH 6.06) for 20 min and endogenous peroxidase activity was blocked using BLOXALL® solution (Vector Laboratories, Peterborough, UK). Non-specific sites were blocked using goat serum for 30 min in an ambient temperature humidified chamber. Tissue sections were incubated in Anti-FKHRL1 (FOXO3a) #F2178 polyclonal primary antibody (Sigma Aldrich, St. Louis, MO) at 4°C overnight. Negative controls were made by incubating sections with goat serum in lieu of a

primary antibody. Sections were then washed and probed with biotinylated anti-rabbit secondary antibody (Vector Laboratories, Peterborough, UK) for 1 h and processed using the VECTASTAIN Elite ABC HRP kit for 30 min per manufacturer's instructions (Vector Laboratories, Peterborough, UK). FOXO3a was detected using 3, 3'-diaminobenzidine (DAB) peroxidase substrate kit (Vector Laboratories, Peterborough, UK) and counterstained with hematoxylin. Imaging was performed using light microscopy (Nikon Eclipse E800).

Estradiol Measurement

Estrogen detection in tissue culture medium was used as a measure of folliculogenesis. Pooled culture medium from Day 1 (24 h) and Day 2 (48 h) was collected after the 48 h incubation period described above. Medium from 3 culture replicates of 6 calves were collected for both HD and 740 Y-P+bpV(pic) treatments. Duplicate aliquots of culture medium were measured for estradiol by radioimmunoassay (RIA). The inter-assay coefficient of variation (CV%) was 4.9%.

Jess Western analysis

Total protein was isolated from 100-200 mg of cultured fetal calf ovarian tissue (4 calves, 2 culture repetitions, HD and 740 Y-P+bpV(pic) treatments and fresh non-fragmented control) via homogenizing in RIPA buffer (R0278; Sigma Aldrich) containing 1X protease (11836170001; Millipore Sigma) and 1X phosphatase inhibitor (78420; ThermoFisher) followed by ultrasonication. Protein lysate were then centrifuged at 15,000 rpm for 15 min at 4ºC to remove the cellular debris. The supernatant was transferred into fresh 1.5 ml centrifuge tube and subjected for protein quantification using DC^{TM} Protein Assay Kit (500111; Bio-Rad, Hercules, CA) following the manufacturer protocol. After quantification, 250 ng/µL of total protein was

used to analyze the protein abundance of target proteins on capillary based Western blot plateform (Jess, ProteinSimple, San Jose, CA) using 12-230 kdD JESS separation module (SM-W004) following the manufacturer's protocol. Protein detection and digital images were collected and analyzed with Compass software (ProteinSimple) and data were reported as area under the peak, which represented the intensity of the signal. The following primary antibodies were used at a 1:20 dilution: anti-FOXO3a (ab109629; Abcam, Cambridge, UK), anti-FOXO3a (phosphor s253) (ab154786; Abcam), YAP (4912S; Cell Signaling Technology, Danvers, MA), phospho-YAP (Ser127) (4911S; Cell Signaling Technologies), AKT (4691S, Cell Signaling Technologies), phospho-AKT (4060S, Cell Signaling Technologies), anti-aromatase (ab124776; Abcam), and anti-FSH-R (nbp2-36489; Novus Biologicals, Centennial, CO). Depending upon the primary antibody, HRP-conjugated anti-mouse (042-205; Proteinsimple) and anti-rabbit (043-426; Proteinsimple) ready-to-use secondary antibodies were used for chemiluminescence based detection of target proteins. Abundance of each protein was normalized by ACTIN and presented as relative fold change between treatment groups and fresh tissue control.

Gene expression of markers of cellular proliferation and folliculogenesis

Expression of the following genes was analyzed (Table 1): estrogen receptor 1 (*ESR1*), follicle stimulating hormone receptor (*FSHR*), anti-m*üllerian* hormone (*AMH*), aromatase *(CYP19)*, aryl hydrocarbon receptor (*AHR*), bone morphogenic protein 15 (*BMP15*), growth differentiation factor 9 (*GDF9*), and marker of proliferation Ki-67 (*MKI67*). Data were normalized using expression of the housekeeping genes 18S ribosomal RNA (*18S*) and glyceraldehyde 3-phosphate dehydrogenase *(GAPDH)*. A total of six biological replicates (ovarian cortical tissue from 6 calves) for each treatment group (chemically activated or fragmented only) and three controls (uncultured cortical tissue) were used for quantitative real-

time PCR snap-freezing in liquid nitrogen in 10 µL PBS + 0.01% PVA. Tissue homogenization was performed using an Argos Technologies® Pellet Mixer (Cole-Parmer, Vernon Hills, IL) to grind the tissue in a BioMasher II® Tissue Grinder (Kimble Chase, DWK Life Sciences, Vineland, NJ) tube followed RNA extraction using RNeasy®Micro Kit (Qiagen) with on-column DNase treatment (Qiagen). Complementary DNA (cDNA) was synthesized using iScript™ cDNA Synthesis Kit (BiORAD, Hercules, CA) following the manufacturer's protocol. The cDNA samples were diluted 1:3.5 using RNase-free water and stored at -40°C until qPCR was run. Each PCR reaction was performed in duplicate using 7.5 µL Power SYBR™ Green PCR Master Mix (Applied Biosystems), 1.5 µL 10 µM primer mix, and 1.5 µL 1:3.5 diluted cDNA sample. The qPCR program was as follows: 50°C for 2 min for first cycle, 95°C for 10 min for second cycle followed by 40 cycles of amplification step at 95°C for 15 s and 58°C for 1 min. A melting curve was analyzed for each experiment to assess the specificity of primer amplification. Normalized data were analyzed and plotted using the comparative C_T method ($2^{\wedge\Delta C_T}$); (Schmittgen and Livak 2008).

Statistical analysis

All statistics were performed using GraphPad Prism® 8.0d (GraphPad Software, La Jolla, CA). Gene expression data were analyzed using one-way ANOVA test with Tukey's HSD *post hoc* test. Jess western data were analyzed using Brown-Forsythe and Welch ANOVA test, with ratios compared using unpaired Student's t-test. Estradiol concentrations were compared by unpaired Student's t-test. Quantitative data are presented as mean ± SEM. P values <0.05 were considered significant for all analyses.

Results

Tissue morphology and viability post vitrification-warming

Stromal and follicular morphology assessed using both hematoxylin and eosin (H&E) showed follicles with intact morphology in fresh non-vitrified (Fig. 5.2), vitrified-warmed (Fig. 5.3), and vitrified-warm in vitro cultured (Fig. 5.4) mature cow ovarian cortical tissue. Fresh tissue sections showed uniform cytoplasm in primordial follicles (Fig. 5.2b,e arrows), close contact between oocytes and granulosa cells (Fig. 5.2d,f arrows), and dense tightly compacted stroma (Fig. 5.2a,c). Vitrified-warmed tissue sections also showed uniform cytoplasm in follicles (Fig. 5.3b, arrows), close contact between oocytes and granulosa cells (Fig. 5.3c), and dense tightly compacted stroma (Fig. 5.3a) as seen in fresh tissue. However, some sections showed evidence of cryodamage such as shrunken oocytes (Fig. 5.3e, arrows) and decompaction of stromal cells (Fig. 5.3e-f, arrows). Vitrified-warmed-cultured tissue sections also showed some morphology similar to fresh tissue (Fig. 5.4g). However, most sections showed some evidence of cryodamage including shrunken oocytes (Fig. 5.4f,h, arrows), pyknotic nuclei (Fig. 5.4a, arrows), abnormal space between the follicle and stroma (Fig. 5.4d, arrow) and decompaction of stromal cells (Fig. 5.4b).

Live/dead staining with calcein-AM and ethidium homodimer-I was used to assess post-thaw follicle viability in mechanically isolated primordial, primary, and secondary follicles from vitrified-warmed ovarian tissue source from 3 fetal calves. Live (green) cells were observed in follicles isolated from tissue pieces from all calves (Fig. 5.5).

Effects of vitrification on expression of cellular proliferation and folliculogenesis associated genes.

Expression of *MKI67*, *AHR*, *GDF9,* and *BMP15* did not statistically differ between fresh non-vitrified, vitrified-warmed, and vitrified-warmed mature cow ovarian cortical tissue (Fig. 5.6).

Localization of FOXO3a after Hippo disruption and PI3K-Akt pathway activation

Localization of Foxo3a in fetal bovine ovarian cortical tissue was assessed by immunohistochemistry (Fig. 5.7-5.9). First, Foxo3a localization in the cytoplasm was observed in non-fragmented fresh and non-fragmented vitrified-warmed follicles (Fig. 5.7a-b). Both cytoplasmic and nuclear localization of Foxo3a was observed in HD (Fig. 5.8a-d) and 740 Y-P + bpV(pic) (Fig. 5.9a-d) treated tissue. Antibody signal was low in all groups, including in non-fragmented tissue, making accurate quantification difficult.

Effects of IVA on Hippo signaling and PI3K-Akt pathway components

Jess analysis showed a significant decrease in both YAP ($p= 0.003$); (Fig. 5.10) and p-YAP ($p= 0.03$); (Fig. 5.11) in both HD and 740 Y-P+bpV(pic) treatment groups compared to fresh controls. No difference was observed in the ratio between YAP and p-YAP (Fig. 5.12) in treatment groups and fresh control. A significant decrease in total Akt ($p= 0.005$); (Fig. 5.13) was observed in both treatment groups compared to fresh controls, while no difference was observed in p-Akt between any group (Fig. 5.14). A significant increase in the ratio of Akt to p-Akt was observed in all groups ($p\leq 0.001$); (Fig. 5.15). No difference was observed in Aromatase between any group (Fig. 5.16).

We were unsuccessful at precipitating clear bands for FSHR, FOXO3a, and p-FOXO3a using Jess, therefore quantitative and comparative analysis could not be performed for these proteins.

Effect of IVA on estradiol production in ovarian tissue

Estradiol concentrations did not differ ($P<0.05$) between HD and 740 Y-P + bpV(pic) treatment groups, 3.2 ± 0.4 versus 3.1 ± 0.3 pg/μL (Fig. 5.17a), nor between biological replicates (Fig. 5.17b).

Effects of IVA on expression of cellular proliferation and folliculogenesis associated genes

Expression of *ESR1, CYP19, FSHR, AHR, GDF9, AMH, BMP15,* and *MKI67* in cortical tissue was similar between IVA treatment groups and control (Fig. 5.18A-F, H). Expression of *CYP19* (Fig. 5.18G) decreased in both IVA treatment groups compared to control (p=0.03).

Discussion

Vitrification and in vitro tissue culture systems can maintain ovarian tissue viability and support ongoing folliculogenesis, and bovine primordial follicles have been found to initiate growth within 2 days of culture (Fortune *et al.* 2010). In our study, adult cow tissue was used to confirm follicle viability after vitrification. This experiment was conducted after all fetal calf tissue used in Experiment 2 had been collected and cryopreserved. Limited amounts of tissue was available from each fetal calf and it was reserved for use in IVA experiments. Therefore, adult cow ovarian tissue was used for preliminary investigations of tissue morphology, viability, and expression of follicular growth and cellular proliferation associated genes. Vitrified-warmed fetal calf tissue was used to determine effects of Hippo signaling pathway disruption and PI3K-Akt-PTEN pathway activation in primordial follicles by evaluating cellular localization of FOXO3a, ratios of phosphorylation of key Hippo and PI3K pathway components YAP and Akt, as well as proteins involved with granulosa cell steroid biosynthesis, and expression of early folliculogenesis and cellular proliferation associated genes. Results from this study indicated that vitrification maintained partial follicle viability, however, tissue morphology was compromised after vitrification and during in vitro culture, thus in vitro activation via Hippo disruption and chemical activation was unsuccessful. Similarly, a recent study of vitrified-warmed bovine cortical tissue showed while follicular morphology was maintained post-warming, a decrease in the percentage of normal follicles was observed after 5 days in in vitro culture compared to those

cultured samples on day 1 (Shahsavari *et al.* 2020b). As well they observed higher levels of degeneration and tissue damage in all vitrified-warmed groups compared to controls on day 1. Interestingly, by day 5, similar levels of degeneration were then observed post-culture between vitrified-warmed and fresh controls indicating that in vitro culture had similar negative effects on tissue, regardless of tissue status prior to culture (Shahsavari *et al.* 2020b). In the present study, vitrification didn't immediately show detrimental effects on morphology or follicle viability, however it is possible that non-immediate effects of cryodamage may have impacted tissue viability after 48 h in vitro culture. Unfortunately, we were not able to culture fresh tissue for comparison.

Activated follicles are responsible for secretion of many growth factors and hormones involved with autocrine and paracrine signaling. Oocytes in the primary and secondary stage secrete transforming growth factor β (TGFβ) family proteins, GDF9 and BMP15, which promote newly forming granulosa cell proliferation (Dong *et al.* 1996; Galloway *et al.* 2000). Growing follicles produce transcription factor AHR, which is involved in estradiol biosynthesis, as well as MKI67, a well-established marker of cellular proliferation (Hur *et al.* 2009; Hernandez-Ochoa *et al.* 2010). No differences in expression were observed in genes *AHR*, *BMP15*, *GDF9*, and *MKI67* in vitrified or cultured tissue compared to controls, indicating maintenance of tissue but not proliferation. It is possible that gene expression in tissue that was vitrified did not differ from fresh controls due to immediate processing for PCR after warming, however that is unclear as similar expression levels were observed in vitrified-warmed cultured tissue, despite morphological evidence of stromal and follicular degeneration after in vitro culture.

Mechanical signaling or mechanotransduction can be triggered by internal or external factors, and plays a critical role in the dynamic changes of the ovarian follicle (Kawashima and

Kawamura 2018). Biomechanical mediators including but not limited to intercellular junctions, cellular polarity and membrane ion channels, and extracellular matrix (ECM) proteins keep primordial follicles in quiescence via Hippo signaling (Shah *et al.* 2018). Disruption of Hippo signaling by mechanical forces dephosphorylates and activate yes-associated protein (YAP) and PDZ-binding motif (TAZ) though a serine/threonine kinase cascade, translocates YAP/TAZ to the nucleus to trigger a proliferative state and promote cellular growth (Pan 2007; Kawamura *et al.* 2013; Xiang *et al.* 2015). Mechanical signaling acts on upstream regulators of YAP/TAZ, Rho-GTPase and Rho-associated protein kinase (ROCK), to regulate Hippo function via actin remodeling (Dupont *et al.* 2011; Dupont 2016). Polymerization of actin from globular actin (G-actin) to filamentous actin (F-actin) facilitates cell shape preservation, adhesion, and locomotion and allows for nuclear localization of YAP and is seen as a trademark of Hippo disruption (Kawamura *et al.* 2013). In our study, decreased levels of both YAP and p-YAP in both treatments compared to fresh controls indicate universal tissue degradation due to in vitro culture rather than a treatment specific effect. No differences observed in phosphorylation ratios (YAP : p-YAP) in any treatment group further indicates that Hippo signaling pathways were not adequately disrupted, thereby imparting no effect on down-stream effectors, including CCN growth factors and BIRC apoptotic proteins which promote granulosa cell proliferation and survival (Pan 2007; Holbourn *et al.* 2008).

The transition of primordial to primary follicle is gonadotropin independent (Fortune *et al.* 2000). In addition to biomechanical cues, it has been shown in mice that certain granulosa cell derived growth factors have been implicated in PI3K-Akt pathway stimulation including the receptor tyrosine kinase, Kit (Hutt *et al.* 2006). When stimulated, PI3K initiates a phosphorylation cascade by which PIP2 is phosphorylated to PIP3, which in turn phosphorylates

and activates Akt. Once activated, p-Akt is translocated to the nucleus where it phosphorylates FOXO3a at the Akt site causing its translocation and retention in the cytoplasm and inhibits FOXO3a dependent transcription resulting in the transition from primordial to primary follicle (Liu *et al.* 2009). In our study, decreased levels of Akt in treatments compared to fresh controls and overall low p-Akt levels all groups are again indicative of negative effects of in vitro culture. Phosphorylation ratios (Akt: p-Akt) in all groups showed significantly lower p-Akt present in cells indicating a failure to stimulate PI3K-Akt signaling through IVA methods, most likely due to impaired tissue function. In the present study, IHC showed predominantly cytoplasmic FOXO3a localization in non-fragmented fresh controls implying PI3K-Akt pathway activation and primordial follicle awakening. As these were fetal calf tissue pieces, mass primordial follicle activation in non-fragmented, non-stimulated tissue is unlikely. While the primary antibody used in this study was confirmed for use in bovine ovarian tissue (Bao *et al.* 2011), these observations along with off-target staining of stromal tissue make these results ambiguous and suggest that the antibody used was not specific enough. Three serine phosphorylation sites associated with FOXO3a phosphorylation by Akt have been shown to be conserved between humans, mice, pigs: Ser ^{Thr32} , Ser^{253}, Ser^{315} (Matsuda *et al.* 2010; Bao *et al.* 2011; Schneider *et al.* 2014). Unfortunately, antibody non-specificity was seen again in protein quantification of FOXO3a and p-FOXO3a Ser^{253}. Different FOXO3a and p-FOXO3a Ser^{253} primary antibodies were used at increased dilutions (1:250, 1:1000), but we were unable to precipitate clear, on-target protein bands of either protein. Therefore, the true effect of our IVA treatments on FOXO3a localization remains unclear.

The ovary is an endocrine reproductive organ. Estradiol is produced through FSH-mediated aromatase (CYP19) activity in granulosa cells in preantral follicles, and is crucial for

oogenesis, cellular proliferation, and folliculogenesis (Kishi *et al.* 2018). In this study, expression of *ESR1, FSHR,* and *AMH* did not differ between IVA treatments and fresh controls, indicating no increase in folliculogenesis of present activated follicles. As AMH is secreted by dormant primordial follicles, steady unchanged expression of *AMH* is further evidence of unsuccessful IVA. Hippo pathway associated components including Yap are known to be involved in steroidogenesis in ovarian cells (Fu *et al.* 2014). In this study, *CYP19* expression decreased in both treatment groups compared to control, however no difference in aromatase protein levels was observed. Estradiol concentration in IVA medium did not differ between treatment groups, and was low compared to what has been reported in previous bovine studies (Fortune *et al.* 2010). However, total tissue volume differed between culture wells making an accurate assessment of the RIA data difficult. Low estradiol concentrations indicate limited aromatase biosynthesis activity in present preantral growing follicles suggesting compromised granulosa cell integrity after in vitro culture.

Since these data were collected, studies in bovine have been able to report successful outcomes from similar protocols and techniques described used here (Grosbois and Demeestere 2018; Telfer *et al.* 2020). Recent studies have shown that abnormal hippo disruption use for Akt-stimulated follicle growth may result in over accumulation of nuclear YAP/TAZ, leading to ovarian tissue abnormalities, tumor progression, and impaired fertility. What impact that has on an in vitro system or gametes produced there from are unknown (Plewes *et al.* 2018). Indeed, proper functioning of the PI3K-Akt pathway has also been linked to bovine oocyte competence, suggesting that both pathways influence not only follicular growth proliferation, but also gamete quality (Andrade *et al.* 2017). It is essential to understand how in vitro effects on these pathways affect oocyte competence if we are to use this technology as an alternative approach for sourcing oocytes.

Conserved pathways involved in primordial follicle activation in mammals make the continuous collective research relevant across species. Excitingly, studies in humans have proposed the use of drug-free IVA without tissue culture (Kawamura *et al.* 2016; Zhai *et al.* 2016; Kawashima and Kawamura 2018). Ovarian cortical tissue was surgically removed from patients with primary ovarian insufficiency (POI), fragmented to induce Hippo disruption, pieces were immediately surgically grafted back into the patient, and mature oocytes were able to be retrieved from antral follicles after in vivo follicle growth (Kawamura *et al.* 2016; Zhai *et al.* 2016). Results presented in our study suggest that vitrified bovine cortical tissue maintains viability after warming and could be used in a simplified drug-free IVA protocol to improve IVA outcomes by eliminating the negative effects resulting from in vitro tissue culture. As seen in other species, fragmented tissue pieces could then be surgically removed, allotransplanted under the oviductal serosa of an ovariectomized recipient, or xenotransplanted under the kidney capsules of severe combined immunodeficiency mice (SCID) mice to support follicular growth (Li *et al.* 2010; Kawamura *et al.* 2013; Ayuandari *et al.* 2016). Competent oocytes could then be aspirated from in vivo matured follicles followed by in vitro maturation and fertilization to create embryos.

At present, improved vitrification protocols to preserve stromal integrity and follicular viability are still needed to make it feasible to biobank vitrified ovarian cortical tissue from valuable domestic or wild Bovidae. Additionally, continued studies are needed to establish successful IVA protocols for bovine ovarian tissue that can result in production of competent gametes. Sourcing female germ cells from primordial follicles preserved in vitrified ovarian tissue may provide a unique alternative solution for obtaining valuable genetic material for use with other ART such as in vitro embryo production to produce healthy offspring.

Table 5.1. Gene symbol, gene description, primer sequences, melting temperature (Tm) , amplicon (pair of bases) and accession number for every gene quantified by real-time quantitative PCR in Experiment 1 and 2.

Supplemental Table 1. Gene symbol, gene description, primer sequences, melting temperature (Tm), amplicon (pair of bases) and accession number for every gene quantified by real-time quantitative PCR in Experiment 1 and 2.

Gene symbol	Gene description	Sequence (5' → 3')	Tm (°C)	Amplicon size (bp)	Accession number
AHR	Aryl hydrocarbon receptor	forward GCAGCCACCATCCATACT reverse TTGGCATCACAACCAGTAGG	55	93	NM_001621.5
MKI67	marker of proliferation Ki-67	forward TTACCTCCCAACACACCTCT reverse GGGCTGCTCCTTGATGATTT	56	96	NM_002417.4
CYP19	aromatase	forward CTGCTGCTCACTGGATTTCT reverse GGCCCAATTCCCAGAAAGTA	60	89	NM_033343.4
BMP15	bone morphogenic protein 15	forward CACATACAGACCCTGGACTTTC reverse GGTGGGAATGAGTTAGGTGAAG	63	106	NM_005448.2
ESR1	estrogen receptor 1	forward GGCTAGAGGATCCTCATGATTGG reverse TGTCCAGGAGAAGGTTAGGA	63	87	NM_001122742.2
AMH	anti-müllerian hormone	forward GAAATGGTGCGCTCCTGA reverse CCGGCAGCAAGAGTAACA	60	105	NM_001280541.2
GDF9	growth differentiation factor 9	forward GCATTCCCTCCACCCTAAA reverse GGTGACGGGACAATCTTACA	60	113	NM_001034036.1
FSHR	Follicle stimulating hormone receptor	forward CCTCTGACCTTCATCCAGTTTG reverse CATAGCTGGGCTCATCATCTTC	63	114	NM_174681.2

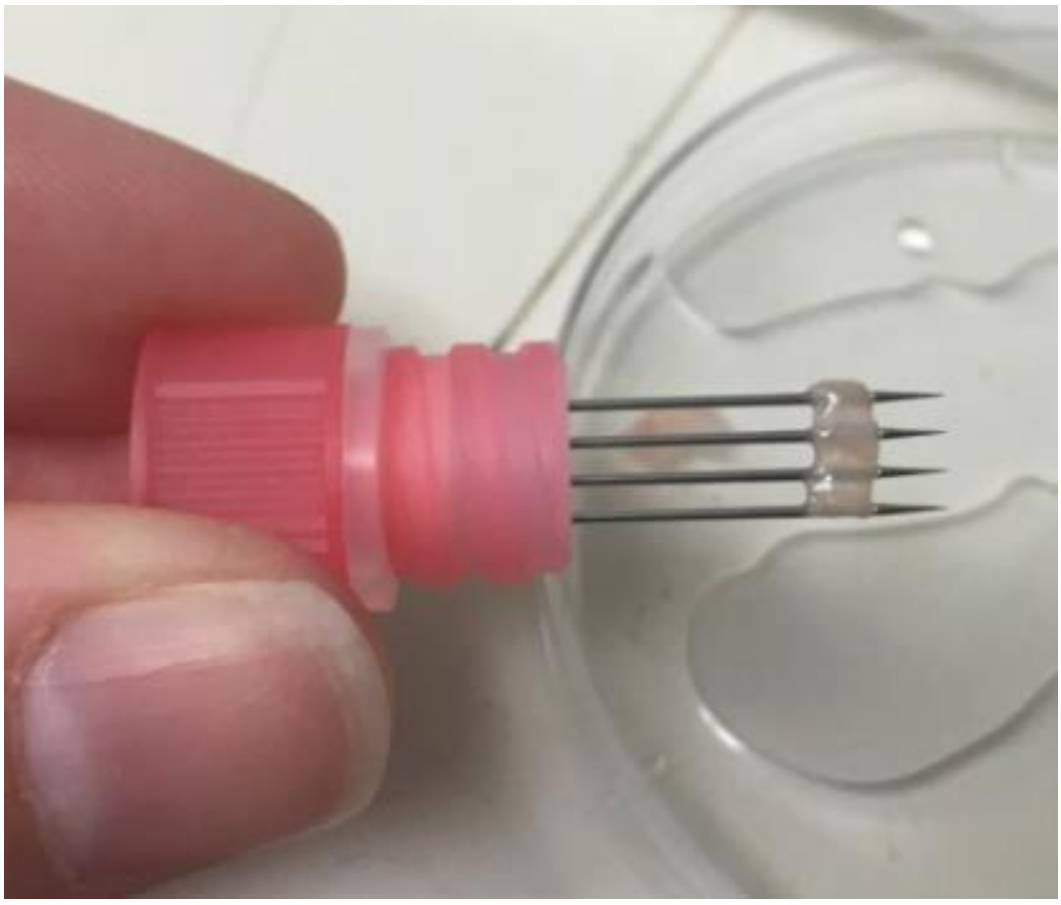

Figure 5.1. Fetal calf ovarian cortical tissue placed on Cryo Device® (Ova Cryo Kit Type M; Kitazato BioPharma, Fuji, Shizouka, Japan) prior to being plunged into liquid nitrogen (LN_2).

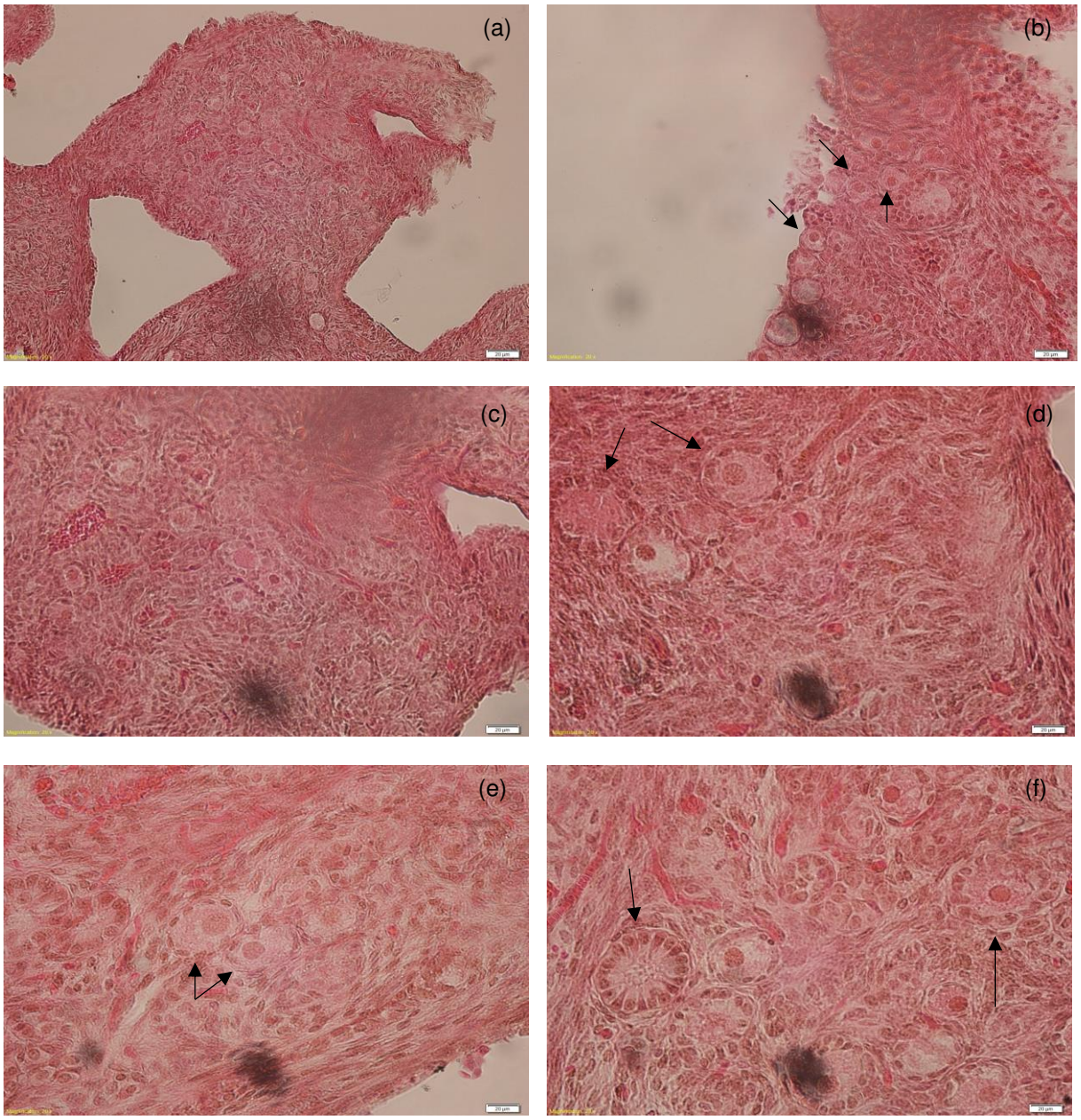

Figure 5.2. Representative images of fresh non-vitrified mature cow ovarian cortical tissue (a-f) show uniform cytoplasm in primordial follicles (b, e, arrows), tight contact between oocytes and granulosa cells in follicles (d, arrows), and dense tightly compacted stroma (f, arrows).

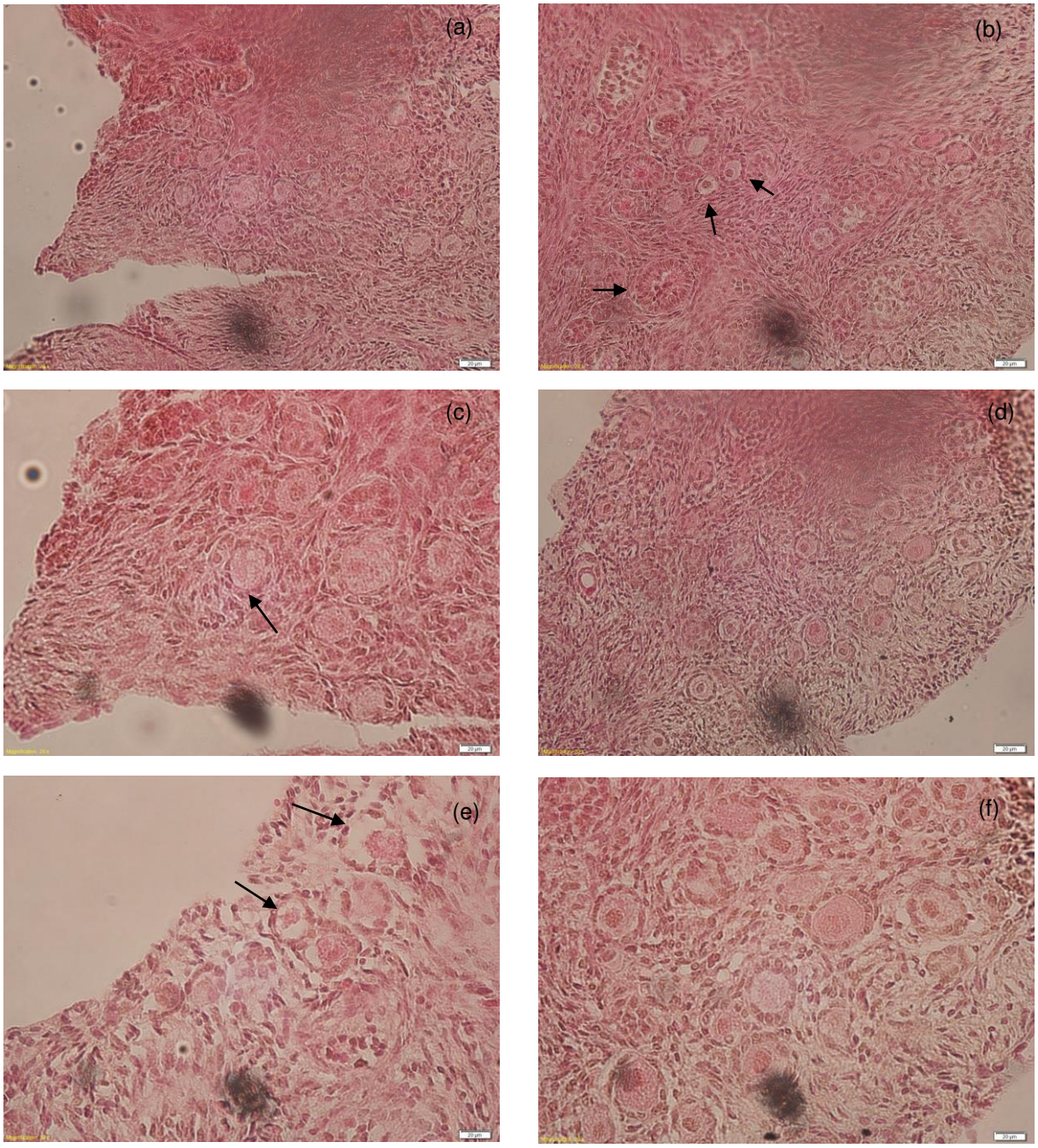

Figure 5.3. Representative images of vitrified-warmed mature cow ovarian cortical tissue (a-f) showing uniform cytoplasm in follicles (b, arrows), tight contact between oocytes and granulosa cells (c, arrow), and dense, compacted stroma (a-c). Evidence of cryodamage include shrunken oocytes (e, arrow), and decompaction of stromal cells (e-f).

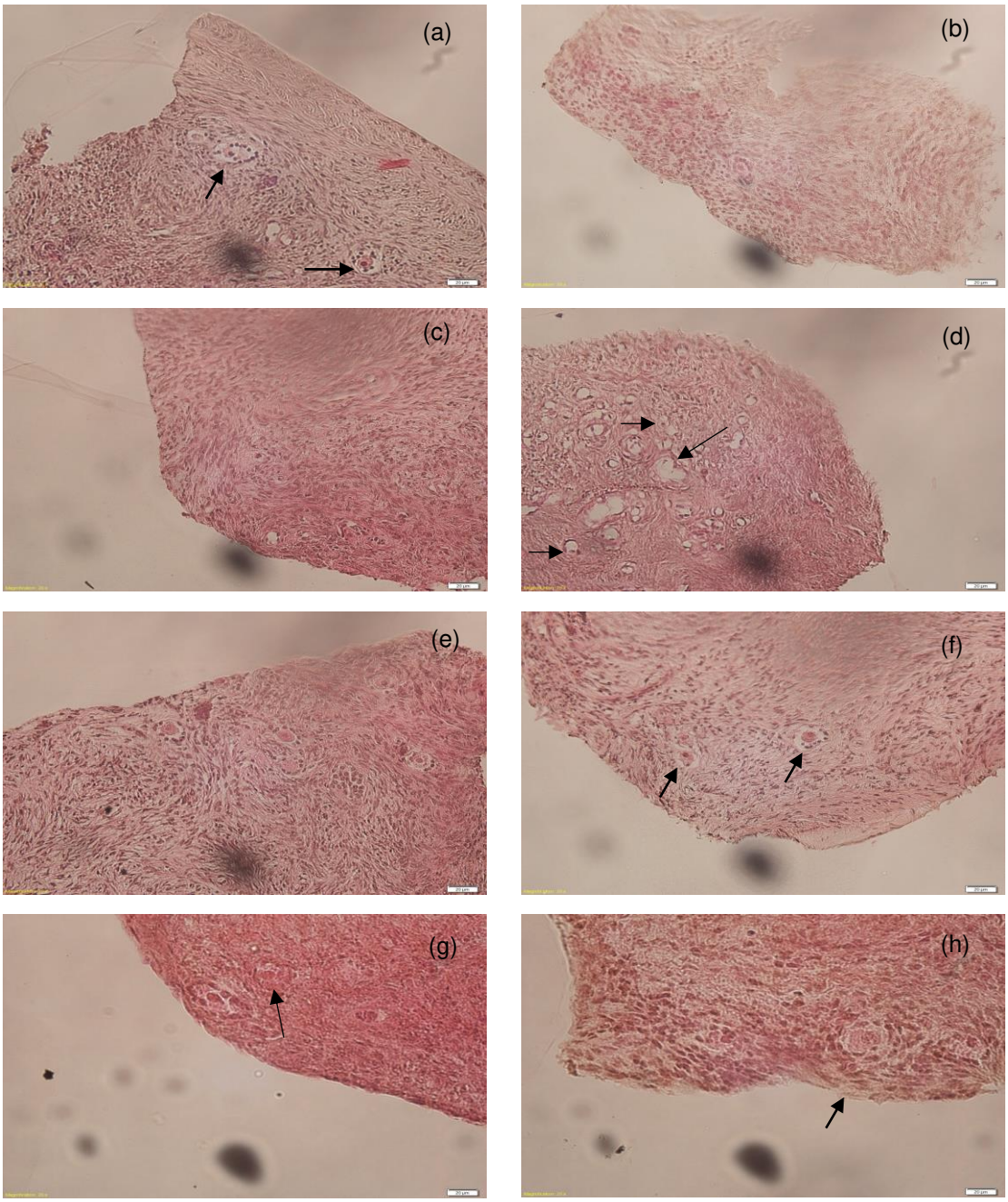

Figure 5.4. Representative images of vitrified-warmed-cultured mature cow ovarian cortical tissue (a-h) showing abnormal morphology as evidence of cryodamage and of in vitro culture including shrunken oocytes (f,h, arrows), pyknotic nuclei (a, arrows) abnormal space between the follicle and stroma (d, arrows) and decompaction of stromal cells (b, e). Fragments showing normal stromal and follicular morphology (g, arrow) were also present.

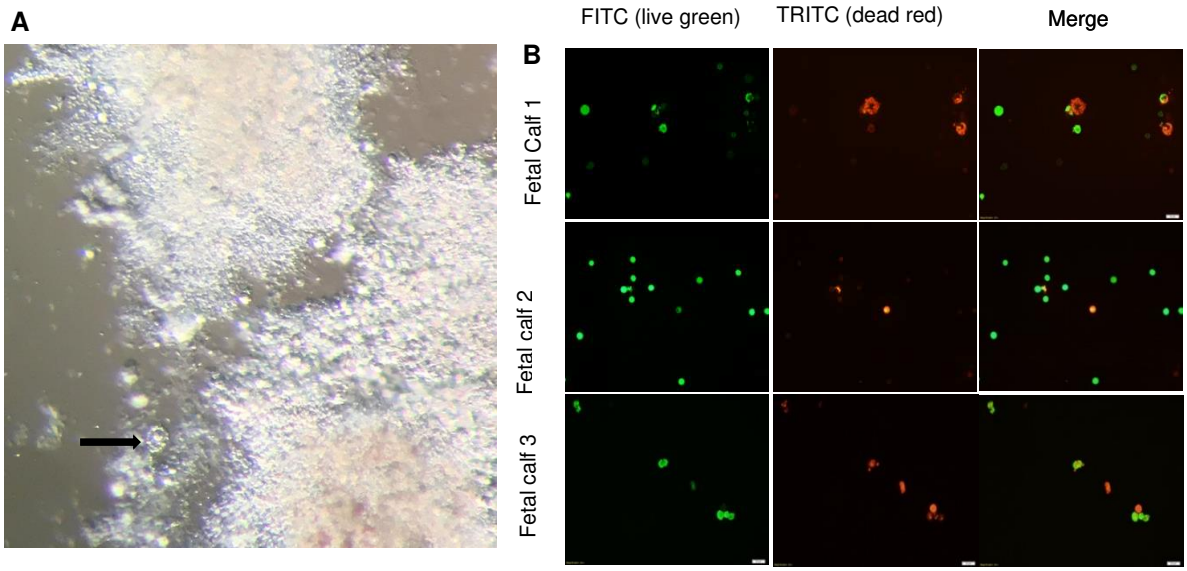

Figure 5.5. Viability assessment of immature follicles (arrow) mechanically isolated from vitrified-warmed fetal calf tissue (A) stained with calcein-AM and ethidium homodimer-I confirmed that live (green) cells were observed in follicles isolated from tissue pieces from three calves (B).

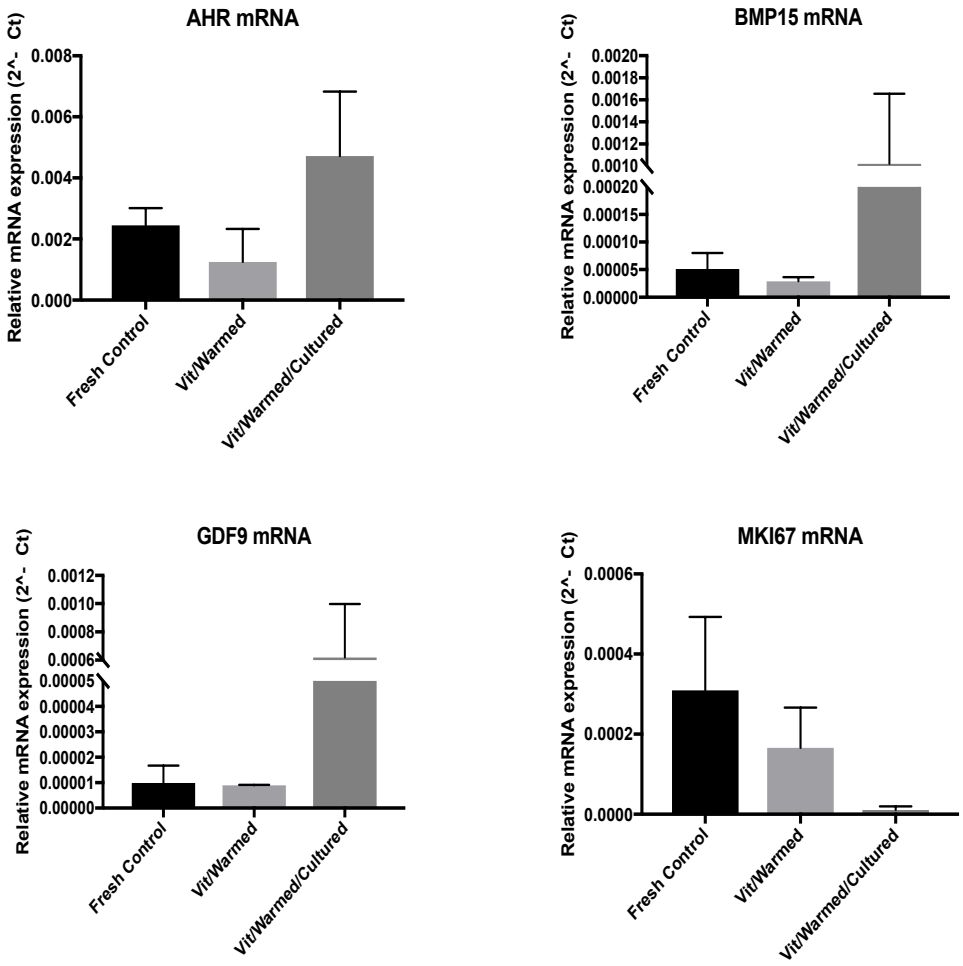

Figure 5.6. Relative expression of AHR, BMP15, GDF9, and MKI67 in fresh non-vitrified, vitrified-warmed, vitrified-warmed-cultured mature cow ovarian cortical tissue.

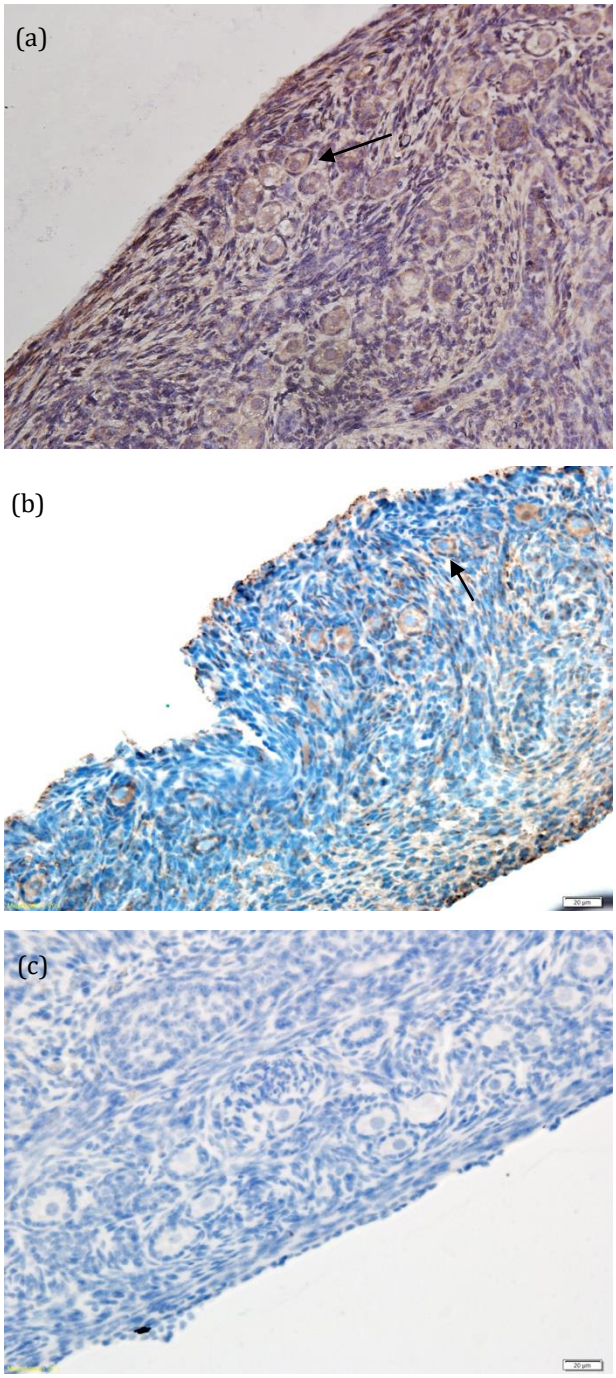

Figure 5.7. Localization of FOXO3a in the cytoplasm of primordial follicles in non-fragmented fresh (a) and non-fragmented vitrified-warmed (b) fetal bovine ovarian cortical tissue (c) no primary antibody control.

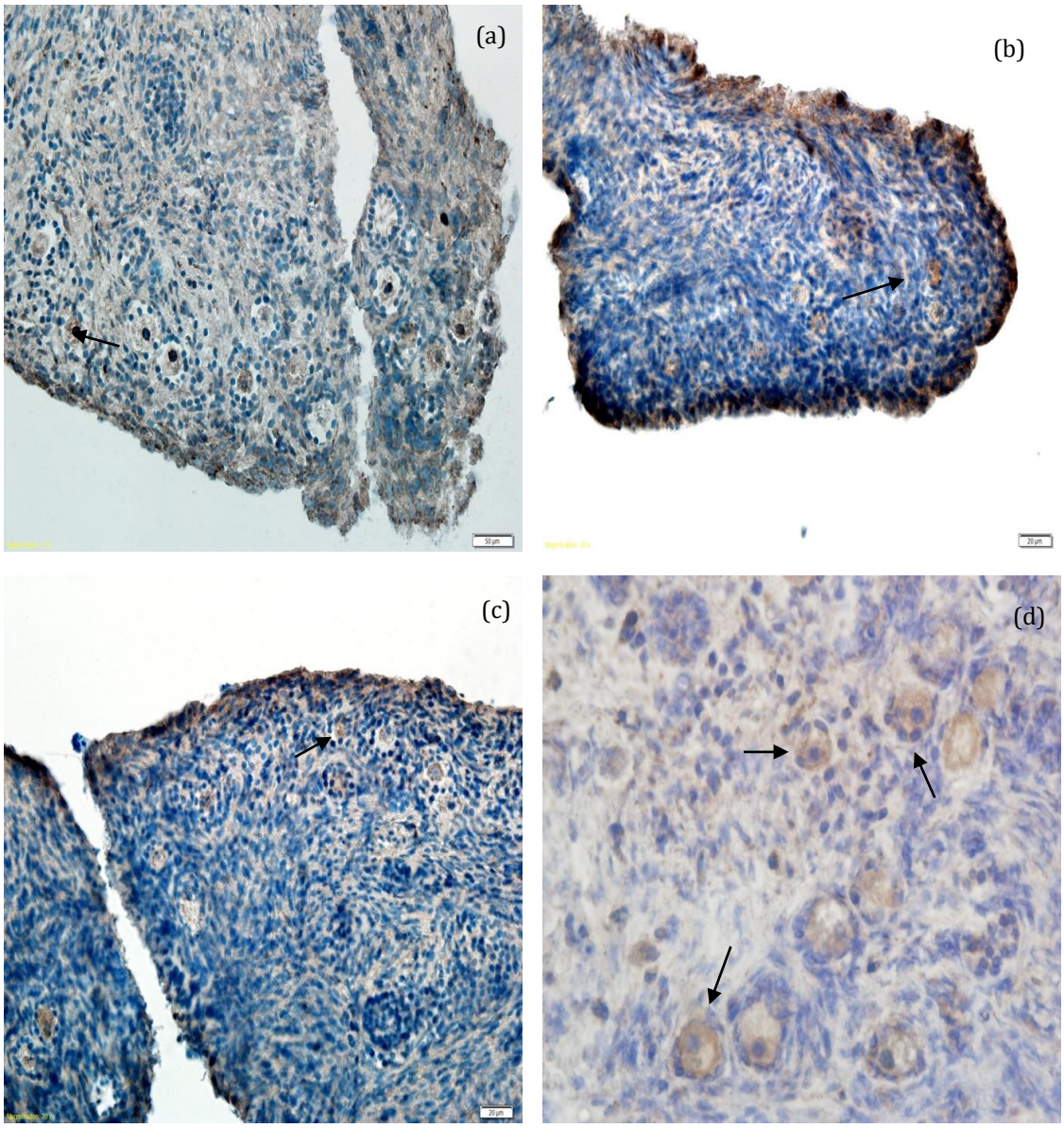

Figure 5.8. Localization of FOXO3a in the cytoplasm (a, b, d, arrows) and nuclei (c, arrow) of primordial follicles in vitrified-warmed fetal calf ovarian cortical tissue fragmented to induce Hippo signaling disruption.

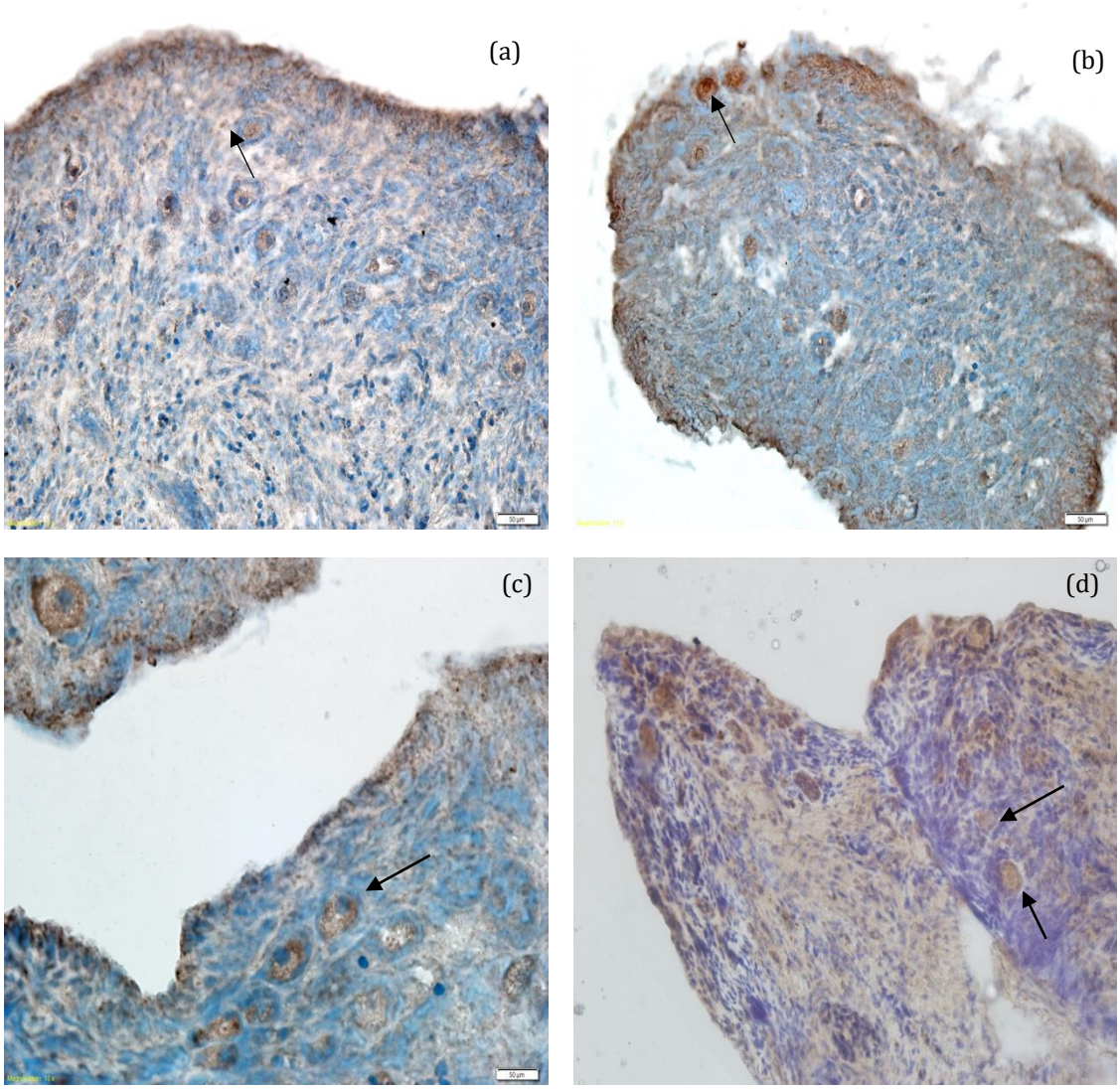

Figure 5.9. Localization of FOXO3a in the nuclei (a-b, arrows) and cytoplasm (c-d arrows) of primordial follicles in vitrified-warmed fetal calf ovarian cortical tissue fragmented and cultured with PI3K activator 740 Y-P and PTEN inhibitor bpV(pic).

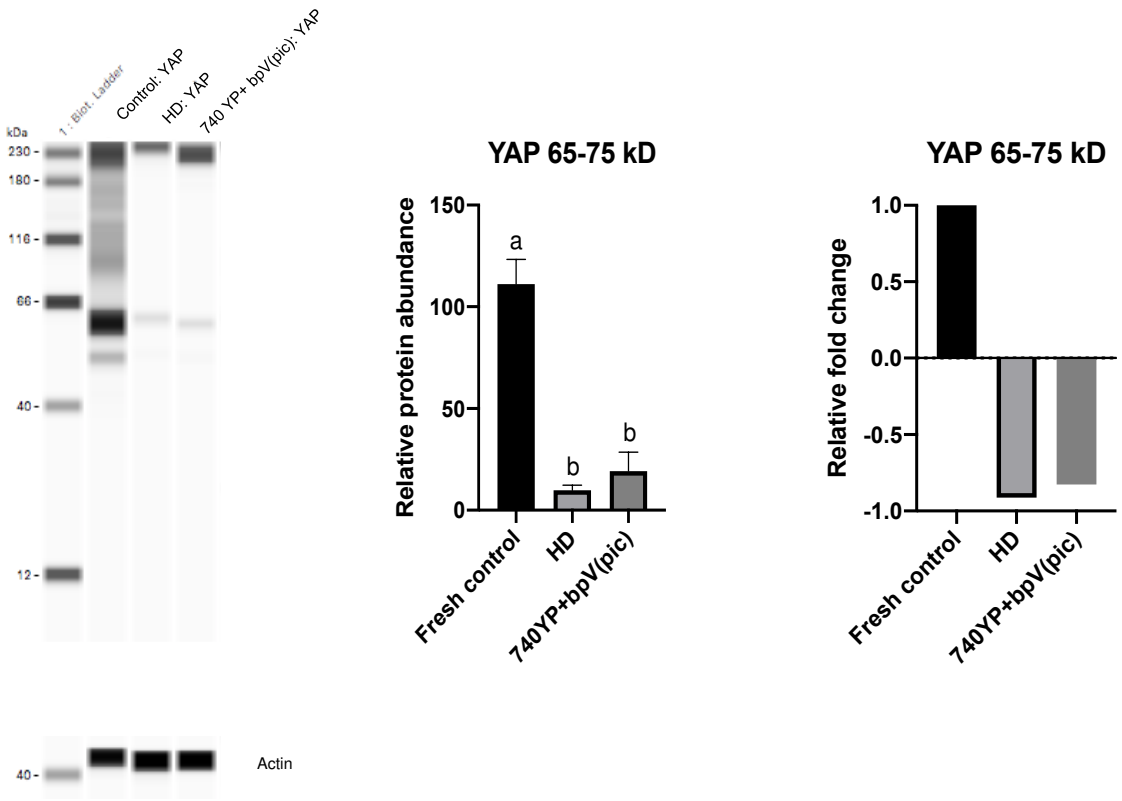

Figure 5.10. (A) Representative protein bands, (B) relative protein abundance, and (C) relative fold change of YAP protein (65-75 kD) in vitrified-warmed fetal bovine ovarian cortical tissue in IVA treatment groups obtained from Jess western analyses. IVA treatment groups: Hippo disruption via tissue fragmentation (HD), chemical activation and fragmentation (740 Y-P + bpV(pic)), and non-fragmented fresh control. Data expressed as mean + SE. Differing superscripts indicate statistical differences ($p<0.05$).

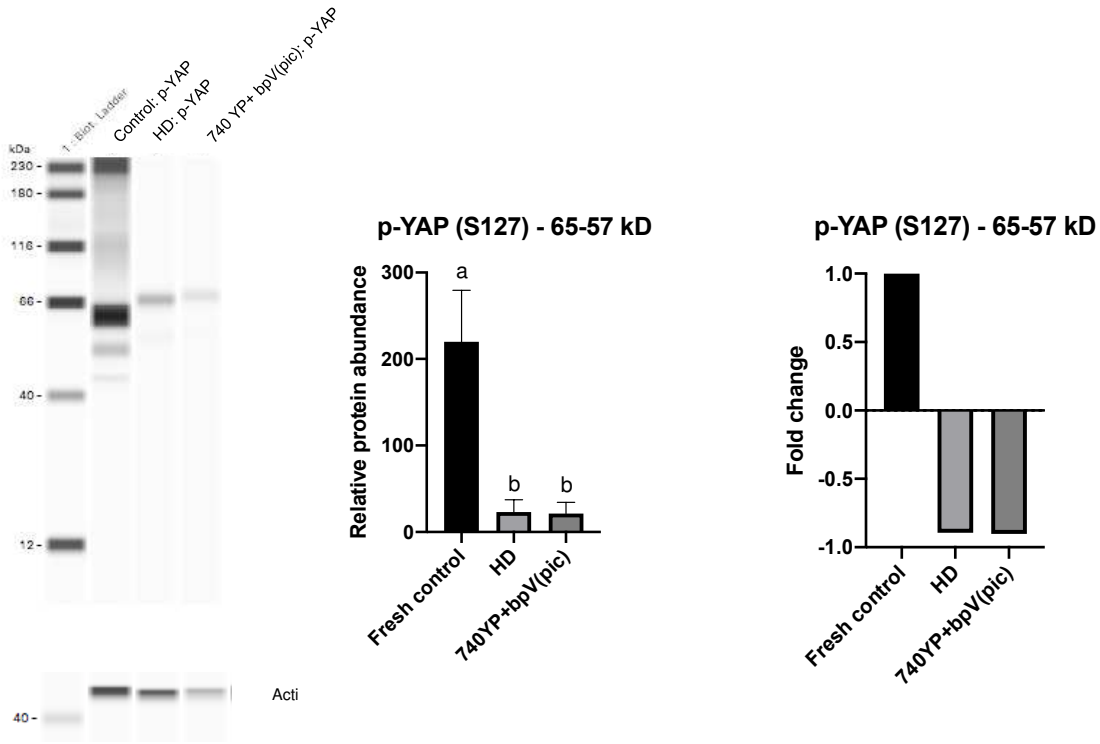

Figure 5.11. (A) Representative protein bands, (B) relative protein abundance, and (C) relative fold change of phosphorylated YAP protein (p-YAP S127) (65-75 kD) in vitrified-warmed fetal bovine ovarian cortical tissue in IVA treatment groups obtained from Jess western analyses. IVA treatment groups: Hippo disruption via tissue fragmentation (HD), chemical activation and fragmentation (740 Y-P + bpV(pic)), and non-fragmented fresh control. Data expressed as mean + SE. Differing superscripts indicate statistical differences ($p<0.05$).

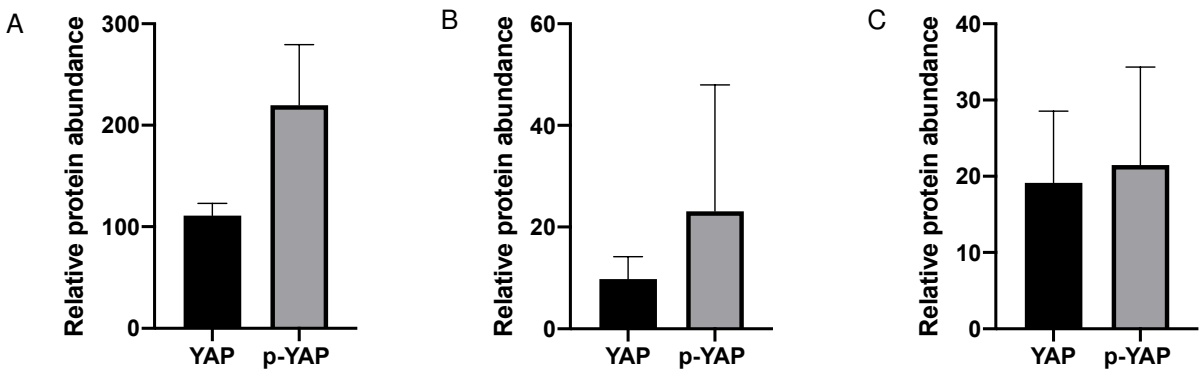

Figure 5.12. Ratio of total YAP protein (YAP) to phosphorylated YAP (p-YAP S127) in fetal calf ovarian cortical tissue from (A) non-fragmented fresh control tissue, vitrified-warmed IVA treatment tissue: (B) Hippo disruption via tissue fragmentation (HD) and (C) chemical activation and fragmentation (740 Y-P + bpV(pic)). Data expressed as mean + SE. Differing superscripts indicate statistical differences ($p<0.05$).

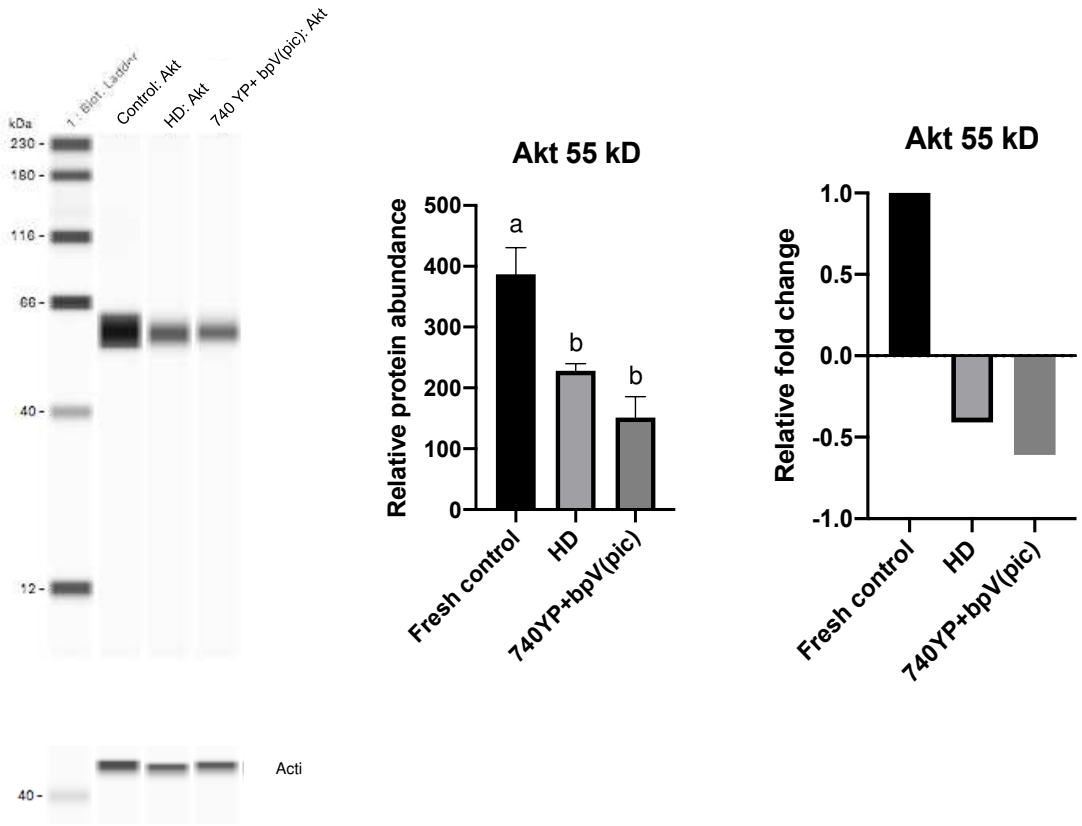

Figure 5.13. (A) Representative protein bands, (B) relative protein abundance, and (C) relative fold change of Akt protein (55 kD) in vitrified-warmed fetal bovine ovarian cortical tissue in IVA treatment groups obtained from Jess western analyses. IVA treatment groups: Hippo disruption via tissue fragmentation (HD), chemical activation and fragmentation (740 Y-P + bpV(pic)), and non-fragmented fresh control. Data expressed as mean + SE. Differing superscripts indicate statistical differences ($p<0.05$).

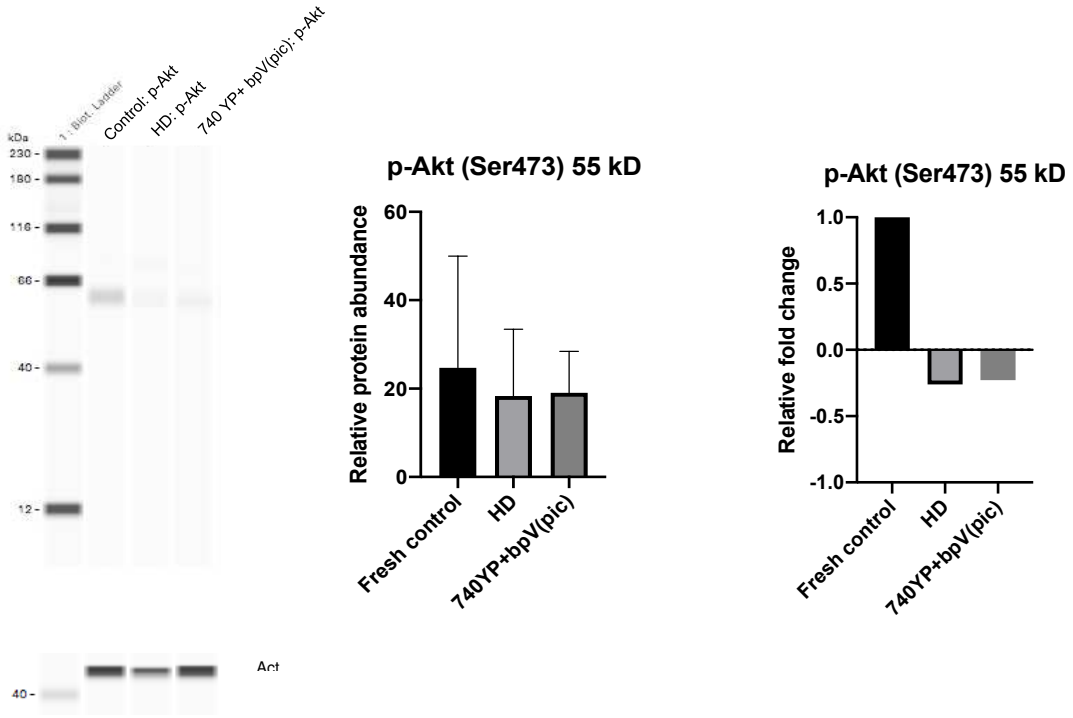

Figure 5.14. (A) Representative protein bands, (B) relative protein abundance, and (C) relative fold change of phosphorylated Akt protein (p-Akt S473) (55 kD) in vitrified-warmed fetal bovine ovarian cortical tissue in IVA treatment groups obtained from Jess western analyses. IVA treatment groups: Hippo disruption via tissue fragmentation (HD), chemical activation and fragmentation (740 Y-P + bpV(pic)), and non-fragmented fresh control. Data expressed as mean + SE.

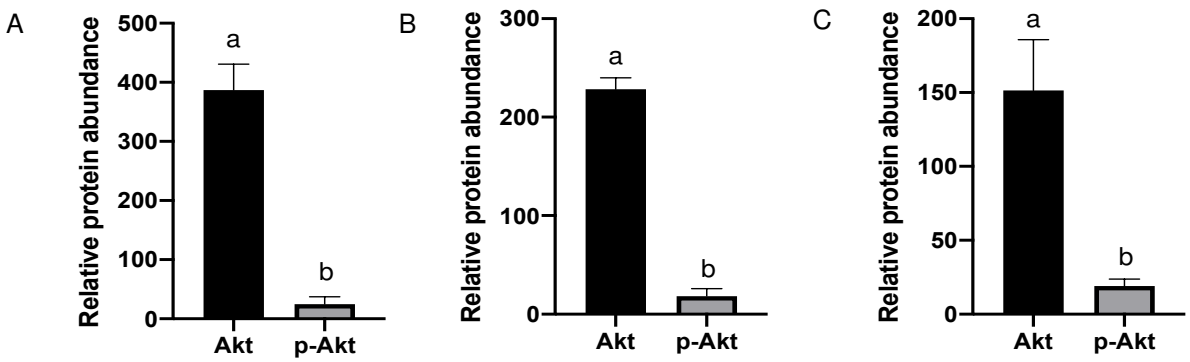

Figure 5.15. Ratio of total Akt protein (Akt) to phosphorylated Akt (p-Akt S473) in fetal calf ovarian cortical tissue from (A) non-fragmented fresh control tissue, vitrified-warmed IVA treatment tissue: (B) Hippo disruption via tissue fragmentation (HD) and (C) chemical activation and fragmentation (740 Y-P + bpV(pic)). Data expressed as mean + SE. Differing superscripts indicate statistical differences ($p<0.05$).

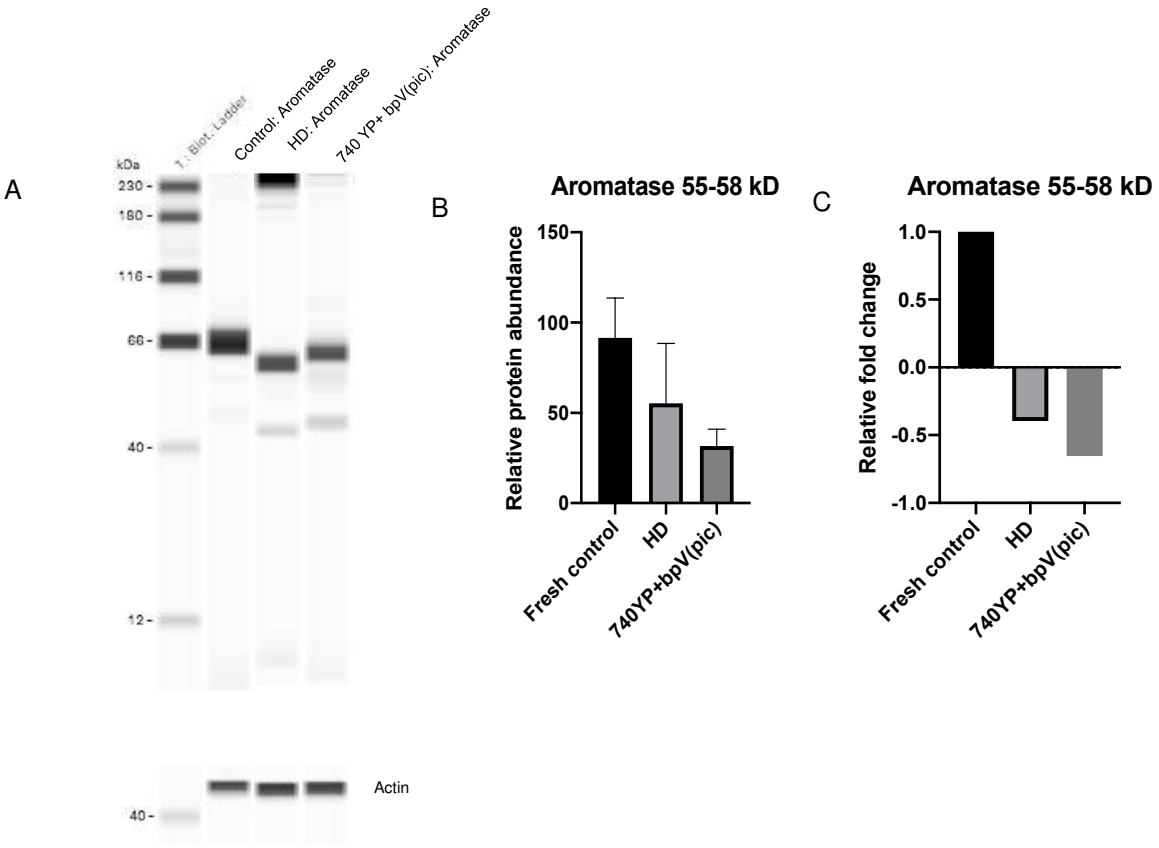

Figure 5.16. (A) Representative protein bands, (B) relative protein abundance, and (C) relative fold change of Aromatase protein (55-58 kD) in vitrified-warmed fetal bovine ovarian cortical tissue in IVA treatment groups obtained from Jess western analyses. IVA treatment groups: Hippo disruption via tissue fragmentation (HD), chemical activation and fragmentation (740 Y-P + bpV(pic)), and non-fragmented fresh control. Data expressed as mean + SE.

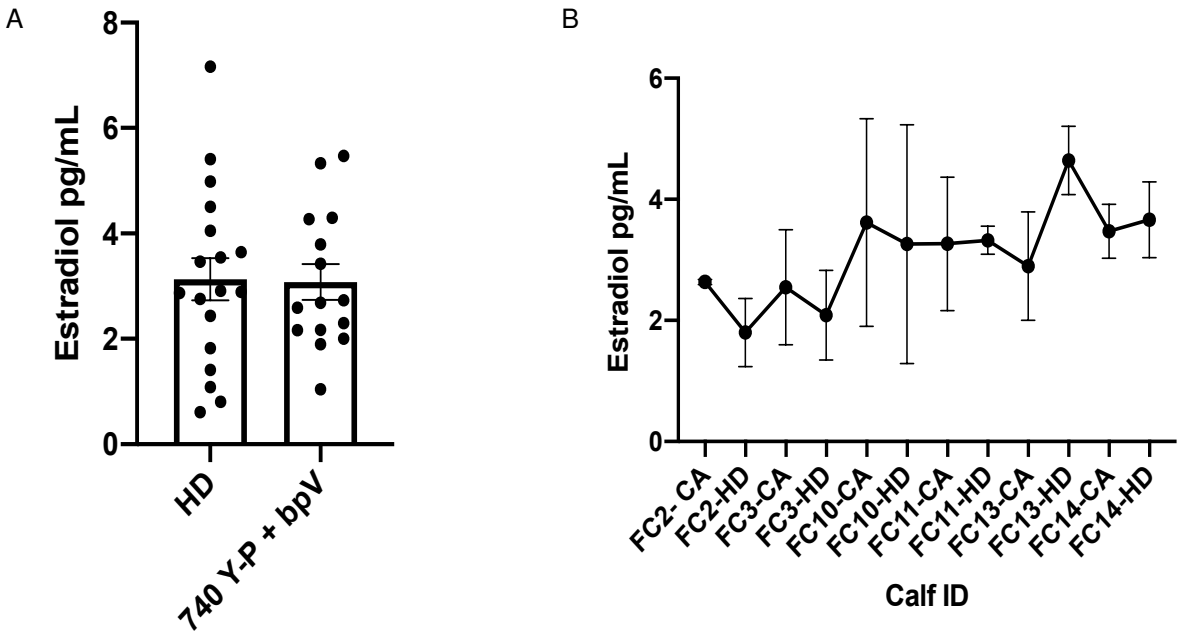

Figure 5.17. (A) Estradiol (pg/mL) in IVA culture medium collected after 48 h tissue culture of vitrified-warmed fetal bovine ovarian cortical tissue from (A) IVA treatment groups: Hippo disruption via tissue fragmentation (HD), chemical activation and fragmentation (CA, 740 Y-P + bpV(pic)) and from (B) six individual fetal calves (FC-2, 3, 10, 11, 13, 14) was determined by RIA. Data are expressed as mean ± SEM.

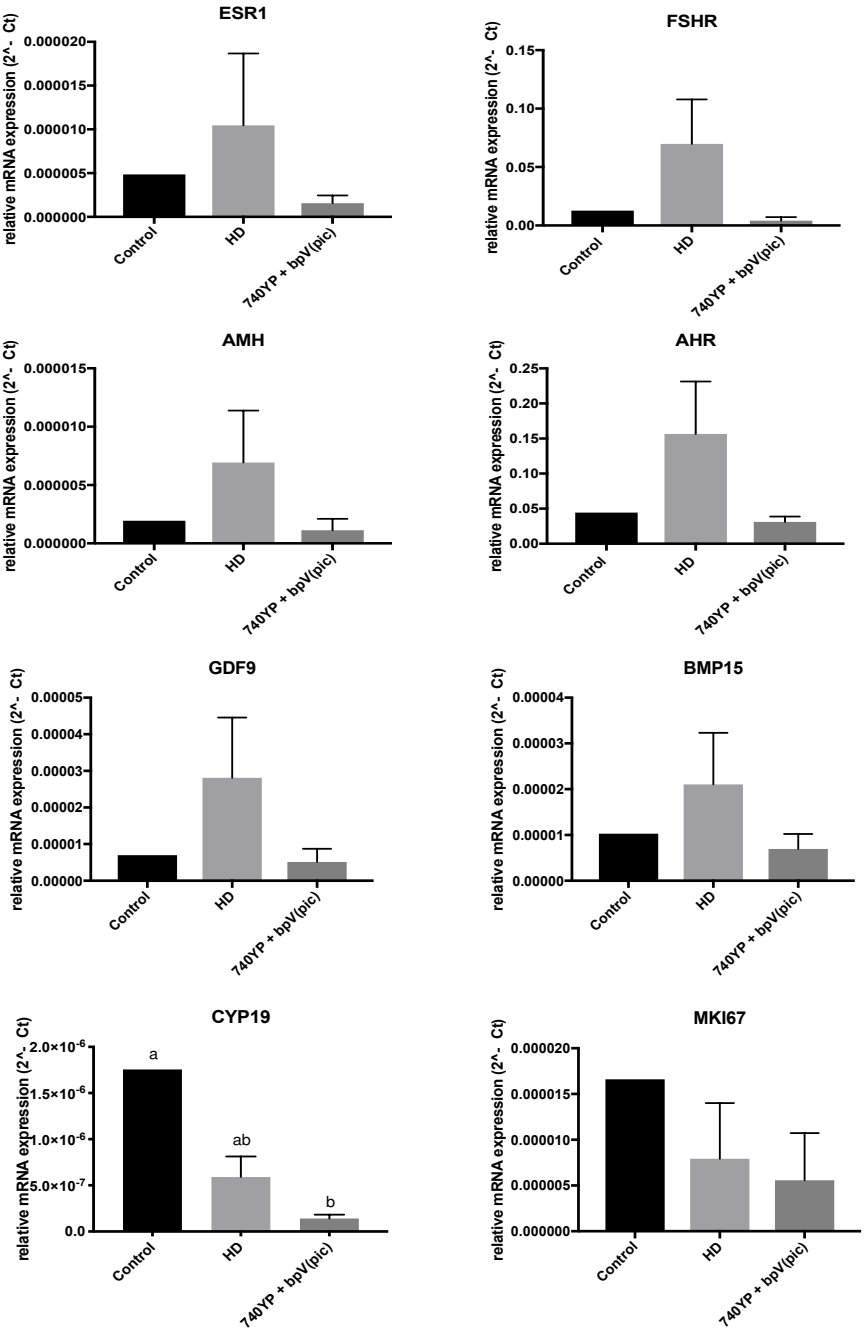

Figure 5.18. Relative expression of *ESR1, FSHR, AMH, AHR, GDF9, BMP15, CYP19 and MKI67* in vitrified-warmed fetal bovine ovarian cortical tissue in IVA treatment groups: Hippo disruption via tissue fragmentation (HD), chemical activation and fragmentation (740 Y-P + bpV(pic)), and a non-fragmented fresh control. Differing superscripts indicate statistical differences ($p<0.05$).

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CONCLUSION

The ongoing decline in global biodiversity due to habitat loss, population fragmentation, and limited contact between animals in remaining populations, has resulted in the loss of genetic diversity within species. However, this is not exclusive to non-domestic species. Genetic losses in domestic bovids are also occurring at an increasing rate as large scale animal agriculture increasingly pushes for uniformity in individuals to increase efficiency in production systems, resulting in a loss of biodiversity in domestic species. As more species face extinction, herd managers are incorporating assisted reproductive technologies (ARTs) into conservation strategies to improve reproductive outcomes and ensure long-term preservation of valuable genetic material. However, routine implementation of ARTs, particularly in non-domestic species, has been difficult, and the number of live offspring born from these attempts are much lower compared to those seen in species such as humans, mice, and livestock. Limiting factors such as a lack in species-specific knowledge of the basic reproductive biology, level of invasiveness and difficulty of the techniques, and cost all limit the rate at which these technologies can be broadly used successfully.

ARTs can be used as tools for diversifying sources of gametes and production methods for gametes and embryos within a conservation program. Gametes can be successfully collected from live superstimulated females or from male and female animals post-mortem for use in vitro embryo production (IVP) systems. The value of ARTs in conservation has increased through the development of cryopreservation techniques and systems for indefinite storage of genetic material including gametes, embryos, tissue and somatic cells in genome resource banks (GRBs) for current and future applications. The materials stored within these biorepositories can represent even higher amounts of genetic diversity than may be available in the breeding

populations represented in conservation herds and can be used to augment herd genetics over many generations.

Our findings from the projects described here showed that ARTs can be used successfully to generate and cryopreserve gametes and embryos from genetically valuable animals using reproductive materials collected post-mortem. Techniques including IVP, embryo vitrification, and embryo transfer can be modified from known domestic animal protocols (cattle) and used successfully in other bovid species (bison), and transfer of IVP embryos can result in live young. While, oocyte cryopreservation could be tremendously useful in conservation programs to preserve female genetics, protocols for bovine oocyte and ovarian tissue vitrification are still experimental and require continued research before they can be implemented with success. Our findings echo those previously described by many who are trying to implement ARTs as tools in conservation strategies. These techniques require continued development if they are to be used to produce live animals in any significant scale. However, for species on the brink of extinction, this may be the current best option for genetic preservation. Methods proven successful in one species are not always equally successful in others. Development of species-specific techniques in less accessible, non-domestic species is a slow process and continues to be challenging due to difficulties in obtaining appropriate research materials or animals, and due to the cost/logistically prohibitive nature of the research.

While these techniques may prove to be an important tool for reproductive assistance and genetic preservation, it is important to consider how ARTs can be effectively implemented to have real, lasting impacts in the big picture of conservation. Interdisciplinary approaches that include scientific, political, and public communities, as well as ethics committees seem to be the most successful. Multigroup strategies that encourage average people to care about specific

conservation initiatives and prioritize their success can have greater effects to change individual or local practices and lifestyles, to prevent further loss of species and habitat. These strategies have proven successful for other conservation efforts such as habitat restoration, habitat allocation for conservation purposes, and pressure to enforce consequences for habitat destruction or poaching. We saw the benefits of this strategy first-hand during the creation of the Laramie Foothills Bison Conservation Herd. The collaborative efforts of representatives from local government agencies, the USDA, and Colorado State University, inspired a local interest in creating a conservation herd on city land. This herd was established, partially through the use of ARTs, with the goal of supporting genetically valuable brucellosis-free bison on land designated for habitat and species conservation. Reproductive science is just one aspect of many in the collaborative efforts that can conserve biodiversity in both captive and wild populations. While ARTs may be an important tool for conservation, ethical considerations for the applications of ART in conservation is critical. ARTs are being used as final attempts at recovery for species at the brink of extinction, often without time to consider the ethical implications of their application. However, discussions regarding a way to measure and account for the ethics of these projects are getting more attention in the scientific literature. Academic groups are developing tools for determining the ethical assessment of ARTs in the context of conservation, such as the Ethical Assessment Tool (ETHAS). In this example, integrated, multilevel and standardized self-assessment are specifically customized for individual procedures, and can generate an ethical acceptability ranking (totally, partially, not acceptable) and a risk rank (low, medium, high), thereby allowing for implementation of measures to address or manage issues beforehand. These types of assessments aim to create an ethical baseline for the use of ARTs in conservation strategies, to ensure that these efforts continue to be supported by all groups

involved in the larger conservation initiative. ARTs have tremendous use as tools to preserve genetic biodiversity, and when implemented with full consideration, this technology can be an important component for accomplishing the larger goals of conservation.

APPENDIX

APPENDIX 1: TIMING OF SUPEROVULATION AND EMBRYO COLLECTION IN NORTH AMERICAN BISION

Bison from Yellowstone National Park have a unique genetic history that makes them a conservation priority for the species. Unfortunately, there is a high prevalence of the zoonotic disease brucellosis in the herd. The goal of this study was to determine the best day to collect embryos after superovulation of bison for the purpose of transferring the embryos into disease-free recipients and generating genetically valuable, *Brucella*-free offspring. Five *Brucella*-positive bison cows from Yellowstone National Park were synchronized and superovulated (i.e. induced to ovulate more than one egg in a single reproductive cycle) using two injections of follicle stimulating hormone (FSH) over a 48 h period (total of 400mg i.m.) and one injection of prostaglandin with the last FSH injection (25mg i.m.). After final injections, females were immediately moved to a pen with multiple bulls for natural breeding. Video recordings were captured for 4 days to determine when cows were bred (Fig. 1). Reproductive tracts were collected from females 7 (n=3) or 8 (n=2) days after observed breeding. Embryos were recovered from the oviduct and uterus separately to determine the location of embryos 7 or 8 days after breeding by flushing 30-120 mL of lactated ringers through the oviduct or uterus with a 16-gauge needle (Fig. 2). Embryos were isolated from the collected media and evaluated for stage of development and quality before being frozen in straws using a slow freeze method. Superovulated females were bred 48-60 hours after the last FSH injection. Embryos were recovered from four of the five cows. Embryos collected 7 days post-breeding reached the morula and 16-cell stage, while embryos collected 8 days post-breeding were at the blastocyst stage of development (Fig. 3). All embryos were found in the uterine horns, except in one female, which had two unfertilized ova in the oviducts 7 days post-breeding. The mean number

of total embryonic structures recovered from bison on day 7 and 8 were 4±4 and 3±2.8, respectively. The mean number of viable embryos, unfertilized ova, and degenerate embryos recovered from bison on day 7 and 8 were, 2±3.4, 3±1.7, 0.3±0.6 and 2±2.8, 0±0, 1±0, respectively. Two embryos were thawed by holding the straw in air for 8 sec followed by submersion in a warm water bath (35°C). Embryos were expelled from the straw and washed through a 0.2% trypsin solution per IETS guidelines prior to transfer to recipient bison in September of 2015. Pregnancy rates will be determined in spring of 2016. Superovulation can increase the number of embryos recovered from genetically valuable bison females, however hormonal stimulation alters the natural timeline of reproductive receptivity. Determining when females enter estrus and embryos enter the uterus in bison could improve the success rates of embryo recovery. This study suggests bison embryos should be collected 8 days post breeding or artificial insemination to ensure all embryos have moved into the uterus. Superovulation coupled with the embryo washing technique can increase the reproductive efficiency of bison providing a mechanism by which genetics from Yellowstone bison with brucellosis can be preserved, ultimately resulting in offspring without brucellosis.

Figure A1.1. Camera footage capturing bison breeding activity.

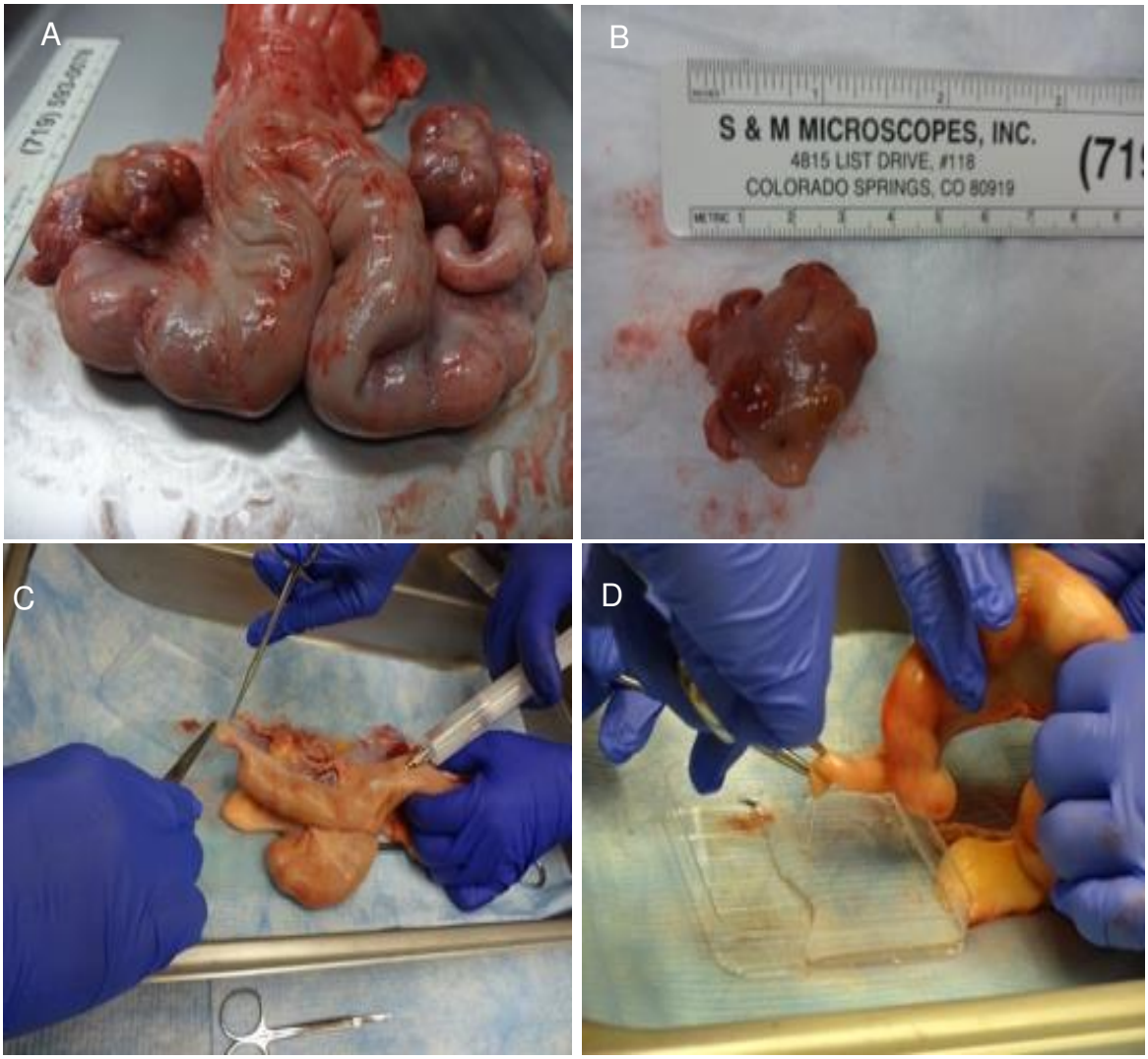

Figure A1.2. (a) Bison reproductive tract prior to embryo flushing, (b) bison ovary with multiple ovulation points (CL), (c) flushing of uterine horns separately with lactated Ringer's solution, (d) collection of flush medium containing embryos.

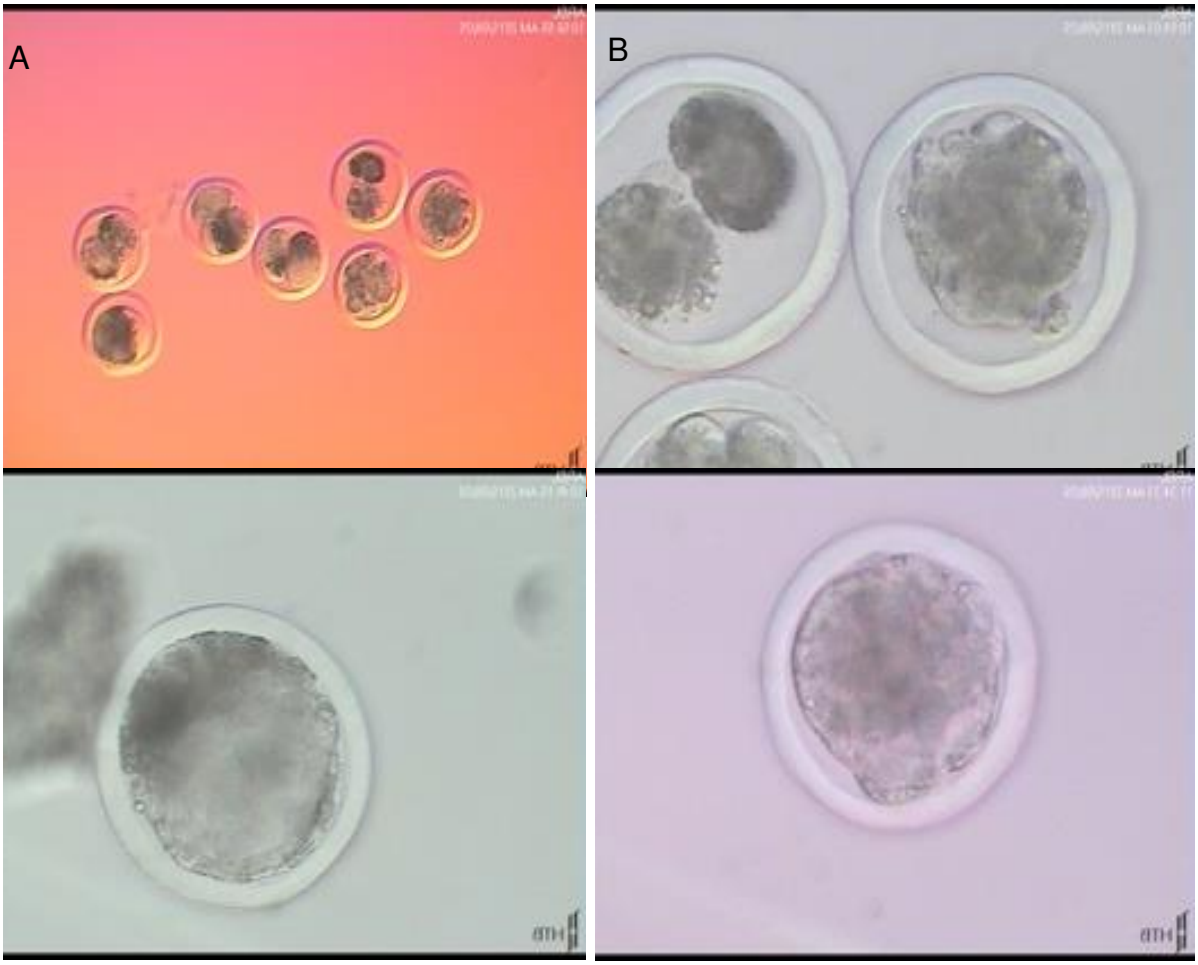

Figure A1.3. (a) Day 7 in vivo derived bison embryos, (b) Day 7 morula and degenerate 2-cell embryo, (c) Day 8 grade 1 blastocyst (d) Day 8 early blastocyst.

APPENDIX 2: SUPPLEMENTAL TABLES

Supplemental Table 1
Meiotic competence of plains bison oocytes produced from cumulus oocyte complexes (COC) derived from different sized diameter follicles over 12 months. Oocytes from <2 mm, 2-5 mm, or >5 mm follicles were matured in vitro for 23 h at 38.5°C in 5% CO_2 in air. Nuclear status was determined using fluorescence microscopy and oocytes were classified as still having a germinal vesicle (GV), having undergone germinal vesicle breakdown (GVBD), or having completed meiosis II (MII) (Experiment 1).

End point	Follicular Diameter		
	<2 mm	2-5 mm	>5 mm
January (2018)			
COC submitted to IVM (n)	76	55	36
GV	12/76 (15.8%)	6/55 (10.9%)	3/36 (8.3%)
GVBD	30/76 (39.5%)	16/55 (29.1%)	9/36 (25.0%)
MII	34/76 (44.7%)[a]	33/55 (60.0%)[ab]	24/36 (66.7%)[b]
February (2018)			
COC submitted to IVM (n)	43	69	27
GV	4/43 (9.3%)	5/69 (7.2%)	1/27 (3.7%)
GVBD	12/43 (27.9%)	13/69 (18.8%)	4/27 (14.8%)
MII	27/43 (62.8%)	51/69 (73.9%)	22/27 (81.5%)
March (2019)			
COC submitted to IVM (n)	46	52	30
GV	15/46 (32.6%)	9/52 (17.3%)	4/30 (13.3%)
GVBD	17/46 (37.0%)	15/52 (28.8%)	9/30 (30.0%)
MII	14/46 (30.4%)[a]	28/52 (53.8%)[b]	17/30 (56.7%)[b]
April (2018)			
COC submitted to IVM (n)	72	54	18
GV	14/72 (19.4%)	13/54 (24.1%)	3/18 (17.6%)
GVBD	18/72 (25.0%)	15/54 (27.8%)	2/18 (11.1%)
MII	40/72 (55.5%)	26/54 (48.1%)	13/18 (72.2%)
May (2018)			
COC submitted to IVM (n)	55	112	31
GV	3/55 (5.5%)	11/112 (9.8%)	2/31 (6.5%)
GVBD	23/55 (41.8%)	30/112 (26.8%)	6/31 (19.4%)
MII	29/55 (52.7%)	71/112 (63.4%)	23/31 (74.2%)
June (2018)			
COC submitted to IVM (n)	60	135	36
GV	13/60 (21.7%)	18/135 (13.3%)	7/36 (19.4%)
GVBD	14/60 (23.3%)	42/135 (31.1%)	7/36 (19.4%)
MII	33/60 (55.0%)	75/135 (55.6%)	22/36 (61.1%)
July (2018)			
COC submitted to IVM (n)	53	120	46
GV	15/53 (28.3%)	27/120 (22.5%)	5/46 (10.9%)
GVBD	20/53 (37.7%)	28/120 (23.3%)	7/46 (15.2%)
MII	18/53 (34.0%)[a]	65/120 (54.2%)[b]	34/46 (73.9%)[bc]
August (2018)			
COC submitted to IVM (n)	87	125	34
GV	17/87 (19.5%)	26/125 (20.8%)	2/34 (5.9%)
GVBD	32/87 (36.8%)	40/125 (32.0%)	7/34 (20.6%)
MII	38/87 (43.7%)[a]	59/125 (47.2%)[a]	25/34 (73.5%)[b]
September (2018)			
COC submitted to IVM (n)	35	53	27
GV	13/35 (37.1%)	4/53 (7.5%)	2/27 (7.4%)
GVBD	16/35 (45.7%)	19/53 (35.8%)	6/27 (22.2%)
MII	6/35 (17.1%)[a]	30/53 (56.6%)[b]	19/27 (70.4%)[bc]
October (2018)			
COC submitted to IVM (n)	59	47	17
GV	18/59 (30.5%)	6/47 (12.8%)	2/17 (11.8%)
GVBD	25/59 (42.4%)	20/47 (42.6%)	3/17 (17.6%)
MII	16/59 (27.1%)[a]	21/47 (44.7%)[ab]	12/17 (70.6%)[bc]
November (2017)			
COC submitted to IVM (n)	113	170	16
GV	24/113 (21.2%)	14/170 (8.2%)	2/16 (12.5%)
GVBD	60/113 (53.1%)	49/170 (28.8%)	5/16 (31.3%)
MII	29/113 (25.7%)[a]	107/170 (62.9%)[b]	9/16 (56.3%)[b]
December (2018)			
COC submitted to IVM (n)	63	54	21
GV	11/63 (17.5%)	5/54 (9.3%)	3/21 (14.3%)
GVBD	25/63 (39.7%)	17/54 (31.5%)	4/21 (19.0%)
MII	27/63 (42.9%)	32/54 (59.3%)	14/21 (66.7%)

[abc] Within rows, values with different superscripts significantly differ ($P < 0.05$)

Supplemental Table 2
Meiotic competence of plains bison oocytes produced from cumulus oocyte complexes (COC) collected from 2-5mm follicles and matured in vitro for different lengths of time over 12 months. Oocytes were matured in vitro for 24h, 29 h, or 34 h at 38.5°C in 5% CO_2 in air. Nuclear status was determined using fluorescence microscopy and oocytes were classified as still having a germinal vesicle (GV), having undergone germinal vesicle breakdown (GVBD), or having completed meiosis II (MII) (Experiment 2).

End point[1]	Length of time of in vitro maturation		
	24 h	29 h	34 h
January (2018)			
COC submitted to IVM (n)	40	-	27
GV	2/40 (5.0%)	-	1/27 (3.7%)
GVBD	12/40 (30.0%)	-	9/27 (33.3%)
MII	26/40 (65.0%)	-	17/27 (63.0%)
February (2018)			
COC submitted to IVM (n)	34	-	36
GV	4/34 (11.8%)	-	5/36 (13.9%)
GVBD	8/34 (23.5%)	-	3/36 (8.3%)
MII	22/34 (64.7%)	-	28/36 (77.8%)
March (2019)			
COC submitted to IVM (n)	43	47	-
GV	7/43 (16.3%)	5/47 (10.6%)	-
GVBD	11/43 (25.6%)	8/47 (17.0%)	-
MII	25/43 (58.1%)	34/47 (72.3%)	-
April (2018)			
COC submitted to IVM (n)	54	44	-
GV	4/54 (7.4%)	4/44 (9.1%)	-
GVBD	18/54 (33.3%)	8/44 (18.2%)	-
MII	32/54 (59.3%)	32/44 (72.7%)	-
May (2018)			
COC submitted to IVM (n)	60	63	-
GV	15/60 (25.0%)	8/63 (12.7%)	-
GVBD	15/60 (25.0%)	18/63 (28.6%)	-
MII	30/60 (50.0%)	37/63 (58.7%)	-
June (2018)			
COC submitted to IVM (n)	42	36	-
GV	1/42 (2.4%)	1/36 (2.8%)	-
GVBD	14/42 (33.3%)	8/36 (22.2%)	-
MII	27/42 (64.3%)	27/36 (75.0%)	-
July (2018)			
COC submitted to IVM (n)	49	51	-
GV	8/49 (16.3%)	6/51 (11.8%)	-
GVBD	14/49 (28.6%)	12/51 (23.5%)	-
MII	27/49 (55.1%)	33/51 (64.7%)	-
August (2018)			
COC submitted to IVM (n)	38	33	-
GV	6/38 (15.8%)	4/33 (12.1%)	-
GVBD	13/38 (34.2%)	9/33 (27.3%)	-
MII	19/38 (50.0%)	20/33 (60.6%)	-
September (2018)			
COC submitted to IVM (n)	53	54	-
GV	4/53 (7.5%)	1/54 (1.9%)	-
GVBD	19/53 (35.8%)	19/54 (35.2%)	-
MII	30/53 (56.6%)	34/54 (63.0%)	-
October (2018)			
COC submitted to IVM (n)	47	51	-
GV	6/47 (12.8%)	5/51 (9.8%)	-
GVBD	20/47 (42.6%)	15/51 (29.4%)	-
MII	21/47 (44.7%)	31/51 (60.8%)	-
November (2017)			
COC submitted to IVM (n)	26	-	25
GV	3/26 (11.5%)	-	3/25 (12.0%)
GVBD	11/26 (42.3%)	-	9/25 (36.0%)
MII	12/26 (46.2%)	-	13/25 (52.0%)
December (2018)			
COC submitted to IVM (n)	40	38	-
GV	12/40 (30.0%)	6/38 (15.8%)	-
GVBD	13/40 (32.5%)	16/38 (42.1%)	-

Supplemental Table 3
In vitro production of plains bison embryos produced from cumulus oocyte complexes (COC) collected from 2-5 mm follicles and matured in vitro for different lengths of time over 12 months. Oocytes were matured for 24h, 29 h or 34 h, fertilized, and cultured in vitro (Day 0 = day of in vitro fertilization). Blastocyst rates are presented as blastocysts/oocyte (Experiment 2).

End point	Length of time of in vitro maturation		
	24 h	29 h	34 h
January (2018)			
COC submitted to IVF (n)	211	-	230
Cleaved oocytes	104/211 (49.3%)	-	105/230 (45.7%)
Blastocysts on Day 7	17/211 (8.1%)[a]	-	4/230 (1.7%)[b]
Blastocysts on Day 8	22/211 (8.6%)[a]	-	4/230 (1.7%)[b]
February (2018)			
COC submitted to IVF (n)	198	-	184
Cleaved oocytes	56/198 (28.3%)	-	53/184 (28.8%)
Blastocysts on Day 7	15/198 (7.6%)[a]	-	5/184 (2.7%)[b]
Blastocysts on Day 8	17/198 (8.6%)[a]	-	5/184 (2.7%)[b]
March (2019)			
COC submitted to IVF (n)	172	201	-
Cleaved oocytes	96/172 (55.8%)	117/201 (58.2%)	-
Blastocysts on Day 7	14/172 (8.1%)	16/201 (8.0%)	-
Blastocysts on Day 8	17/172 (9.9%)	34/201 (8.5%)	-
April (2018)			
COC submitted to IVF (n)	237	212	-
Cleaved oocytes	130/237 (54.9%)[a]	91/212 (42.9%)[b]	-
Blastocysts on Day 7	18/237 (7.6%)	7/212 (3.3%)	-
Blastocysts on Day 8	18/237 (7.6%)	10/212 (4.7%)	-
May (2018)			
COC submitted to IVF (n)	261	377	-
Cleaved oocytes	159/261 (60.9%)	250/377 (66.3%)	-
Blastocysts on Day 7	14/261 (5.4%)	13/377 (3.5%)	-
Blastocysts on Day 8	26/261 (10.0%)[a]	21/377 (5.6%)[b]	-
June (2018)			
COC submitted to IVF (n)	170	163	-
Cleaved oocytes	132/170 (77.7%)	122/163 (74.9%)	-
Blastocysts on Day 7	10/170 (5.9%)	7/163 (4.3%)	-
Blastocysts on Day 8	15/170 (8.8%)	13/163 (8.0%)	-
July (2018)			
COC submitted to IVF (n)	196	177	-
Cleaved oocytes	133/196 (67.9%)[a]	139/177 (78.5%)[b]	-
Blastocysts on Day 7	25/196 (12.8%)	18/177 (10.2%)	-
Blastocysts on Day 8	31/196 (15.8%)	20/177 (11.3%)	-
August (2018)			
COC submitted to IVF (n)	118	112	-
Cleaved oocytes	83/118 (70.3%)	84/112 (75.0%)	-
Blastocysts on Day 7	13/118 (11.0%)	11/112 (9.8%)	-
Blastocysts on Day 8	15/118 (12.7%)	14/112 (12.5%)	-
September (2018)			
COC submitted to IVF (n)	148	147	-
Cleaved oocytes	97/148 (65.5%)	97/147 (66.0%)	-
Blastocysts on Day 7	11/148 (7.4%)	5/147 (3.4%)	-
Blastocysts on Day 8	12/148 (8.1%)	5/147 (3.4%)	-
October (2018)			
COC submitted to IVF (n)	145	134	-
Cleaved oocytes	84/145 (57.9%)	91/134 (67.9%)	-
Blastocysts on Day 7	10/145 (6.9%)	4/134 (3.0%)	-
Blastocysts on Day 8	13/145 (9.0%)	8/134 (6.0%)	-
November (2017)			
COC submitted to IVF (n)	239	-	266
Cleaved oocytes	145/239 (60.7%)	-	148/266 (55.6%)
Blastocysts on Day 7	26/239 (10.9%)[a]	-	10/266 (3.8%)[b]
Blastocysts on Day 8	32/239 (13.4%)[a]	-	14/266 (5.3%)[b]
December (2018)			
COC submitted to IVF (n)	136	124	-
Cleaved oocytes	52/136 (38.2%)	46/124 (37.1%)	-
Blastocysts on Day 7	7/136 (5.2%)	3/124 (2.4%)	-
Blastocysts on Day 8	9/136 (6.6%)	4/124 (3.2%)	-