BUREAU OF LAND MANAGEMENT
OR-WA State Office
Grants & Cooperative Agreements

PROJECT PROPOSAL

Instructions: A Project Proposal must be submitted with the Standard Form (SF) 424 Application for Financial Assistance for all Financial Assistance Agreements. A new proposal must be included with any request for modification which involves a revision to funding, project scope, period of performance, or key personnel.

Agreement or Funding Opportunity No.: L14AS00048 Date: 08/21/15

Organization Name: Colorado State University

Project Title: Re-immunization of Free-Ranging Horses with GonaCon Immunological Vaccine: Effects on Reproduction, Side-Effects, and Population Performance

Current Funding Estimated Period of Performance (PoP): **08/24/15** to 8/23/20

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* If a specific start work date is needed, contact your BLM Program Officer. Do not start work without prior approval from the Grants Management Officer.

1. Purpose, Objectives, and Relevance:
(Describe why the project is needed, the applicant's objectives, how the applicant's objectives support their mission, and how this project benefits the general public.)

BACKGROUND

1. Re-immunization

In many areas of the western United States, overabundant and rapidly expanding populations of feral horses (*Equus caballus*) pose a significant dilemma for natural resource managers. The Wild Free-Roaming Horses and Burros Act of 1971(P.L.92-195) provided protection for feral horses and burros (*Equus asinus*) on most federal lands and established guidance for their management as a wildland species (Wagner 1983). There is, however, wide-spread concern among state, federal, and private land management agencies that unregulated feral horse populations are severely altering native plant communities and limiting the abundance and diversity of habitat resources allocated for native wildlife and other domestic livestock species.

Current population control methods such as utilizing periodic roundups and adoption or sale of excess animals, or maintaining excess feral horses in long-term holding facilities are expensive, resource intensive, and unsustainable. Clearly, more efficient, cost effective, and humane approaches to reducing feral horse densities on public lands are needed. Controlling the fertility of female horses offers a potential non-lethal alternative to conventional methods (National Research Council 2013).

A promising immunological approach to contraception in feral horses involves immunization against the neuropeptide gonadotropin releasing hormone (GnRH). Scientists at the National Wildlife Research Center (NWRC) have conjugated synthetic GnRH peptides to a highly immunogenic carrier protein that, when combined with a potent adjuvant, stimulates the host's immune system to produce antibodies that bind to endogenous GnRH. This, in turn, prevents synthesis and secretion of important downstream reproductive hormones necessary for reproduction. Animals generally return to fertility as antibodies concentrations decline (Powers et al. 2011).

Multiple years of infertility have been achieved in captive and free-ranging wild ungulates with a single inoculation with the GnRH-based vaccine, known as GonaCon. This vaccines has been experimentally tested and found to provide multiple years of infertility after a single application in white-tailed deer (*Odocoileus virginianus)(* Miller et al. 2008, Gionfriddo et al. 2011*a*), bison (*Bison bison)(* Miller et al. 2004), elk (*Cervus elaphus)(* Killian et al. 2009, Powers et al. 2011, 2014), wild pig (*Sus scrofa)(* Massei et al. 2012), and feral horses (Killian et al. 2008, Gray et al. 2010, Baker et al. 2013). However, multiple years of infertility are only experienced in a fraction of vaccinated animals. In free-ranging elk, there was approximately a 90% treatment effect the first year after vaccination but that dropped to 50% by the second year and by the third year of the study, there was no measureable response (Powers et al. 2014). Similarly, during the first 3 years of our current investigation in feral horses at THRO, we observed a 25-35% decrease in foaling in treated versus control mares for the first and second years of the study but no effect by year three (Baker et al. 2013).

Repeat vaccinations generally result in a more profound and longer-lasting antibody production due to the anamnestic response (Tizard 1982). Therefore, we expect longer- lasting contraceptive effects in re-vaccinated mares. The single-injection GonaCon vaccine is unique in that the formulation initiates high antibody titers that remain elevated in some applications; however, to our knowledge, no research has been conducted to evaluate booster doses of this vaccine in any mammalian species.

Booster immunizations using a variety of GnRH vaccines in domestic horses have been shown to improve contraceptive efficacy and to suppress behavioral and physiological estrus (Garza et al.1986, Elhay et al. 2007, Botha et al 2008). However, these GnRH vaccines differ from GonaCon in that they incorporate different protein carrier molecules and adjuvants, and are formulated for short duration (< 1 yr.) contraceptive effectiveness that is generally achieved by using a primary immunization followed 35 days later by a booster inoculation.

While a single vaccination is often preferred from a management perspective, GonaCon vaccine may prove to be more effective if repeat vaccinations are delivered on a periodic basis. Efficacy data collected from 25 mares treated with single application of GonaCon in 2009, at Theodore Roosevelt National Park (THRO) revealed a moderate 2-year decline of approximately 30% in foaling rates, with all mares regaining fertility by three years post-primary vaccination treatment (Baker et al. 2013). Surprisingly, re-vaccination of these same mares in the fall 2013 (four years post-primary vaccination) has resulted to date, in complete infertility during the 2015 foaling season (the first season to expect a re-vaccination effect on fertility). Clearly, these results are both statistically and biologically significant, as well as encouraging from a fertility control perspective.

If these results persist over time and these mares remain infertile, it would lend support to our hypothesis that re-vaccination with GonaCon, even four years post-primary vaccination produces a strong anamnestic response in horses that stimulates anti-GnRH antibodies and suppresses fertility. At present, however, it is premature to predict how many of these re-vaccinated mares failed to conceive during the 2014 breeding season and will not foal or regain fertility during 2015 and beyond. It is possible that the booster vaccination simply delayed the estrous cycle in these mares, which could result in foals being born later in the foaling season.

While these findings are tentative and inconclusive, they suggest that repeat vaccinations

are likely needed to achieve high efficacy of GonaCon vaccine in free-ranging horses and these effects have not been investigated or determined. Thus, our proposed research offers a unique opportunity to address this question at THRO and will have relevance, not only to feral horses, but also to other wild ungulates that have been treated with a single treatment of GonaCon vaccine. Our proposed research will begin to define the vaccination schedule needed to maintain infertility in free-ranging horses and whether or not long-term or permanent sterility is a possible outcome. We will investigate the safety and efficacy of a repeat vaccination under the hypothesis that this vaccine will be more efficacious and longer-lasting than the original primary immunization.

2. Remote Dart Delivery

Fundamental to practical field application of GonaCon vaccine in free-ranging horses is a safe, reliable, and effective method of administering a single dose of the vaccine to free-ranging horses by means of a syringe dart. Many contraceptive agents have been successfully applied via syringe dart or biodegradable implant to an assortment of wild ungulate species including white-tailed deer (Turner et al. 1992, Jacobsen et al. 1995, DeNicola et al. 1997), elk (Shideler et al. 2002, Baker et al. 2005), feral horses (Kirkpatrick et al.1990, Roelle and Ransom 2009), and elephants (*Loxodonta Africana*) (Delsink et al. 2002). However, to our knowledge, evaluation of remotely-delivered GonaCon vaccine is limited to one field investigation with white-tailed deer (DeNicola unpublished data). Although dart performance in this study was less than expected, it provided important basic information regarding optimum dart configuration and delivery ballistics. Using this preliminary data, technicians at Pneu-Dart, Inc. developed a prototype dart configuration for delivering this highly viscous vaccine formulation to free-ranging horses.

We tested this GonaCon-specific dart delivery system with captive feral horses at the 2013 scheduled roundup at THRO. Eleven adult mares (2-4 years of age), that had not been previously vaccinated, were held in small paddocks and remotely darted in the biceps femoris muscle with 2 ml (2000 µg) of GonaCon vaccine. All darts were weighed (± 0.01g) before and after injection to determine the precise dose delivered. Darting distance varied from 10-15 m. Nine out of 11 darts delivered, on average, 95% of the GonaCon vaccine formulation. Two darts failed to discharge possibly due to low muzzle velocity. All darts appeared to dispense the vaccine deep into the muscle mass and none of the darts were observed to bounce without penetration, partially discharge, blow-out, or show evidence of subcutaneous delivery of the vaccine. The two horses in which the darts failed to discharge were subsequently re-treated and the second darts successfully delivered a full dose. With 85% of the 2015 foaling season complete, 7/11 (63%) of these mares have not foaled. In contrast, only 16% of the untreated mares have not foaled to date. A dependable dart delivery system for administering GonCon remotely to free-ranging horses is critical to the determination of an optimum re-vaccination schedule in our proposed study. If successful, this technology will potentially provide resource managers with an alternative strategy for managing this feral horse population.

3. Biological Side-Effects

Evaluation of the biological side-effects of GonaCon vaccine treatments have been reported for numerous wild ungulate species including white-tailed deer (Curtis et al. 2008, Gionfriddo et al. 2011*b*), elk (Powers et al. 2011, 2012, 2014), bison (Miller et al. 2004) and feral horses (Baker et al. 2013). Results from these investigations generally conclude that GonaCon does not cause serious adverse effects on general health, body condition, existing pregnancy, neonatal health, major organ systems, or fertility of male and female offspring of females treated during pregnancy.

Granulomatous intramuscular injection-site lesions, that occasionally break and drain as abscesses, are the only adverse effect of vaccination consistently reported in these studies. The formation of these injection site lesions may be necessary for stimulation of a strong immune

response and infertility. GonaCon vaccine contains AdjuVac; a water-in-oil based adjuvant developed from a USDA approved Johnes disease vaccine called Myocopar™ (Fort Dodge Animal Health). AdjuVac contains killed *Mycobacterium avium,* which is needed to induce a rapid, strong, and sustained contraceptive response (Miller et al. 2008*a,* Perry et al. 2008). This combination of water - in- oil emulsion and killed mycobacteria results in a highly potent adjuvant that stimulates both humoral and cellular immunity (Warren et al. 1986).

Vaccines, like GonaCon, that contain mycobacteria may induce strong immune responses because of the formation of a repository or depot at the injection site (Fukanoki et al. 2000). In response to the presence of the depot, a granuloma forms as the immune system attempts to isolate the foreign material. The continued existence of this depot, which initiates a chronic inflammatory response, likely provides a long-term source of antigen stimulation and persistent antibody production. We speculate that this is the mechanism by which a single vaccination can provide multiple years of infertility in a portion of the population in many species that have been studied.

However, even with this prolonged antigenic stimulation, the immune response from a single vaccination does not consistently provide multiple years of infertility in all or even a high proportion of animals (Powers et al. 2014, Baker et al. 2013). In all studies, where post-mortem examinations were performed, prevalence of injection-site inflammation and granulomas were present but in some species, such as white-tailed deer and elk, they were not apparent antemortem (Curtis et al. 2008, Powers et al. 2011, Gionfriddo et al. 2011*b*).

In contrast to these species, injection site reactions in feral horses, following GonaCon vaccination at THRO, are readily observable as subcutaneous swellings. In past studies at THRO (2009-2013), all injection site reactions appeared to be confined to the general gluteus muscle where the vaccine was first hand-injected. Reactions to the vaccine were first observed 30 days post-treatment in 17.2% (5/29) of mares and by the second breeding season, 79.3% (23/29) of treated females showed some evidence of inflammation or swelling at the injection site. Saline control mares displayed no evidence of injection site reactions. Swellings of various sizes (marble to baseball size) were most common, followed by nodules, and rarely a draining abscess. Most of these reactions were observable for three years post-treatment, then began to resolve and become less visible by year 4 (many that could not be visually observed were still manually palpable at the 2013 roundup).

However, similar to other studies where injection site reactions have been evaluated, we did not observe any clinical evidence of lameness, impaired mobility, depression, or decreased health or fitness in any animal that was associated with GonaCon vaccine treatment. While results from the above investigations are generally consistent relative to the effects of GonaCon-induced injection site reactions, they are also limited to the consequences of a single vaccination usually delivered by hand-injection.

At the 2013 THRO round-up, GonaCon –treated mares were re-vaccinated, four years post-primary vaccination, with a booster dose on the opposite side in the biceps femoris muscle. This investigation is in progress but thus far, injection site reactions appear to be less apparent than those observed following the 2009 vaccination (Baker et al. unpublished). At this time, the cumulative effects of re-vaccination are unknown and the potential for more intense immune reactions with additional doses of this vaccine delivered by syringe dart is a consideration (Broderson 1989, Roelle and Ransom 2009).

4. Behavioral Side-Effects

Behavioral side-effects of GonaCon vaccination in wild ungulates have not been extensively investigated (Gray et al. 2010, Baker et al. 2012, Ransom et al. 2014). Given the physiological mechanism of action, GonaCon vaccine has the potential to suppress fertility and diminish the reproductive behaviors typically associated with estrus. However, in GonaCon-vaccinated female elk (Powers et al. 2011) and free-ranging horses (Gray et al.

2010, Baker et al. 2012, Ransom et al. 2014) such behaviors were maintained throughout the first breeding season after immunization and were not different from untreated females.

In a previous study at THRO during 2009-2010, daily activity patterns, social interactions, and reproductive behaviors were similar for GonaCon treated and control mares (Baker et al. 2012, Ransom et al. 2014). But, since GonaCon only prevented conception in 50% of treated mares (n = 28), behavioral observations were limited to only 14 infertile females. Thus, rigorous quantitative investigation into the potential effects of GonaCon treatment on feral horse behavior is missing from the assessment of this immunocontraceptive as a potential management tool. Inferences to free-ranging feral horse populations are, therefore not definitive and deserve further investigation.

In an attempt to further our understanding of the behavioral side-effects GonaCon vaccine, we conducted behavioral observations during the first breeding season (2014) following re-vaccination of these same mares at THRO in 2013. We measured the effects of this vaccine on sociosexual behavior, harem dynamics, and activity budgets of treated (n = 25) and control (n =25) horses. To date (July 20 2015), none of the re-vaccinated mares have foaled, whereas 84% (21/25) of the control mares have done so. As a result of higher vaccine efficacy in treated mares, our sample size increased by 44% and offered a more thorough investigation into potential effects of GonaCon treatment on feral horse behaviors. We postulated that based on the assumed mechanism of action of GonaCon that re-vaccination would suppress reproductive behaviors in treated females compared to controls.

5. Population Modeling

We will integrate contraceptive efficacy and population monitoring data at THRO to estimate parameters and unobserved states in a Bayesian hierarchal model (Dulberger et al. 2010, Monello et al. 2014, Hobbs and Hooten 2015, Hobbs et al. 2015, Rahio et al. in review).We will use the model to evaluate the population-level effects of GonaCon on the free-ranging horse population at THRO. We will forecast the consequences of alternative contraceptive strategies on population performance with rigorous evaluation of uncertainty. There is an urgent need to extend studies of efficacy of individuals to populations (Ransom et at. 2014). A key extension of our experimental research is to determine the effects of different GonaCon delivery regimes on the growth rate of the THRO population.

OBJECTIVES:

The primary objectives of this research are:

a) To begin to determine the optimum and most effective re-vaccination schedule with GonaCon vaccine for suppressing reproductive rates in free-ranging horses, the duration of effectiveness, and the return to fertility following treatment.

b) To determine the safety and physiological side-effects (if any) in feral horses following re-vaccination with GonaCon including visual assessment of general health, body condition, injection site reactions, effects on current pregnancy, and neonatal health and survival.

c) To determine the effects of GonaCon vaccination on the behavioral side-effects (if any) in free-ranging horses including quantitative assessment of the effects on daily activity patterns and social interactions.

d) To develop and test a safe and effective dart configuration and injection system for remotely administering GonaCon vaccine to free-ranging horses by means of a syringe

dart.

e) To develop a Bayesian model to forecast the consequences of different GonaCon vaccine treatments on feral horse population dynamics at THRO.

HYPOTHESIS:

H1: Female feral horses re-vaccinated with GonaCon will show significantly ($P \leq 0.05$) lower reproductive (yearly pregnancy and foaling) rates than non-treated control mares and contraceptive efficacy of re-vaccinated mares will be greater and longer lasting than that observed following the initial immunization.

Rationale: An immune response is a physiologic reaction to a foreign substance or antigen; especially one mediated by lymphocytes and involving recognition of antigens by specific antibodies or previously sensitized lymphocytes. Vaccines rely on the anamnestic response for optimal function. This response is a renewed rapid production of antibodies on the second (subsequent) encounter with the same antigen. This reaction is possible through memory cells that store information regarding the recognition of an antigen based upon previous exposure. Booster or repeat vaccinations generally result in a more rapid and stronger immune reaction to a second inoculation with the same antigen (Tizard 1982). However, the optimum re-vaccination schedule for GonaCon vaccine in feral horses or any other ungulate species has not yet been investigated or determined.

2. Technical Approach:

(Describe how the project will be conducted. The project design must contain enough detail to show the development of the project, including the relationship between the partners, milestones, and objectives. Clearly describe the techniques, procedures, and methodologies to be used; the data collection, analysis, and means of interpretation; the expected results and/or outcomes; and the procedures for evaluating project effectiveness, including appropriate performance measures and the probabilities of obtaining them.)

EXPERIMENTAL DESIGN AND METHODS

Study area and experimental horses

Theodore Roosevelt National Park (THRO) is located near the town of Medora in southwestern North Dakota (45º 55’ N/103º 31’W) and consists of two units that are separated by approximately 115 km of federally and privately owned rangeland. The South Unit of the park, where this study will be conducted, comprises 19,000 ha and consists of eroded badlands with gullies and ravines separated by upland plateaus and small erosion-resistant buttes (Laird 1950). All feral horses used in these experiments are free-ranging and permanently reside in this unit of the park.

At present, there are approximately170 horses divided into roughly 10-15 individual bands and bachelor groups. Horses and bison are confined to the South Unit by a 1.8 to 2.4-m woven wire boundary fence. Feral horse history, distribution, habitat use, and population management at THRO have been previously described (Marlow et al. 1992). Individual horses are known by unique markings and band affiliations. Age and reproductive genealogy data for each animal has been retained in a database since 1993. The approximate date of birth (± 30 days) is known for each horse. Photographs have been taken of each mare from birth to adulthood to assist in the

identification of individual horses.

Experimental treatments

In order to determine the optimum re-vaccination schedule for GonaCon vaccine in free-ranging horses at THRO, we propose four post-primary vaccination treatment intervals of: a) four years, b) two years, c) one year, and d) six months (Table 1). The numbers of experimental treatments are limited by the availability of adult mares currently residing in the park. All experimental mares participating in these experiments have been assimilated into various bands such that each band contains one or more individuals from these treatment groups as well as untreated control mares.

Table 1. Summary of primary and secondary vaccination schedules and sample sizes for each experimental group of feral horses treated with GonaCon Immunological Vaccine or saline at THRO.

RE-VACCINATION TREATMENT	SAMPLE SIZE (N)	DATE OF PRIMARY VACCINATION	DATE OF SECONDARY VACCINATION
FOUR YEARS POST-PRIMARY	25	OCT - 2009	SEPT - 2013
TWO YEARS POST-PRIMARY	11	SEPT - 2013	SEPT - 2015
ONE YEAR POST-PRIMARY	16	SEPT - 2015	SEPT - 2016
SIX MONTH POST-PRIMARY	16	SEPT - 2015	MAR - 2016
SALINE CONTROL	25	OCT – 2009	SEPT - 2013

A description of each treatment group, the method of treatment application, and pertinent measurements and observations are presented below:

1) Four-year post-vaccination group. This experimental group was initially established and treated during the scheduled roundup at THRO in 2009. Ongoing measurements of foaling rates and biological side-effects following re-vaccination in 2013 are currently being conducted and will provide a four-year post-primary re-vaccination treatment group (n = 25) and control group (n = 25).

Experimental animals and treatment application: During a scheduled NPS gather and removal in September 2013, horses were herded by helicopter into permanent corrals and handling facilities. Fifty, adult mares (5-19 years of age) (25 GonaCon -treated: 25 saline-control) that had been previously vaccinated with a single inoculation of GonCon- or saline solution in October 2009 were identified and retained within the park for this experiment. Band stallions were also retained. All mares were identified individually using a photographic data base of pelage color and band association, as well as, previously implanted passive integrated transponder (PIT) tags. General health, pregnancy status, and body condition of each animal was assessed while horses were restrained in a hydraulic squeeze chute. Pregnancy status and approximate stage of gestation were determined using rectal palpation of the reproductive tract and transrectal ultrasound imaging (Bucca et al. 2005). Up to 50 mls of blood was collected and serum removed, frozen, and archived for future anti-GnRH antibody analyses (Powers et al. 2011). We collected

hair samples from all horses to assess the genetic status of the population and fecal samples for pregnancy determination and prevalence of endoparasites. Body condition of mares was assessed and scored visually according to methods described by Henneke et al. (1983). Mares in the treatment group received an intramuscular booster inoculation, by hand-syringe, containing 2000μg (2 ml) of GonaCon (synthetic GnRH conjugate Blue Carrier protein and emulsified in AdjuVac™ adjuvant (Miller et al. 2008) in the middle gluteus muscle on the opposite side from the primary vaccination. Mares in the control group were injected in the same way with an equal volume of saline solution. These treatments and procedures were identical to the ones used in 2009 except that injections were given on the right side of the body in 2013 rather than the left to allow differentiation from the previous injection site.

2) Two-year post-vaccination group. This vaccine treatment was applied at the 2013 scheduled roundup at THRO to investigate remote delivery of GonaCon vaccine. Re-vaccination of these mares in 2015 will provide a two-year post-vaccination treatment group.

Experimental animals and treatment application. Based on the promising results from the captive trial conducted in 2013, we will extend our evaluation of a remote dart delivery system of GonaCon from a controlled captive setting to a field test with these same mares that are now free-roaming in their respective bands at THRO. This field application will also provide an additional cohort of mares that have been re-vaccinated two years post-primary vaccination. During September 2015, the eleven mares that were previously administered a primary dose of GonaCon vaccine by means of syringe dart delivery, will be located in the park and re-immunized using the same dart configuration and delivery ballistics as that used for the captive trials in 2013. Each dart will be numbered and correspond to an individual mare. We will determine darting efficacy by measuring the precise dose of the vaccine delivered to each mare. This will be done by weighing each dart (± 0.01g) before and after injection. We will measure dart retention time in each animal and dart performance (i.e. failure rate, partial discharge, blow-out, bounce)**.** In the case of darts that fail to discharge or partially inject the vaccine, the animal will be re-darted until the full dose has been delivered. We will also record each animal's behavioral response to dart injection.

3) One year post-vaccination group and 4) six-month post-vaccination group. Including these two additional re-vaccination treatments will hopefully allow us to more clearly define the optimum re-immunization schedule for GonaCon vaccine in feral horses. However, we have no prior immunological evidence to support these time periods as being optimum or different from each other. These intervals were selected primarily on the basis of practical field application of the vaccine. It would generally be infeasible to locate and treat horses via remote dart delivery during the winter months (December-February) at THRO. Therefore, shorter time periods such as three months (which was the minimum time required for maximum antibody production in elk) (Powers et al. 2011) are not practical. Re-vaccination of mares at the 6 month interval will be conducted in March 2016 and for mares in the one-year interval group during September 2016.

Experimental animals and treatment application. Thirty-two free-ranging mares (1.5-3.5 years of age) will be selected for these treatment groups. A randomized complete block design consisting of either a one year or six-month GonaCon- re-vaccination group will be used in this analysis. Mares will be paired on the basis of age and pregnancy status such that animals within each block (n = 16 blocks of 2 mares each) will be as similar as possible. Within each pair, a mare will be randomly assigned to each experimental group. The general health, pregnancy status, and body condition of each mare will be determined in the field by trained biologist familiar with these animals. Pregnancy status will be determined by fecal estrogen assay (Baker et al. unpublished data). Body condition of all study mares will be evaluated visually and scored on a scale of 1 (very thin) to 9 (very fat) (Henneke et al. 1983). During September 2015, all 32 mares will receive a primary

vaccination with GonaCon vaccine via remote dart delivery. Approximately 6 months (March 2016) following the initial vaccination, 16 mares will be re-vaccinated with GonaCon and 1 year later (September 2016) the remaining 16 mares will be similarly treated. All horses will receive the re-vaccination treatment using remote dart delivery.

Field Measurements:

***Effects on reproduction*.** We will determine the effectiveness, duration of effects, and reversibility of a second immunization with GonaCon on reproduction during 2015-2020 (or beyond, if necessary) by comparing foaling and pregnancy rates of treated and control mares. Annual foaling rates will be estimated by observing all mares, at least weekly, during the breeding season (April – August) and documenting the presence of new foals and estimating approximate date of birth. We will continue to monitor reproductive rates in all experimental mares during 2015-2020 or until the magnitude of the difference in foaling rates between treatment and control mares is less than 50% or funding is no longer available. Supplementary to foaling rates, we will also collect fecal samples during approximately mid-gestation (October-February) and determine fecal estradiol concentrations to estimate pregnancy rates of all mares (Baker et al. unpublished data).

***Biological side-effects*.** In conjunction with the above measurements, we will assess the safety and side effects of a second immunization with GonaCon. In both treatement and control groups of horses, we will evaluate the effects (if any) on general health, body condition, existing pregnancy, neonate survival and injection site reactions at weekly intervals during the breeding season and opportunistically throughout the year. In addition, we will observe all experimental mares for presence or absence of lameness (limping, gait alteration, reluctance to stand or bear weight, and evidence of swelling or discharge) at the site/side of vaccine injection. We will classify injection site reactions into four categories according to the scoring system of Roelle and Ransom (2009). Both the previous injection site in 2009 and the one in 2013 will be evaluated each year in conjunction with foaling observations.

Behavioral side-effects. **We evaluated the effects of GonaCon vaccine on the daily activity patterns and social interactions of the four-year post vaccine group during March – August 2014. We used a restricted randomized design to balance observations as much as possible among all experimental animals while also trying to observe the behavior of each mare at least 6-8 times per month. We located bands containing selected mares by vehicle, foot, or horseback. Observations were balanced across time of day and conducted from distances of 50-100m with the aid of binoculars and spotting scopes. Each sampling period consisted of 20 min of continuous observation. We used a combination of instantaneous scan sampling procedure to record time budget data and all-occurrence sampling to record reproductive behaviors (Altmann 1974). We followed field and analytical methods described by Ransom and Cade (2009) to develop a herd-specific ethogram for selected behaviors at THRO. We will compare behavioral observations of GonaCon-treated mares and control mares the first breeding season following primary vaccination in 2010 and following re-vaccination in 2013. Statistical analysis of data followed those described by Ransom et al. (2014). Analysis of these behavioral data will be completed during spring of 2016 and a draft manuscript will be submitted to a peer-reviewed journal for publication (Ransom et al., in preparation).**

Statistical analysis

Our power analysis was originally developed for the four-year post-treatment group but offers an approximation of statistical power needed to detect a treatment effect for other

treatments as well. We used a fixed sample size of available mares (n = 50, equally divided into 2 groups of 25 each), to estimate statistical power (1-β) for detecting a treatment effect (0.9 – 0.2) over time. We then used a 1-sided, two-sample t-test with a normal approximation together with software program SYSTAT 12.02.00 (SYSTAT Software, Inc.) to estimate the power for detecting effect sizes that vary from 0.20-0.90 (Kang and Kim 2004) (Table 2). Our current 2-year mean effect- size (difference between mean foaling rates in treatment [0.485] and control [0.759] groups) is 0.274. If repeat vaccination does not improve contraceptive efficacy, we will have little power to detect a difference between treatment groups and will conclude there is little effect due to re-vaccination. However, if revaccination increases effect size to 0.6 or better we will have sufficient power to detect these effects.

We will determine the efficacy of re-vaccination treatments by comparing the proportion of fertile females in each treatment group with control females in the original four-year post-vaccination group combined across all foaling seasons. Females will be classified as being fertile, or infertile on the basis of the presence of a foal at heel, or fecal estrogen concentrations indicating pregnancy. We will use a linear mixed model analysis with restricted maximum likelihood estimation to determine treatment effects on fertility rates. A chi-squire test will be used to test for differences among fertility rates, foal survival, and seasonality of births. We define the foaling season to include March, April, May, June, and July. Results will be shown as means ± standard errors when appropriate.

We will also explore using Bayesian beta-bimodal (similar to the one used by Monello et al. 2014 to estimate elk survival) to examine the size of treatment effects. Power will be less of an issue in this approach because we will be able to show the probability distribution of differences attributable to treatment.

Table 2. Power calculations and corresponding contraceptive treatment effect size for the GonaCon field experiment with free-ranging mares at Theodore Roosevelt National Park.

Total Sample Size	Group Sample Size	THRO Foaling Rate	Effect Size	Alpha	Power (1-β)
50	25	0.759	0.9	0.1	0.977
50	25	0.759	0.8	0.1	0.949
50	25	0.759	0.7	0.1	0.898
50	25	0.759	0.6	0.1	0.817
50	25	0.759	0.5	0.1	0.706
50	25	0.759	0.4	0.1	0.570
50	25	0.759	0.3	0.1	0.425
50	25	0.759	0.2	0.1	0.290

Limitations in study design

One difficulty in this study is that, to our knowledge, there are no published data regarding the optimum re-vaccination schedule for GonaCon vaccine in horses or any other wild or domestic ungulate. Thus, while we may have adequate sample sizes to detect treatment differences between GonaCon-treated and control groups, our sample sizes may be inadequate to detect small differences among the four post-primary treatment groups. This limitation is due to the restricted availability of additional female horses at THRO for this experiment.

Moreover, the control group of mares used to compare treatment effects in this study was originally selected in 2009 to be as similar as possible to the four-year re-vaccination group.

However, it is not necessarily representative of the re-vaccinated mares selected for the subsequent treatments. If this study was implemented in captivity, more appropriate control groups could have been established. Additionally, a more complex study design that incorporated different vaccination time-points and regimes could have more accurately determined the optimal time point for re-vaccination.

Our study was implemented to compliment practical management efforts at THRO that are determined by having reasonable access to study horses for treatment application. Regardless of efficacy outcome, this study will provide valuable information. If re-vaccination at these intervals is not successful, our study will provide important information on the utility of this vaccine. If it is successful, the vaccine may have more wide-spread utility than previously observed.

Performance Measures and Reporting:

2015 - 2016

1. Collect and summarize four-year post-primary vaccination foaling rate estimates for GonaCon-treated mares and control mares for the 2015 and 2016 foaling seasons.

2. Collect and summarize data pertinent to foaling rates and side-effects of GonaCon-treated mares for the two-year post-primary vaccination group for the 2015 and 2016 foaling seasons.

3. Select and document successful re-vaccination of mares in the two-year post-primary vaccination group (11 mares) and primary vaccination of mares in the one-year (16 mares) and six month (16 mares) post-vaccination groups (September 2015).

4. Document successful re-vaccination of mares in the six month revaccination group during March 2016 and for the one-year group in September 2016.

5. Compare foaling rates on all vaccination schedules to their pregnancy rates estimated via fecal estrogen analysis.

6. Provide data analysis summarizing the effects of GonaCon vaccine on daily activity patterns and social interactions of feral horses at THRO during 2015-2016.

BUDGET

Table3. Yearly budget, by category, for proposed research at Theodore Roosevelt National Park 2015-2020.

Category	Year 1	Year 2	Year 3	Year 4	Year 5
Personnel	$40,898	$29,300	$29,878	$34,847	$67,473
Fringe benefits	$7,626	$5,866	$5,991	$7,033	$15,722
Travel	$3,003	$2,946	$1,964	$1,964	$1,964
Equipment	$ 0	$ 0	$ 0	$0	$ 0
Supplies	$4,550	$1,950	$1,950	$1,950	$1,950
Other	**$** 0	$ 0	$ 0	$1,000	$5,000
Direct costs	$56,077	$40,062	$39,783	$46,794	$92,109
Indirect costs	$9,813	$7,011	$6,962	$8,189	$16,119
Total costs	$65,890	$47,073	$46,745	$54,983	$108,228

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3. Qualifications, Experience, and Past Performance:

(Describe who will carry out the project activities. List all project personnel, including consultants, contractors, sub-recipients, etc., if known. Describe their responsibilities and the amount of time each will dedicate to the project. Briefly describe how their experience and qualifications are appropriate to successfully achieve the stated objectives.)

Dan L. Baker, Affiliate Faculty, Research Scientist, Colorado State University, Department of Biomedical Sciences/Animal Reproduction and Biotechnology Laboratory: will coordinate all project activities, study design, data collection and analysis, personnel management, reporting, interagency coordination. Dr. Baker has been the project leader in the evaluation of GonaCon in feral horses at Theodore Roosevelt National Park (THRO) since 2009. Prior to that (2006-2013) he was involved with similar research with this contraceptive vaccine in captive and free-ranging elk in Rocky Mountain National Park (ROMO) (50%).

Jenny G. Powers, Wildlife Veterinarian, National Park Service: attending veterinarian, assist with study design, and assessment of biological side-effects of GonaCon vaccine. Dr. Powers has been involved with the evaluation is this contraceptive agent at THRO since 2009 and was

involved in similar research with captive and free-ranging elk in ROMO. Much of her previous research has been focused on the efficacy and physiological side-effects of various contraceptive agents. She will also facilitate animal care and use approval from NPS for this project.

Blake E. McCann, Wildlife Biologist, National Park Service, Theodore Roosevelt National Park: liaison and on-site project manager at THRO, study design, will lead efforts in dart delivery of GonaCon in free-ranging horses, will provide in-kind support for this research effort (i.e. vehicles, office space, housing for field technicians) and coordinate research activities with ongoing NPS operations. Dr. McCann has been involved with the evaluation of GonaCon since 2013 has been instrumental in the design and evaluation of a GonaCon-specific dart configuration and ballistic system for feral horses.

N. Thompson Hobbs, Professor, Senior Research Scientist, Colorado State University (CSU), Department of ESS, Natural Resource Ecology Laboratory: will lead efforts to model effects of fertility control on feral horse population dynamics; provide statistical analysis of data, and coordinate administrative services and support for this project within NREL. Dr. Hobbs has been involved with several projects modeling the effects of fertility control on wild ungulates. He is currently working on a Bayesian state-space model of population dynamics of white-tailed deer to evaluate alternatives for population management including fertility control (5%).

Jason E. Bruemmer, Professor, Colorado State University, Department of Animal Science, Equine Reproduction Laboratory: provide technical expertise on reproductive physiology of feral horses, study design, interpretation of data, and manuscript preparation. Dr. Bruemmer has been involved with this investigation since 2009 and has provided pregnancy assessment of experimental mares at the 2009 and 2013 roundups. We have incorporated his mare pregnancy criteria and body condition scoring system into our field measurements.

Terry M. Nett, Professor, Colorado State University, Department of Biomedical Sciences, Animal Reproduction and Biotechnology Laboratory: provide laboratory services for fecal estrogen assay. Dr. Nett has been involved with this research project since 2009, as well as, similar research with this vaccine in captive and free-ranging elk and domestic horses. He is a leading authority on reproductive endocrinology and GnRH metabolism in mammals (1%).

Kathleen M. Eddy, Laboratory and field research technician, Colorado State University, Department of Biomedical Sciences Animal Reproduction and Biotechnology Laboratory: Lead responsibility for developing and validating a fecal estrogen assay for pregnancy determination in horses; this assay will supplement foaling rate measurements to assess pregnancy status and treatment responses in experimental mares at THRO. In addition, she will assist with fecal collections and other field measurement (5%).

Douglas C. Eckery, Senior Scientist and Project Leader, USDA, APHIS, Wildlife Services, National Wildlife Research Center: will be primarily responsible for providing 100- 2ml doses of GonaCon-Equine vaccine packaged in 3ml plastic syringes for this study.

APPENDIX

Institutional Animal Care and Use Permits

G. HUMANE CARE AND USE OF ANIMALS

BLM Wild Horse and Burro Program
Proposal for Collaborative Research Effort / Grant Application

Privileged Communication

Title of proposal: Evaluation of Re-Immunization with GonaCon-Equine™ on Reproduction and Side-Effects in Feral Horses

Investigators: Baker, Dan L.; Nett, Terry M.; Powers, Jenny G; Ransom, Jason I; Bruemmer, Jason E; Hobbs, N. Thompson; McCann, Blake E.

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) and the "Animal Welfare Act of 1966" (PL 89-544) as amended.

Signature: _____ Date 4/30/14
Terry Engle, Ph.D., Chair, CSU Institutional Animal Care and Use Committee

Name of Institution: Colorado State University

NOTE: This completed form must be in receipt of the BLM WH&B Research Advisory Team before the initiation of funding or collaborative work can commence. Private individuals must seek local/regional institutional approval.

United States Department of the Interior
NATIONAL PARK SERVICE
Biological Resource Management Division
1201 Oakridge Drive, Suite 200
Fort Collins, Colorado 80525

National Park Service
Institutional Animal Care and Use Committee
Animal Research Protocol Approval

Principal Investigator(s): Dan Baker/ N. Thompson Hobbs
Telephone: 970.556.8518
Electronic Mail: danbaker@colostate.edu

Region: Midwest Region

Protocol Approval Number: MWR_THRO_Baker_Horse_2013.A3
Project Title: Remotely-delivered GnRH Vaccine (GonaCon-Equine) in Free-Ranging Horses: A Preliminary Investigation

Approval Date: 9/23/2013

Effective Date: 9/23/2013

Questionnaire Dates; Years 1 and 2 (if applicable): 9/23/2014, 9/23/2015

Expiration/Re-Submittal Date: 9/23/2016

Funding Agency(ies): None

Species: Horse (*Equus caballus*)

Number(s) of Animals: 10 horses/year, 30 total horses over three years

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This project study was reviewed by the National Park Service Institutional Animal Care and Use Committee. The following action(s) were taken:

Project Status: Approved

Midwest Region/ Intermountain Region/ NPS IACUC Chair: Dan Licht /s/, Mike Wrigley /s/, John Bryan /s/