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ABSTRACT

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 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 15 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 while mitigating diseases like brucellosis.

1. Introduction

North American bison (*Bison bison*) are an iconic species of cultural, conservation, and commercial importance. In the late 1800s, 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) [1–4]. 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

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loss and subject to the forces of natural selection) [5,6]. 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 [7]. 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 [7]. 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 [8]. 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 processes 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 [9–11]. 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 [12]. *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 [13]. 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%) [14,15]. 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 [16].

Bison are seasonal breeders, entering estrus during the late summer or early autumn in North America [17]. 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 [18,19]. 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 take place in winter, can potentially be used for IVP and embryo transfer to produce live offspring [16,18,19].

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 [20,21]. 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.

2. Materials and methods

2.1. 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.

2.2. In vitro embryo production

2.2.1. 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 (HCDM-M) with 10% BSA and 10 mg/mL gentamicin sulfate [16,22]. 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).

2.2.2. 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 [22]. The same equilibration conditions were used for all embryo culture media . IVM medium was supplemented as follows: 15 ng/mL NIDDK-oFSH-20, 1 μg/mL USDA-LH-B-5, 1 μg/mL estradiol 17β, 50 ng/μL epidermal growth factor, and 0.1 mM cysteamine [22]. 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 [22]. 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 [23]. Motile sperm were isolated by separating thawed semen through a 45/90 Percoll® gradient [24]. 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 0.5×10^6 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 [22]. 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 [22]. 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 [22]. 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 [25]. All spent CDM-2 media and degenerate embryos from the IVP process were cultured for presence of *Brucella abortus*.

2.3. 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 of modified H1 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 [26]. Embryos were placed onto Cryotop® devices (Kitazato, Japan) in <1 μL V2 and plunged into liquid nitrogen [26].

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 cryostorage at Colorado State University and are effectively a biobank of Yellowstone bison embryos.

2.4. 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 [13]. 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 [13].

2.5. 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 [25]. While other synchronization methods used with bison have been previously described, they require daily or frequent animal handling and examination [27]. 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.

2.6. 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 were 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).

3. Results

3.1. 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).

Table 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. Blastocyst development per COC and per cleaved embryo was calculated based on numbers of embryos on Day 8. Number of COC collected presented as mean ± SEM.

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%)

[ab] Within rows, superscripts differ to indicate significance (P < 0.05, Chi-square test).

3.2. 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).

3.3. 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 (Fig. 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 first months 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.

4. 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 non-pregnant 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 [28]. These studies similarly reported a reduction in oocyte yield from pregnant females due to restricted follicular growth on the ovary supporting the CL [28]. 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

Table 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. Blastocyst development per COC and per cleaved embryo was calculated based on numbers of embryos on Day 8. Number of COC collected presented as mean ± SEM.

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%)

[ab] Within rows, superscripts differ to indicate significance ($P < 0.05$, Chi-square test).

Fig. 1. A healthy female calf (IVF1) was born in May 2017 to a recipient bison cow (pictured with the calf here) 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.

to that of mature cows [29]. 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 [27,30]. 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 effects 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 have have poor developmental potential [30]. A seasonal effect on in vitro fertilization success has been described in wood bison, inferring reduced oocyte competence during the non-breeding season [18]. 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 [19]. 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 Montana. 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 [31]. 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 [31].

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 [32]. The success of assisted reproductive technology (ART) in these species has relied heavily on work done in domestic species of similar taxa [33]. 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 [16,34–36]. 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 [32]. Translocating animals is stressful and potentially dangerous for bison, can increase risk of disease transmission, and disrupt a herd's social order [37]. 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 [38,39]. 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, 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.

CRediT authorship contribution statement

Hayley M. Benham: Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Matthew P. McCollum:** Investigation, Writing - review & editing. **Pauline Nol:** Investigation, Writing - review & editing. **Rebecca K. Frey:** Resources, Writing - review & editing. **P. Ryan Clarke:** Resources, Writing - review & editing. **Jack C. Rhyan:** Resources, Conceptualization, Writing - review & editing. **Jennifer P. Barfield:** Resources, Investigation, Supervision, Methodology, Conceptualization, Writing - review & editing, Funding acquisition.

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