United States Department of the Interior
BUREAU OF LAND MANAGEMENT
Wild Horse and Burro Program

Wild Horse and Burro (WHB) Research Proposal to the BLM

(PRIVILEGED COMMUNICATION)

This application is for research projects that involve any handling of live animals, or that are requesting funding from BLM for completion of the research. If an applicant only seeks to use tissues, DNA or other material from samples that have already been or will be collected during the normal course of other BLM activities, or from wild horses or burros that may die or be euthanized for reasons unrelated to the project proposed here, then the applicant should instead complete a "Research Proposal for Tissue Collection or Use" form, available from the research coordinator (pgriffin@blm.gov).

Under no circumstance may wild horse or wild burro specimens be sold, bartered or traded. Under no circumstances may specimens or DNA from BLM-administered WHB be transferred or used for research purposes without prior approval from the BLM Wild Horse and Burro Program Division Chief. Guidelines in italics may be deleted prior to submission.

A. COVER PAGE

1a. Evaluation of a vaccine against ovarian growth factors as a single dose, long-lasting immunocontraceptive
TITLE OF PROPOSED PROJECT (90 Character Maximum)

1b. [redacted]
NAME OF APPLICANTS / INVESTIGATORS (Principal-Investigator LAST NAME, FIRST NAME; Co-Investigators LAST NAME, FIRST NAME)

2a. [redacted]
NAME OF PRINCIPAL INVESTIGATOR (PI)

2b. [redacted] 2c. [redacted]
POSITION TITLE EMAIL

2d. USDA APHIS WS NWRC 2e,f. [redacted]
INSTITUTION AND DEPARTMENT PHONE FAX

2g. ADDRESS:

3a. THIS PROPOSAL IS A: (Mark one only) _X_ NEW APPLICATION ___ CONTINUATION ___ UNPLANNED EXTENSION

3b. FOR COMPLETION, A FUNDING REQUEST IS:

	INCLUDED and REQUIRED	INCLUDED but NOT REQUIRED	NOT INCLUDED
	X		

3c. AMOUNT OF FUNDING REQUESTED: If additional years are requested, please note amount for each year

$ 82,960.81	$ 76,544.06	$ 78,776.53
FIRST YEAR	SECOND YEAR	THIRD YEAR

3d,e. DATES OF PROPOSED PROJECT:

November 2019	October 15, 2022
START	END

AGREEMENT: It is understood and agreed by the undersigned if this proposal / application is approved, whether or not a grant is made, it will be according to the terms of the proposal and the stipulations set forth in any accompanying instructions from BLM made at the time of proposal approval. In addition, a written agreement appropriate for the nature of the proposed work (e.g., Memorandum of Understanding, Assistance Agreement, Task Order, letter of agreement) will be required to outline the obligations of the researchers and the BLM in the conduct of the study.
PRINCIPAL INVESTIGATOR ASSURANCE: I agree to accept responsibility for the conduct, completion and reporting of the study proposed here and to provide the agreed upon progress and final reports.

4a. SIGNATURE OF PRINCIPAL INVESTIGATOR: [redacted] DATE: 07/16/2019

CERTIFICATION AND ACCEPTANCE: I certify that the statements made in this application are true and complete to the best of our knowledge, and I accept the obligation to comply with the above agreement. I understand that the Principal Investigator and his/her department will be responsible for any expenses incurred by this project which exceed the approved funding amount.

4b. OFFICIAL SIGNING FOR ORGANIZATION: [redacted] DATE: 07/15/2019

4c. ADDRESS: _4101 LaPorte Ave., Fort Collins, CO 80521

4d. [redacted]
EMAIL

4e,f. [redacted]
PHONE FAX

BLM Wild Horse and Burro Program
Wild Horse and Burro (WHB) Research Proposal to the BLM

Privileged Communication.

B. ABSTRACT OF PROPOSED PROJECT AND LIST OF ALL STUDY PERSONNNEL

Abstract: Evaluation of a vaccine against ovarian growth factors as single dose, long-lasting immunocontraceptive.

[redacted] **USDA APHIS WS National Wildlife Research Center, Co- PI project planning, data collection and analysis, 2 days/month (12.11% effort)**

[redacted] **Colorado State University, Co-PI project planning, data collection and analysis, 2 days/ month (12.11% effort)**

Remarkably, equine fertilization rates approach 100%, including in those considered wild. According to Bureau of Land Management (BLM), 81,000+ wild horses and burros live on public lands with an additional 44,000 in off-range holding. At stake is the declining health of public lands and equids that reside there. So, unlike other livestock species it is not enhancing reproduction that we propose but rather **the need for effective contraception designed to manage wild horse populations.** Successful reproduction depends on an adequate number of healthy oocytes. Physiological mechanisms controlling follicular growth and number of oocytes released at ovulation involve a complex systemic and local exchange of signals between the oocyte and surrounding cells. Both GDF-9 and BMP-15 are critical for follicular growth in several species and that these two oocyte-specific factors cooperate in a species-specific way to regulate oocyte maturation and communication with adjacent granulosa cells. Both GDF-9 and BMP-15 have been identified in equine oocytes. Therefore, it is likely that these regulatory proteins function similarly in horses. Our intent is to disrupt their function and attenuate oocyte development, leading to premature depletion and/or disruption of ovulation. Technology is available to produce single dose vaccines that can induce a strong immune response for an extended period of time (> 1 year). However, with the antigens currently being used (i.e. GnRH and pZP) antibody levels need to be maintained for many years, and thus, life-long booster injections are also needed. Thus, the challenge we face is to identify antigens that can cause long-term sterility in a shorter time period. If our hypothesis is correct, a combination of GDF9 and BMP15 may be such an antigen. We have completed the 3rd year of a study in which mares (n=10/treatment) were vaccinated against either GDF9 or BMP15 for the first two years resulting in a 50% decrease in ovulations and altered follicular growth. In the third year, an additional 10 mares were vaccinated against the combination of GDF9 and BMP15. For the duration of year three, **no mares ovulated** or even developed a follicle larger than 12mm in diameter. Based on our findings we propose to immunize mares with the combined vaccine using a long-lasting adjuvant (AdjuVac-+ liposomes) and allow them access to fertile stallions for a period of three years. Data will be collected monthly on pregnancy status, progesterone levels and antibody titers.

C. PROJECT PROPOSAL

1. Goals / Objectives / Hypotheses:

As the population of wild horses and burros continues to increase, it is evident that reproductive control will be the favored mechanism employed to curtail increasing numbers, while allowing for herd maintenance on public lands. Reproduction in all mammals including horses depends on an adequate number of healthy oocytes present in primordial follicles within ovaries and their ability to develop into mature follicles and ovulate. The total number of oocytes contained in ovaries of horses is established before birth. Follicular recruitment and growth is constant and irreversible. Ovulation is key to fertility in that it is the only way an oocyte becomes available for fertilization. Although the mechanisms that initiate these processes is metered, they have not been completely elucidated. However, it is known that communication by the oocyte is critical in each of these steps. Additionally, two key regulatory proteins produced exclusively by the oocyte have been identified. These are growth and differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15). To date their *exact* function is unknown, but their dysregulation has resulted in premature oocyte depletion and altered ovulation in other species. **Our working hypothesis is that vaccination against both of these proteins (GDF9 and BMP15) will result in long-term sterility through premature oocyte depletion and/or inhibition of ovulation**. To test this hypothesis, we propose to vaccinate mares against GDF9 and BMP15 in a single dose and track hormonal profile and fertility.

Hypothesis: We hypothesize that immunizing mares in a single dose against a combination of GDF9 and BMP15, two oocyte specific regulatory proteins, will result in long-term sterility.

2. Specific Aims:

Specific Aim 1: Determine whether vaccination against a combination of GDF9 and BMP15, utilizing AdjuVac+liposome, sufficiently stimulates the equine immune system so that all primordial and primary oocytes are exposed to anti-GDF9 and BMP15 antibodies, thus resulting disrupted follicle maturation and sterility.

Specific Aim 2: Determine whether mares vaccinated against GDF9 and BMP15 with a single dose are rendered infertile for at least a 3-year period.

3. Background and Significance/Preliminary Studies:

Successful reproduction in mammals depends on an adequate number of healthy oocytes present in primordial follicles within the ovaries. The total number of oocytes contained in the ovaries is established either before or shortly after birth (Eckery et al., 1996; Peters et al., 1976; Sawyer et al., 2001; Tingen et al. 2009). Each primordial follicle consists of an oocyte surrounded by a few granulosa cells. Every day a certain number of primordial follicles begins to grow. Growth is evidenced by an increase in the size of the oocyte and proliferation of the granulosa cells. Initiation of growth is a committed step and follicles cannot return to a non-growing state. Consequently, nearly all follicles end up dying at some stage with <0.1% going on to ovulation. The entry of follicles into the growth phase is tightly controlled to ensure that an adequate number of follicles

is available throughout the reproductive life of the animal (Monniaux et al., 2014; Reddy et al., 2010). Consequently, a continual non-renewable loss of the finite supply of oocytes occurs throughout puberty and adult life; which in some species eventually leads to complete depletion of the oocytes (e.g. menopause in humans) and sterility. Disruption of this finite supply of oocytes, either through genetic mutations (Di Pasquale et al., 2004) or exposure to harmful substances (Hoyer et al., 2014) can also cause permanent sterility.

The physiological mechanisms controlling follicular growth and the number of oocytes released at ovulation involve a complex exchange of endocrine signals between various organs and the ovaries, and very importantly, a local exchange of molecules between the oocyte and its surrounding somatic cells within the ovaries (Binelli and Murphy, 2010; McNatty et al., 2007b; Peters et al, 1976). Communication between these two cells types is crucial for the survival and growth of the follicles (Gilchrist et al., 2004; Matzuk et al., 2002). Significant discoveries have shown that the oocyte itself produces two key regulatory growth factors, namely growth and differentiation factor 9 (GDF9) and bone morphogenetic factor 15 (BMP15), that are essential for regulating follicular growth and ovulation rate (Cong et al., 1996; Galloway et al., 2000). These discoveries also led to a paradigm shift in recognizing that the oocyte acts to control the follicle and plays a major role in its own growth and ovulation.

Both GDF9 and BMP15 are critical for early follicular growth in several species (Eckery et al., 2002; Juengel et al., 2002; Juengel and McNatty, 2005; Shimasaki et al., 2004). These two oocyte-specific growth factors cooperate in a species-specific manner to regulate oocyte maturation and communicate with the adjacent granulosa cells (Lin et al., 2012). In sheep, ewes that have double copy mutations in the genes encoding either GDF9 or BMP15 are sterile, but otherwise healthy. In these animals, follicles do not progress beyond the first, or primary, stage of growth. Interestingly, in animals that have only a single copy mutation in either of these genes, and thus produce essentially only half the amount of protein, ovulation rate is increased (Galloway et al., 2000; McNatty et al., 2005). In mice, deletion of GDF9 leads to sterility, but if they don't express BMP15 they are only sub-fertile and often have decreased litter size (Moore and Shimasaki, 2005). Mutations in GDF9 or BMP15 have also been found in women with premature ovarian failure (Di Pasquale et al., 2004; Pouresmaeili and Fazeli, 2014).

Immunization against GDF9 and BMP15

In a series of experiments conducted in sheep, ewes could be made infertile after immunization against either GDF9 or BMP15 (McNatty et al., 2007a). Moreover, specific regions of each growth factor were identified that were important for the biological activity of the respective proteins. This enabled the production of effective peptide vaccines that were specific to those regions. The homologous regions were identified in cows and deer, and found to have 100% amino acid sequence identity to the respective regions in sheep. In cows, vaccination against these peptide regions showed variable results, wherein some animals' reproductive cycles were suppressed and in others there was an increase in ovulation rate (Juengel et al., 2009). In deer (Eckery et al., 2014), animals vaccinated against BMP15 were not made infertile, rather they became more fecund. This may have occurred because the biological activity of BMP15 was only partially blocked and caused an increase of ovulation rate similar to the effect in sheep that have a single copy mutation. Deer vaccinated against GDF9 were made more fecund in the first year, but were made infertile in years 2 and 3. The effects observed in the first year could have been because of the timing of vaccination in relation to onset of breeding. Regardless, it appears that GDF9 was only partially

inhibited during the first breeding season. Results from all of these studies demonstrate that vaccination against GDF9 and BMP15 has the potential to control fertility in a range of species.

Genes for both GDF9 and BMP15 have been identified in the mare (Wade et al., 2009) and shown to be expressed in equine oocytes (Campos-Chillon et al., 2015). Therefore, it is likely that these growth factors have similar functions in horses. Very recently we tested the hypothesis that vaccination against either GDF9 or BMP15 would alter reproductive function in the mare. We determined that while BMP15 immunized mares experienced a 50% decrease in ovulations, those vaccinated against GDF9 did not differ from controls, yet both groups demonstrated altered estrous activity and significant decrease in follicle size prior to ovulation (Davis et al., 2018).

There are two ways that can be considered to cause oocyte depletion. The first would be to use an agent to directly kill the primordial follicles. The second would be to trigger a mass activation (growth) of the primordial follicles. Because the initiation of primordial follicular growth is a committed step, this would result in all follicles dying prematurely, leading to oocyte depletion. We hypothesize that blocking follicular growth at the primary stage through *vaccination against both GDF9 and BMP15* will prevent ovulation and cause an increase in the number of follicles that are recruited to grow. This will increase follicular turnover, and as a consequence cause premature oocyte depletion and permanent sterility.

3. Preliminary Data

We have now passed the midway point (and summer solstice) of the final year of our BLM sponsored study in which mares (n=10) were vaccinated against both GDF9 and BMP15. This was based on the results (described earlier; Davis et al., 2018) for the first two years in which mares were vaccinated against either GDF9 or BMP15, resulting in a 50% decrease in ovulations and demonstrated disruption of estrous behavior and follicular growth. In this third year, an additional 10 mares were vaccinated against the combination of GDF9 and BMP15. During that year through April of the following year, **no mares have ovulated** or even developed a follicle larger than 12mm in diameter (Figure 1.). In mares, follicles normally reach 40-50 mm in diameter before ovulation.

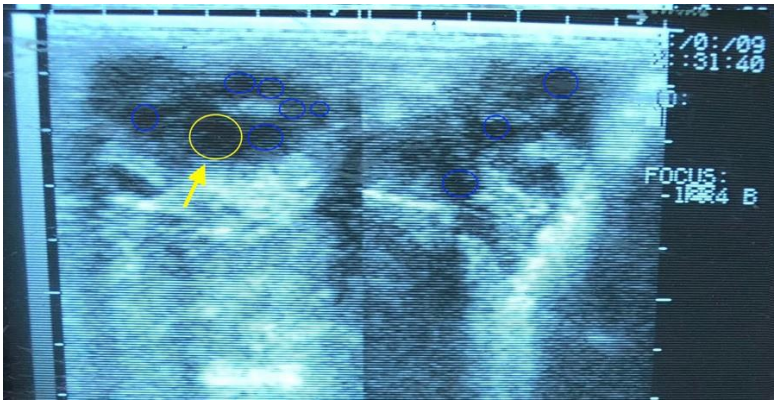

Figure1.
A sonogram of the left and right ovary of a mare on June 29, 2018 following vaccination against both GDF9 and BMP15. Note no follicle larger than 6mm (arrow pointing to circle around largest follicle; blue circles around smaller follicles) and no luteal tissue.

These data, although immensely encouraging, have been generated from mares vaccinated and boostered over the course of the trial utilizing a mild adjuvant (Petgel A, Seppic, France) which has required frequent boosters (see Figure 2.).

The technology is now available to produce vaccines that can induce a strong immune response for an extended period of time (> 1 year) after a single dose. However, with the antigens

currently being used (i.e. GnRH and ZP) antibody levels need to be maintained for many years, and thus, booster injections need to be given over the life of the animal. Thus, one of the challenges we face is to identify antigens that can cause permanent sterility in a shorter time period. If our hypothesis is correct, a combination of GDF9 and BMP15 will be such antigens and with a minimally prolonged titer, the entire population of oocytes could be effected rendering the mare permanently infertile. Our preliminary data suggest that although the titer levels of anti-GDF-9 and BMP-15 returned to pre-treatment levels by the end of the first year, 90% of the mares remained anovulatory during the second year. These data support our hypothesis that the initial antibody titer has successfully altered response in the entire population of oocytes, implying the possibility for permanent sterility.

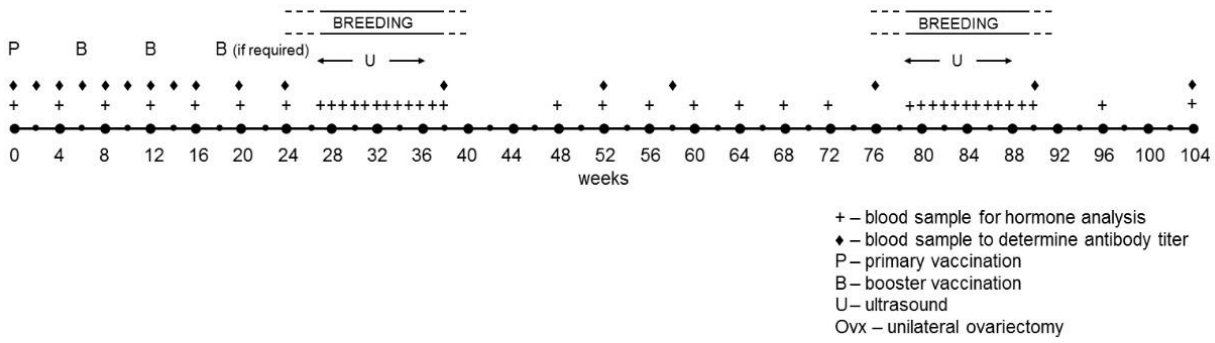

Figure 2. Timeline from previous experiments. Note need for frequent boosters (B).

4. ExperimentalApproach:

This study serves to determine whether a single vaccination against the oocyte specific proteins GDF9 and BMP15 formulated with the appropriate adjuvant (AdjuVac+liposome) will result in permanent sterility. Thirty-two mature mares recently removed from the BLM managed range will be the subjects of this study. The starting target score for Henneke body condition will be at least 4 for both mares & stallions. Mare and stallion ages at the start of the study will be between 3-10 years old.

Specific Aim 1: Determine whether vaccination against a combination of GDF9 and BMP15, utilizing AdjuVac+liposome, sufficiently stimulates the equine immune system so that all primordial and primary oocytes are exposed to anti-GDF9 and BMP15 antibodies, thus resulting in disrupted follicle maturation and sterility.

Rationale: AdjuVac+liposome is expected to sufficiently stimulate the immune system to produce antibodies, (specifically anti-GDF9 and BMP15) for the period of time required to permanently disrupt the local signaling of these oocyte specific proteins. In doing so, there will be life-long

disruption of folliculogenesis/oocyte development and therefore lack of subsequent ovulation/fertility.

Methodology: All mares (n=32) will be treated with a single intramuscular injection. Treated mares (n=16) will receive the newly prepared vaccine against GDF9 and BMP15 while control mares (n=16) will receive adjuvant alone. The BMP15 antigen will be made using a peptide representing the first 24 amino acids of the mature protein (GenBank: GQ183562.1). This region is ~80% identical to the homologous regions of the protein sequence in sheep. The GDF9 antigen will be made using a peptide representing amino acids 20-33 of the mature protein (NCBI Reference Sequence: NC_009157.2). This peptide region is 100% identical to the homologous regions of the protein sequence in sheep. Peptides will be synthesized and conjugated to a carrier protein (keyhole limpet hemocyanin) to make them immunogenic. Each dose of vaccine will consist of 2 ml of the vaccine formulation containing 500 ug of each of the peptide carrier protein conjugates and AdjuVac + liposome. AdjuVac is a mineral oil-based adjuvant that is used in the production of GonaCon Immunocontraceptive Vaccine, which is registered by the EPA for use in wild female horses and burros. The liposome component will be provided by SpayVac-for-Wildlife, Inc. In April 2018, SpayVac-for-Wildlife, Inc. entered into an agreement with IMV Inc. to license two proprietary vaccine delivery platforms, VacciMax® and DepoVax®, for use in contraceptive vaccines for use in populations of overabundant, feral and invasive animals. The combining of a mineral oil-based adjuvant with this liposome technology has previously been shown to be effective in horses (Roelle et al., 2017; Bechert et al., 2013; Killian et al. 2008).

Blood sampling and analyses – Blood samples will be collected via jugular venipuncture monthly starting immediately prior to vaccination and serum harvested to measure antibody responses and progesterone concentrations. For this and all other veterinary procedures, APHIS and CSU researchers will rely on BLM to identify an attending, on-call veterinarian who will be available for project oversight at all times.

Antibody measurements – Antibody responses will be measured using a standard ELISA routinely used in our laboratory (Davis et al., 2018).

Progesterone – Concentrations of the steroid hormone will provide evidence of ovulation and luteal function. Progesterone will be measured using radioimmunoassay at the Colorado State University Reproductive Endocrinology Laboratory.

Specific Aim 2. Determine whether mares vaccinated against GDF9 and BMP15 are rendered infertile for at least a 3-year period.

Rationale: In the previous and on-going studies, mares were exposed to a stallion 3 times/week and had their ovarian activity evaluated at least once weekly or more frequently, specifically whenever the mares showed significant signs of estrus. Those studies were designed to serve as proof-of-concept so that we could demonstrate the effects of vaccination, specifically on behavior and ovarian activity. This study is designed to be the next-step toward practical application by specifically demonstrating infertility, whilst being exposed to fertile stallions.

Methodology: Thirty-two mares will be treated (as described above) on December 1. Mares will then be housed in four pens (n=8 mares/pen; 4 treated + 4 controls). Pregnancy status and ovarian activity will be evaluated monthly by transrectal ultrasonography by one of the co-PIs (CSU; see Biographical Sketch). On February 15, prior to the first expected ovulation of the year, a stallion will be introduced into each pen. Multiple stallions will be used and rotated on a regular basis (e.g. every 2 months), where a single stallion be housed with each group of mares for the duration of the study. Stallions will be between 3 and 12 years of age. In cases where a mare does become pregnant the mare will remain on study for the duration of the study; BLM will take appropriate measures for the care and handling of pregnant mares and their foals. Regardless, the same number of control animals will be present at the start of each year of the study.

.

5. Timeline

The study will be conducted over a three year period starting December 1. All 32 mares will be vaccinated and initial blood samples will be acquired on that date. Stallions will be identified over the next two months. Selected stallions will be housed with the mares starting Feb 15. Serum samples will be collected from mares monthly over the three-year period. Antibody responses will be determined bi-monthly while hormone concentrations will be evaluated at the end of each year to minimize intra-assay variation.

5. Statistical Methods:

A power analysis was conducted, to determine sample size requirements, based on the assumption that non-treated mares may foal at a rate of 80%, treated mares may foal at a rate as high as 40%, using a type I error rate (α) of 0.05 and a type II error rate (β) of 0.1. Based on expected P-values and confidence intervals for a 2-tailed z-test, a sample size of 12 treated mares and 12 control mares is adequate to discern the differences noted in the assumptions stated above. However, allowing for the possibility that a small number of mares may drop out of the study for unrelated reasons, we propose a sample size of 16 treated mares and 16 untreated control mares.

Differences in pregnancy rate will be calculated by chi-square.

6. Anticipated effects:

We expect that specific antibodies against both GDF9 and BMP15 will be produced by the vaccinated mares within two weeks of vaccination and stay elevated for at least a year. The titers may decline over the next two years, but the protein signaling should be sufficiently disrupted to alter oocyte maturation and follicular development and ovulation. This should result in prolonged (permanent) anovulatory period in the mares. The prolonged anovulatory period will result in sterility. In the event that more than 60% of treated mares are fertile in year 1 or year 2,

BLM will initiate a discussion with the researchers on the merits of continuing the study. The study could be discontinued, or changes could be made such as no longer exposing any mares in the study to stallions, but continuing to be examine mares with ultrasound to assess ovulatory outcomes.

7. Pitfalls and Limitations:

Potential pitfalls are most likely limited to animal selection. As we propose to use both mares and stallions captured and removed from BLM controlled range lands, we will not be fully aware of their reproductive capacity prior to the initiation of the study. However, mare fertility will more likely be known. For males, multiple stallions will be used and rotated on a regular basis to ensure mares are exposed to a fertile stallions during most of the study. Because a power calculation reveals that a sample size of two would be adequate to test our hypothesis, we have chosen to include 4 pens of mares in this case.

8. Reporting:

Your signature below affirms your agreement to provide a final report, following the format provided at the end of this template, to be received by the BLM research coordinator no later than 3 months after completion of the project. If your project will take more than one year to complete, BLM requires a progress report once per year, following the format provided at the end of this template. The first progress report shall be received by the BLM WHB research coordinator no later than one year after receipt of samples. BLM will require the return of specimens unless timely reporting is completed. For museum collections, a copy of accession documents and signature of assurance that the specimens are secure will serve as the final report. Different reporting requirements may be established if a BLM assistance agreement is established for this project, in which case the reporting requirements of that agreement would take precedence over those outlined here.

Signature of Principal Investigator: [redacted]

9. References:

Bechert U, Bartell J, Kutzler M, Menino Jr. A, Bildfell R, Anderson M, Fraker M (2013) Effects of Two Porcine Zona Pellucida Immunocontraceptive Vaccines on Ovarian Activity in Horses. J Wildlife Mgt 77:1386–1400.

Binelli M, Murphy BD (2010) Coordinated regulation of follicle development by germ and somatic cells. Reprod Fertil and Devel 22:1-12.

Cauchard S, Bertrand F, Barrier-Battut I, Jacquet S, Laurentie M, Barbey C, Laugier C, Deville S, Cauchard J (2014) Assessment of the safety and immunogenicity of *Rhodococcus equi*-secreted proteins combined with either a liquid nanoparticle (IMS 2014) or a polymeric (PET Gel A) water-based adjuvant in adult horses and foals – identification of promising new candidate antigens. Vet Immunol Immunopathol 157: 164-174.

Campos-Chillon F, Farmerie TA, Bouma G, Clay C, Carnevale EM (2015) Effects of aging on gene expression and mitochondrial DNA in the equine oocyte and follicle cells. Reprod Fertility and Dev 27:925-933.

Davis KA, Klohonatz KM, Mora DSO, Twenter HM, Graham PE, Pinedo P, Eckery DC, Bruemmer JE (2018) Effects of immunization against bone morphogenetic protein-15 and growth differentiation factor-9 on ovarian function in mares. Anim Reprod Sci 192:69-77.

Dewailly D, Andersen CY, Balen A, Broekmans F, Dilaver N, Fanchin R, Griesinger G, Kelsey TW, La Marca A, Lambalk C, Mason H, Nelson SM, Visser JA, Wallace WH, Anderson RA (2014) The physiology and clinical utility of anti-Mullerian hormone in women. Hum Reprod Update Jan 29 [Epub ahead of print].

Di Pasquale E, Beck-Peccoz P, Persani L (2004) Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene. Am J Hum Gen 75:106-111.

Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM (1996) Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature 383:531-535.

Dube JL, Wang P, Elvin J, Lyons KM, Celleste AJ, Matzuk MM (1998) The bone morphogenetic protein 15 gene is X-linked and expressed in oocytes. Mol Endocrinol 12:1809-1817.

Eckery DC, Tisdall DJ, Heath DA & McNatty KP (1996) Morphology and function of the ovary during fetal and early neonatal life: a comparison between the sheep and brushtail possum (*Trichosurus vulpecula*). Anim Reprod Sci 42:551-561.

Eckery DC, Whale LJ, Lawrence SB, Wylde KA, McNatty KP, Juengel JL (2002) Expression of mRNA encoding growth differentiation factor 9 and bone morphogenetic protein 15 during follicular formation and growth in a marsupial, the brushtailed possum (*Trichosurus vulpecula*). Mol Cell Endocrinol 192:115-126.

Eckery D, Miller L, Killian G, DeNicola, T (2014) Effects of vaccination against GDF9 and BMP15 on fertility and ovarian function in white-tailed deer. Proceeding of the Vertebrate Pest Conference, Waikaloa, HI.

Galloway, SM, McNatty KP, Cambridge LM, Laitenen MPE, Juengel JL, Jokiranta TS, McLaren RJ, Luiro K, Dodds KG, Montgomery GW, Beattie AE, Davis GH, Ritvos O (2000) Mutations in an oocyte-derived growth factor (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. Nat Genet 25:279-283.

Gilchrist RB, Ritter LJ, Armstrong DT (2004) Oocyte-somatic cell interactions during follicular development in mammals. Anim Reprod Sci 82-83:431-446.

Hoyer PB, Keating AF (2014) Xenobiotic effects in the ovary: temporary versus permanent infertility. Expert Opin Drug Metab Toxicol 10:511-23.

Juengel JL, Hudson NL, Heath DA, Smith P, Reader KL, Lawrence SB, O'Connell AR, Laitinen MPE, Cranfield M, Groome NP, Ritvos O, McNatty KP (2002) Growth differentiation factor 9 and bone morphogenetic protein 15 are essential for ovarian follicular development in sheep. Biol Reprod 67:1777-1789.

Juengel JL, McNatty KP (2005) The role of proteins of the transforming growth factor- b superfamily in the intraovarian regulation of follicular development. Hum Reprod Update 11:143-160.

Juengel JL, Hudson NL, Berg M, Hamel K, Smith P, Lawrence SB, Whiting L, McNatty KP. (2009) Effects of active immunization against growth differentiation factor 9 and/or bone morphogenetic protein 15 on ovarian function in cattle. Reprod 138:107-14.

Killian G, Thain D, Dieh NK, Rhyan J, Miller L (2008) Four-year contraception rates of mares treated with single injection porcine zona pellucida and GnRH vaccines and intrauterine devices. Wildlife Research 35: 531–539.

Lin JY, Pitman-Crawford JL, Bibby AH, Hudson NL, McIntosh CJ, Juengel JL, McNatty KP (2012) Effects of species differences on oocyte regulation of granulosa cell function. Reprod 144:557-67.

Matzuk MM, Burns KH, Viveiros MM, Eppig JJ (2002) Intercellular communication in the mammalian ovary: oocytes carry the conversation. Science 296:2178-80.

McNatty KP, Smith P, Moore LG, Reader K, Lun S, Hanrahan JP, Groome NP, Laitinen M, Ritvos O, Juengel JL (2005) Oocyte-expressed genes affecting ovulation rate. Mol Cell Endocrinol 234:57-66.

McNatty KP1, Reader K, Smith P, Heath DA, Juengel JL (2007a) Control of ovarian follicular development to the gonadotrophin-dependent phase: a 2006 perspective. Soc Reprod Fertil Suppl.;64:55-68.

McNatty KP, Hudson NL, Whiting L, Reader KL, Lun S, Western A, Heath DA, Smith P, Moore LG, Juengel JL (2007b) The effects of immunizing sheep with different BMP15 or GDF9 peptide sequences on ovarian follicular activity and ovulation rate. Biol Reprod 76:552-560.

Moore RK, Shimasaki S (2005) Molecular biology and physiological role of the oocyte factor, BMP-15. Mol Cell Endocrinol. Apr 29;234(1-2):67-73.

Moore RK, Erickson GF, Shimasaki S (2004) Are BMP-15 and GDF-9 primary determinants of ovulation quota in mammals? Trends Endocrinol Metab. 15:356-61.

Monniaux D, Clément F, Dalbiès-Tran R, Estienne A, Fabre S, Mansanet C, Monget P (2014) The ovarian reserve of primordial follicles and the dynamic reserve of antral growing follicles: what Is the link? Biol Reprod Mar 5 [Epub ahead of print].

Peigné M, Decanter C. (2014) Serum AMH level as a marker of acute and long-term effects of chemotherapy on the ovarian follicular content: a systematic review. Reprod Biol Endocrinol 12:26.

Peters H, Byskov AG, Himelstein-Braw R, Faber M (1976) Follicle growth: the basic event in the mouse and human ovary. J Reprod Fertil 45:559-566.

Pouresmaeili F, Fazeli Z (2014) Premature ovarian failure: a critical condition in the reproductive potential with various genetic causes. Int J Fertil Steril 8:1-12.

Reddy P, Zheng W, Liu K (2010) Mechanisms maintaining the dormancy and survival of mammalian primordial follicles. Trends Endocrinol Metab 21:96-103.

Roelle JE, Germaine SS, Kane AJ, Cade BS (2017) Efficacy of SpayVac as a Contraceptive in Feral Horses. Wildlife Society Bulletin 41:107–115.

Sawyer HR, Smith P, Heath DA, Juengel JL, Wakefield St J, McNatty KP (2001) Formation of ovarian follicles during fetal development in sheep. Biol Reprod 66:1134-1150.

Shimasaki S, Moore RK, Otsuka F, Erickson GF (2004) The bone morphogenetic protein system in mammalian reproduction. Endocrine Rev 25:72-101.

Tingen C, Kim A & Woodruff TK (2009) The primordial pool of follicles and nest breakdown in mammalian ovaries. Mol Hum Reprod 15:795-803.

Wade CM, Giulotto E, Sigurdsson S, Zoli M, Gnerre S, Imsland F, LearTL, Adelson DL, Bailey E, Bellone RR, Blocker H, Distl O, Edgar RC, Garber M, Leeb T, Mauceli E, MacLeod JN, Penedo MC, Raison JM, Sharpe T, Vogel J, Andersson L, Antczak DF, Biagi T, Binns MM, Chowdhary BP, Coleman SJ, Della Valle G, Fryc S, Guerin G, Hasegawa T, Hill EW, Jurka J, Kiialainen A, Lindgren G, Liu J, Magnani E, Mickelson JR, Murray J, Nergadze SG, Onofrio R, Pedroni S, Piras MF, Raudsepp T, Rocchi M, Roed KH, Ryder OA, Searle S, Skow L, Swinburne JE, Syvanen AC, Tozaki T, Valberg SJ, Vaudin M, White JR, Zody MC, Lander ES, Lindblad-Toh K (2009) Genome sequence, comparative analysis, and population genetics of the domestic horse. Science 326: 865-867.

10. Appendices:

Following are two abstracts accepted for presentation at the 2019 Equine Science Society Symposium in Asheville NC June 4-7, 2019

Contraceptive vaccination for mares and its effects on cyclicity and estrous behavior

[redacted]

Overpopulation is an issue for wild horses due to limited forage and decreasing water sources. Sterilizing mares without surgical ovariectomy would be cost effective and safer. There are currently no vaccines that cause permanent sterility in mares. Bone Morphogenetic Protein 15 (BMP-15) and Growth Differentiation Factor 9 (GDF-9) are oocyte-specific proteins involved in follicular development from primordial activation through ovulation. This study investigated the effects of a combination vaccine consisting GDF-9 and BMP-15, on mare cyclicity and estrous behavior. We hypothesized that immunization against the combination of these two factors would result in no ovarian cyclicity. Mature Quarter Horse type mares (n=10/group) were used. The experiment was conducted February through September 2018. All mares were vaccinated 5 times starting at week 0, 6, 12, 18, and 24. Ten mares received the vaccine consisting of both peptides and adjuvant, while control mares received adjuvant. Ovarian activity and ovulations were recorded by trans-rectal ultrasonography once a week and estrous behaviors were evaluated three days a week by interacting individually with a stallion. Follicle diameters were measured, and estrous behavior was scored on a 5-point scale (0=hostile toward stallion–5=actively seeking stallion). Jugular blood samples were collected weekly, and serum was aspirated for investigation of the progesterone. Control mares cycled normally with ovulations associated with estrus. None of the 10 treated mares ovulated or grew a follicle larger than 12mm during the experiment and progesterone in serum samples were 1ng/mL ≥. Future research will focus on the active duration of the vaccination to determine the length of effectiveness. This vaccination could serve as a long-term contraceptive in wild horse herd populations.

Contraceptive, Mare, Ovulation

Estrous behavior and ovarian function in mares vaccinated against Bone Morphogenetic Protein-15 and Growth Differentiation Factor-9

[redacted]

Colorado State University Fort Collins Colorado, National Wildlife Research Center, USDA-APHIS, Fort Collins Colorado

As wild horse populations have reached unsustainable levels on rangelands, a long-term contraceptive has become an option for maintaining healthy populations. Recently, Bone Morphogenetic Protein-15 (BMP-15) and Growth Differentiation Factor-9 (GDF-9) have been identified as oocyte specific growth factors in the mare. These factors are associated with all stages of follicular growth from recruitment to ovulation. The purpose of this study was to analyze the effects associated with vaccination against BMP-15 or GDF-9, specifically on the mare's ovarian cycle and estrous behavior. Both BMP-15 and GDF-9 are oocyte-specific growth factors, and it was hypothesized that BMP-15 and GDF-9 would have an effect on the mare's ovarian cycle, but there would be no effect on the estrous behavior displayed. In this study, 15 mature stock-type mares of proven fertility were assigned to three different groups (n=5/group): control mares, mares vaccinated against BMP-15, and mares vaccinated against GDF-9. All mares were vaccinated 4 times at 6 week intervals in order to ensure appropriate antibody titers. Each treatment vaccination formulation contained 1000 µg of peptide-KLH conjugate (either BMP-15 or GDF-9) in 2ml volume. Control mares were vaccinated with KLH alone. Vaccines were administered intramuscularly in the cervical musculature of the neck using a 20-gauge needle. The study was initiated in March 2017, with ovarian activity and estrous behavior being recorded three times a week via transrectal ultrasonography and teasing scores from direct interaction with a stallion, respectively. These evaluations continued until the study was concluded in September, 2017. It was noted that the average follicle size prior to ovulation was smaller in both the BMP-15 and GDF-9 mares as compared to the control group. Estrous behavior did not differ from treatment groups as compared to the control group. Further research will be needed to fully understand the effects of these two growth factors on ovulation, but these results support the potential for BMP-15 and GDF-9 being used as contraceptives for mares in the future.

D. BIOGRAPHICAL SKETCH

Privileged Communication

Name: [redacted]

Education:

Institution and Location	**Degree**	**Year Conferred**	**Scientific Field**
[redacted]	B.S.	1988	Animal Science
	M.S.	1990	Animal Science
	Ph.D.	1995	Physiology (Reproduction)

Honors/Awards:
NICHD, National Research Service Award, Colorado State University
Invited speaker to six international meetings

Major Research Interest:
Ovarian follicular growth, endocrinology and pituitary function, fertility control for management of animal populations

Role in Proposed Project:
Design and planning. Data collection including blood draws and antibody assays. Data analysis, report and manuscript preparation.

Previous and Current Research Support Relating to the Current Proposal:
BLM – Agreement L15AC00144

Research and/or Professional Experience

2018-present	[redacted] USDA APHIS WS National Wildlife Research Center, Fort Collins, CO
2013-present	Project Leader, USDA APHIS WS National Wildlife Research Center, Fort Collins, CO
2013-present	Affiliate faculty, Department of Biomedical Sciences, Colorado State University
2012-present	Affiliate faculty, School of Biological Sciences, Victoria University of Wellington, New Zealand
2012 – 2013	Research Fellow, Department of Clinical Sciences, Colorado State University
2006 – 2012	Associate Professor Research Fellow/Senior Lecturer, School of Biological Sciences, Victoria University of Wellington, New Zealand
2002 – 2006	Senior Scientist, AgResearch, Wallaceville Animal Research Centre, Upper Hutt, New Zealand
1997 – 2002	Scientist, AgResearch, Wallaceville Animal Research Centre, Upper Hutt, New Zealand
1995 – 1997	Post-doctoral Fellow, AgResearch, Wallaceville Animal Research Centre, Upper Hutt, New Zealand

1990 – 1994 Graduate Research Assistant, Department of Physiology, Colorado State University, Ft. Collins, CO

1988 – 1990 Graduate Research Assistant, Department of Animal Science, Brigham Young University, Provo, UT

Relevant publications:

[redacted] (2018). Effects of immunization against bone morphogenetic protein- 15 and growth differentiation factor-9 on ovarian function in mares. *Animal reproduction science, 192*, 69-77.

[redacted] (2018). Reimmunization increases contraceptive effectiveness of gonadotropin-releasing hormone vaccine (GonaCon-Equine) in free-ranging horses (Equus caballus): Limitations and side effects. *PloS one, 13*(7), e0201570.

[redacted] (2014) Effects of vaccination against GDF9 and BMP15 on fertility and ovarian function in white-tailed deer. 26th Vertebrate Pest Conference, Kona, HI.

[redacted] 2014) Novel management methods: immunocontraception and other fertility control tools. In: Putman R (Ed.), Behaviour and Management of European Ungulates, In press.

Name: [redacted]

Education:

Institution and Location	Degree	Year Conferred
[redacted]	B.S.	1987
	M.S.	1991
	Ph.D.	1996

Honors/Awards:

2018 American Society Animal Sciences – Equine Science Award
2012 CSU – [redacted] Outstanding Professor – [redacted]
1998 CSU – [redacted] Faculty Teaching Award

Major Research Interest:

Equine reproduction

Role in Proposed Project:

Design, planning. Data collection includes ultrasound exam of mares, blood draws, data analysis

Previous and Current Research Support Relating to the Current Proposal:

BLM – Agreement L15AC00144

Research and/or Professional Experience:

2010 – present	Professor, Department of Animal Sciences, Equine Sciences, Colorado State University
2002 - 2008	Associate Professor, Department of Animal Sciences, Equine Sciences, Colorado State University
2006 – present	Affiliate Faculty – Graduate College, Texas A&M University
2004 – 2005	Visiting Scientist – Vincent Center for Reproductive Biology, Massachusetts General Hospital, Harvard School of Medicine
1996 – 2002	Assistant Professor, Department of Animal Sciences, Equine Sciences, Colorado State University
1994 – 1996	Graduate Teaching Assistant, New Mexico State University
1990 – 1993	Lecturer, Manager of Horse Center, Department of Animal Science, Texas A&M University

Recent relevant publications:

[redacted] (2018). Effects of immunization against bone morphogenetic protein-15 and growth differentiation factor-9 on ovarian function in mares. *Animal reproduction science, 192*, 69-77.

[redacted] (2018). Vessel sealer and divider instrument temperature during laparoscopic ovariectomy in horses. *Veterinary surgery : VS, 47*(S1), O26-O31.

[redacted] 2018). Reimmunization increases contraceptive effectiveness of gonadotropin-releasing hormone vaccine (GonaCon-Equine) in free-ranging horses (Equus caballus): Limitations and side effects. *PloS one, 13*(7), e0201570.

E. FACILITIES STATEMENT

Privileged Communication

Animal facilities will be provided by BLM.
Ultrasound equipment will be provided by Colorado State University.
Antibody titer assays will conducted by National Wildlife Research Center
Progesterone assays will be conducted by Endocrinology Laboratory at Colorado State University

F. DETAILED BUDGET FOR EACH 12 MONTH PERIOD

Privileged Communication

DATES FOR THIS 12 MONTH PERIOD FROM **November 2019 to October 2020**

Salary & Wages (Describe % effort or hours for each person, in parentheses)

[redacted]	19250.26
Fringe (0.282)	5428.57
Indirect (CSU- APHIS) 0.1	2467.88
Student hourly (150 hrs @14.00/hr)	2100.00
Fringe (0.07)	147.00
Indirect (CSU – APHIS) 0.1	224.70

Category Total: 29618.41

Equipment & Supplies *(Describe and give cost of each item over $100)*
Palpation sleeves, syringes, needles, vacutainer tubes, lube, Estru-Mate, ice, cold packs, disposable pipette tips

Category Total: 2500.00

Animal Costs *(Including board and maintenance)*
Provided by BLM

Category Total: 0.00

Miscellaneous Costs *(assays, etc,)*

Vaccines (BMP15+ GDF9)	16 @ 250 each	4000.00
Vaccines (control)	16 @ 175 each	2800.00
P4 Assay	384 @ 15 each	5760.00
Titer	384 @ 10 each	3840.00
Travel – 2 people/ monthly exams		
Airfare	24 @ 417	10008.00
Car rental/ gas	12 @ 100	1200.00
Mileage (airport)	12 @ 100	1200.00
Lodging	24 @ 114	2736.00
Per Diem	24 @ 66	1584.00

Category Total: 33128.00

Sub Total: 65246.41
Indirect Costs: 17714.40

TOTAL: 82960.81
AMOUNT REQUESTED OF BLM: 82960.81

DATES FOR THIS 12 MONTH PERIOD FROM **November 2020 to October 2021**
(All costs assume 3% increase)

Salary & Wages (Describe % effort or hours for each person, in parentheses)

[redacted]	19827.77
Fringe (0.282)	5591.43
Indirect (CSU- APHIS) 0.1	2541.92
Student hourly (150 hrs)	2163.00
Fringe (0.07)	151.41
Indirect (CSU – APHIS) 0.1	217.80

Category Total: 30506.97

Equipment & Supplies *(Describe and give cost of each item over $100)*
Palpation sleeves, syringes, needles, vacutainer tubes, lube, Estru-Mate, ice, cold packs, disposable pipette tips

Category Total: 2575.00

Animal Costs *(Including board and maintenance)*
Provided by BLM

Category Total: 0.00

Miscellaneous Costs *(assays, etc,)*

P4 Assay	384 @ 15.45 each	5932.80
Titer	384 @ 10.30 each	3955.20
Travel – 2 people/ monthly exams		
Airfare	24 @ 429.51	10308.24
Car rental/ gas	12 @ 103	1236.00
Mileage (airport)	12 @ 103	1236.00
Lodging	24 @ 117.42	2818.08
Per Diem	24 @ 67.98	1631.52

Category Total: 27117.84

Sub Total: 60199.81

Indirect Costs: 16344.25

TOTAL: 76544.06
AMOUNT REQUESTED OF BLM: 76544.06

DATES FOR THIS 12 MONTH PERIOD FROM **November 2021to October 2022**
(All costs assume 3% increase)

Salary & Wages (Describe % effort or hours for each person, in parentheses)

[redacted]	20422.60
Fringe (0.282)	5759.17
Indirect (CSU- APHIS) 0.1	2618.17
Student hourly (150 hrs)	2228.00
Fringe (0.07)	155.96
Indirect (CSU – APHIS) 0.1	238.40

Category Total: 31422.30

Equipment & Supplies
Palpation sleeves, syringes, needles, vacutainer tubes, lube, Estru-Mate, ice, cold packs, disposable pipette tips

Category Total: 2602.81

Animal Costs *(Including board and maintenance)*
Provided by BLM

Category Total: 0.00

Miscellaneous Costs *(assays, etc,)*

P4 Assay	384 @ 15.91 each	6109.44
Titer	384 @ 10.61 each	4074.24
Travel – 2 people/ monthly exams		
Airfare	24 @ 442.40	10617.60
Car rental/ gas	12 @ 106.09	1273.08
Mileage (airport)	12 @ 106.09	1273.08
Lodging	24 @ 120.94	2902.56
Per Diem	24 @ 70.02	1680.48

Category Total: 27930.48

Sub Total: 61955.59

Indirect Costs: 16820.94

TOTAL: 78776.53
AMOUNT REQUESTED OF BLM: 78776.53

Title of proposal: **Evaluation of a vaccine against ovarian growth factors as single dose, long-lasting immunocontraceptive**

Investigators: [redacted]

Pursuant to procedures established by the Bureau of Land Management, Wild Horse and Burro Research Program, I certify that the above described protocol follows guidelines set forth in the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" (#85-23), the "Animal Welfare Act of 1966" (PL 89-544) as amended, or the "Guide for the Care and Use of Agricultural Animals in Research and Teaching (2010)."

Signature:_[redacted]___Date _[redacted]_

Name: _ Chair, Institutional Animal Care and Use Committee

Name of Institution: _____

NOTE: This completed form or a copy of the animal care and use protocols must be in receipt of the BLM WHB Research Advisory Team before any collaborative work handling animals can commence. Private individuals must seek approval from a recognized Institutional Animal Care and Use Committee.