BIOLOGY OF REPRODUCTION **85,** 1152–1160 (2011)
Published online before print 13 July 2011.
DOI 10.1095/biolreprod.110.088237

Effects of Gonadotropin-Releasing Hormone Immunization on Reproductive Function and Behavior in Captive Female Rocky Mountain Elk (*Cervus elaphus nelsoni*)[1]

Jenny G. Powers,[2,4] Dan L. Baker,[4] Tracy L. Davis,[3,4] Mary M. Conner,[5] Anneke H. Lothridge,[4] and Terry M. Nett[4]

Animal Reproduction and Biotechnology Laboratory,[4] Colorado State University, Fort Collins, Colorado
Department of Wildland Resources,[5] Utah State University, Logan, Utah

ABSTRACT

Fertility control is a potential method for managing overabundant wildlife populations; however, current technology is limited by duration of treatment efficacy and unacceptable side effects. The objective of this study was to determine the efficacy of a single immunization with gonadotropin-releasing hormone (GnRH) vaccine to suppress reproductive function in pregnant female elk and to evaluate potential behavioral and pathological side effects of treatment. Eighteen captive adult female elk were randomly allocated to one of two experimental groups. Ten females were administered a conjugated and adjuvanted GnRH vaccine intramuscularly, and eight elk received an adjuvant sham vaccine without conjugated GnRH. We compared success of existing pregnancy, neonatal survival, subsequent fertility, reproductive behavior rates, and side effects of treatment between January 2006 and January 2010. The GnRH vaccination did not affect existing pregnancy or calf survival during the year that it was applied; however, it reduced the proportion of pregnant females for 3 yr. Male precopulatory behavior rates exhibited toward GnRH-vaccinated females tended to be greater than those directed at sham-vaccinated females during the second half of the breeding season, when GnRH vaccinates continued to be proceptive. Strong immune and inflammatory responses, including robust GnRH antibody concentrations in GnRH vaccinates, and sterile pyogranulomatous injection site abscesses in both groups, were consistent with vaccination. In conclusion, this GnRH vaccine resulted in prolonged, albeit reversible, impairment of fertility, and is associated with extended reproductive behaviors and partial suppression of hypothalamic-pituitary-gonadal axis function in captive female elk.

elk, fertility control, follicular development, GnRH/GnRH receptor, gonadotropin-releasing hormone, immunocontraception, immunology, ovary, pregnancy, reproductive behavior

INTRODUCTION

Regulating the abundance of elk populations has become a significant issue for natural resource managers in many areas of North America [1]. This is particularly true for protected environments, such as national parks and conservation areas, where unregulated populations, if left unchecked, can have adverse effects on natural and human-dominated systems. Hunting, culling, and trapping have traditionally been used to regulate animal numbers, but there are a growing number of circumstances where these methods pose significant liability [2], and as a result resource managers are seeking alternative approaches to population control [3].

Fertility control offers a potential nonlethal method for controlling the growth of overabundant elk populations, and considerable research has been directed toward the development of different contraceptive technologies [4, 5]. Models have been developed to characterize effects of fertility control on population dynamics of wild ungulates, including elk [6]. Yet, current technologies for altering wildlife fertility suffer from a variety of technical, physiological, and regulatory challenges [4]. As a result, only modest successes have been achieved in developing a safe, practical, and feasible method of controlling reproduction in free-ranging wild ungulate populations.

A potential alternative to current fertility control products, and one that may overcome some of these challenges, involves active immunization against gonadotropin-releasing hormone (GnRH), a small, 10-amino acid neuropeptide produced in the hypothalamus that signals via receptors on gonadotroph cells in the anterior pituitary. Gonadotropin-releasing hormone, along with ovarian- and pituitary-derived endocrine, paracrine, and autocrine signaling, is responsible for gonadotroph function [7, 8], and ultimately gametogenesis [9]. Gonadotropin-releasing hormone is not generally immunogenic but can be made so by conjugation to a large, highly immunogenic carrier protein. When combined with a potent adjuvant, this vaccine stimulates a persistent immune response, resulting in prolonged antibody production against GnRH, the carrier protein, and the adjuvant [4]. Although there are competing theories of action [10], the prevailing hypothesis suggests that antibodies to GnRH induce transient infertility by binding to endogenous GnRH in the hypothalamic-pituitary portal vessels, thus preventing attachment to receptors on gonadotrophs, as well as suppression of pulsatile luteinizing hormone (LH) secretion. Gonadotropin-releasing hormone-antibody titers are correlated with suppression of the reproductive system and infertility in a variety of species [4].

Numerous GnRH vaccines have been developed and are successful in interrupting the hormonal cascade that controls ovulation. These vaccines have been used in physiologic research for decades. However, the use of GnRH vaccines as contraceptive agents in wildlife management has been limited by effective duration, multiple treatments to stimulate adequate antibody response, and deleterious side effects associated with the use of the controversial Freund complete adjuvant (FCA) [4, 5]. Recently, an alternative adjuvant (AdjuVac) containing

[1]Supported by Morris Animal Foundation grant D05ZO-303.
[2]Correspondence: Jenny Powers, 1201 Oakridge Dr., Suite 200, Fort Collins, CO 80521. FAX: 970 225 3585;
e-mail: Jenny_Powers@nps.gov
[3]Current address: University of Idaho, Department of Animal and Veterinary Science, Moscow, ID 83844.

Received: 29 August 2010.
First decision: 6 October 2010.
Accepted: 7 July 2011.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

mineral oil and mycobacteria derived from a Johne disease vaccine (Mycopar; Fort Dodge Animal Health, Fort Dodge, IA) has been developed and may be as effective as FCA but is associated with fewer lesions [11].

Based on results from studies in captive and free-ranging wild or feral ungulates, a single application of this GnRH vaccine (GonaCon-B) may prove to be a safe and effective multiyear immunocontraceptive agent for free-ranging wildlife. A single application, formulated with the same or a similar GnRH-protein conjugate, has been shown to provide multiple years of decreased fecundity in several ungulate species, including elk [4, 5, 12]. However, few studies have rigorously investigated the secondary effects of this vaccine on social behaviors or ecological consequences in any wildlife species [5].

Elk are polyestrus short-day ovulators, with the peak of breeding season in North America between mid-September and mid-October [13]. Waves of follicle development occur during the ovulatory [14], anovulatory [15], and transitional [16] seasons. Ovulation occurs approximately every 20–21 days [14], and the ovulatory season can persist as late as April, although it is, on average, 142 days, ending in mid-February [16]. Pregnancy rates for mature females (2–12 yr) can approach one calf per female per year in populations that are not forage limited [17]. Approximately 50% of yearling females become pregnant at 16–18 mo of age if an appropriate body mass is achieved (~220 kg, 10% body fat) [13, 17, 18]. Reproductive senescence is not well described, but fertility may decline by 13–17 yr of age [17]; however, effects of body condition are likely more important to fertility than age [18, 19]. Elk are monotocous, and twinning is exceptionally rare [13]. Calving occurs from late May to early July after a gestation of 247–262 days and coincides with spring nutrient flush in most northern latitudes [13, 20].

Application of GnRH vaccine would be most practical during the winter period (December–February), when elk are concentrated in large, primarily single-sex herds [21], and temperatures and snow cover are conducive to chemical or physical capture [22]. At this time, most adult female elk are in midgestation to late gestation, and any contraceptive treatment must be demonstrated to be safe for the developing fetus and health of the neonate [23].

In light of these issues we examined the contraceptive efficacy and potential side effects of GnRH vaccination by testing the following four hypotheses using captive female elk. A single immunization with GnRH vaccine during midgestation in elk will: 1) not affect existing pregnancy; 2) suppress future fertility, with contraceptive effects waning as GnRH antibody concentrations decrease; 3) suppress reproductive behaviors; and 4) be associated with localized inflammatory reactions but not other pathological side effects.

MATERIALS AND METHODS

Animals and Vaccine

This study was reviewed and approved by the Colorado State University (no. 05-187A-01) and the Colorado Division of Wildlife (no. 07-2005) Institutional Animal Care and Use Committees. Animals were housed at the Colorado Division of Wildlife, Foothills Wildlife Research Facility, in Fort Collins, CO (40°35′46″N, 105°9′29″W). Eighteen female elk (1.5–12 yr of age at study onset; 220–275 kg) and two reproductively proven mature male elk (5–7 yr of age; 400–450 kg) were used for this experiment. The majority of experimental elk were long-term residents of the facility, most having been born in captivity. To meet sample size requirements, two free-ranging 3-yr-old females from a local wild population were captured and brought into the captive herd in the spring of 2005. Female elk were trained for repeated handling in isolation pens, alleyways, and a handling chute, and for blood sampling and ultrasound imaging procedures. Female elk were maintained throughout the experiment in two fenced paddocks (5.0 ha) with minimal native forage and were fed a diet of ad libitum alfalfa-grass hay mix, trace mineral blocks, and water, as well as limited pelleted grain supplement. Male elk were similarly maintained in a paddock, physically removed but within sight of females. During breeding seasons (late September–late November), males were maintained with females. All biological samples were collected and hands-on measurements made while female elk were lightly sedated using xylazine hydrochloride (30–250 mg per animal, i.m.; TranquiVed; Vedco Inc., St. Joseph, MO) in a nonsqueeze chute. Tranquilizer effects were reversed after each sampling session with either yohimbine hydrochloride (30 mg per animal, i.v.; Wildlife Pharmaceuticals, Fort Collins, CO) or tolazoline hydrochloride (600 mg per animal, i.m.; Tolazine; Akorn Inc., Decatur, IL).

Experimental vaccines were prepared as previously described [24]. Briefly, the GnRH vaccine consisted of multiple copies of synthetic GnRH peptide linked to hemocyanin protein (Blue Carrier; Biosonda, Santiago, Chile) from the Chilean mollusk (*Concholepas concholepas* [CCH]) and combined with a water-in-oil adjuvant containing killed *Mycobacterium avium* ssp. *avium*. The sham vaccine was similarly prepared but without conjugated GnRH.

Experimental Protocol

Data were collected between January 2006 and January 2010. At the start of the experiment, all 18 females were pregnant (80–100 days), as determined by serum pregnancy-specific protein B assay [25], rectal palpation [26, 27], and/or transrectal ultrasound [28]. One sham-vaccinated female (20 mo of age) aborted her pregnancy by late February 2006.

Approximately half of the females had been previously exposed to a *Brucella abortus* strain 19 vaccine. To avoid potential confounding anamnestic response effects, which may arise from antigenic stimulation with similar intracellular pathogens (*B. abortus* and *M. avium*) [29] or age-related fertility effects [17], we blocked sample units (female elk) with respect to brucella vaccination history and age (1, 2–10, or ≥11 yr of age) and then randomly assigned animals to either the GnRH vaccine (n = 10) or sham vaccine (n = 8) group. The GnRH-vaccinated females were administered 1.5 mg of GnRH-Blue Carrier protein conjugate with adjuvant (1.5 ml) into the left biceps femoris muscle using a handheld 3-ml syringe and a 3.8-cm, 18-gauge needle. Sham-vaccinated females received a similar volume of carrier protein and adjuvant but without conjugated GnRH. Injection site location was similar in all animals, with placement consistently at the leading edge of the cream-colored rump patch and at the same height as the tuber ischii. To facilitate pretreatment ultrasound examination as described below, hair was removed from the injection site and surrounding skin using electric clippers with a no. 40 blade, and the area was cleaned using isopropyl alcohol.

Females were divided into two pens (each pen with five GnRH vaccinates and four sham vaccinates) and placed in separate paddocks. In late September of each year (2006–2009) one male was placed in each pen for 62–65 days. Females remained in the same pen throughout the study, whereas males were rotated between pens each year to minimize effects due to potential random differences in male fertility and individual male-female interactions.

Pregnancy and Ovarian Measurements

We investigated the effects of GnRH immunization on the existing corpora lutea, pregnancy, and calving (2006) by comparing monthly (January–May 2006) serum progesterone concentrations, calving success, and calf survival and growth. Calves were weighed 12–24 h after birth, and then at 2- to 4-wk intervals for the first 3 mo of life. Calves remained with their dams until weaning on 1 September 2006. Subsequent pregnancy rates were determined each January during a 4-yr period (2007–2010) using methods described above. Once pregnancy was confirmed, abortion was induced using two doses of prostaglandin $F_{2\alpha}$ 6 h apart (25 mg, i.m.; Lutalyse; Pharmacia & Upjohn, Kalamazoo, MI) [30, 31].

We measured ovarian follicular and luteal structures in GnRH- and sham-vaccinated females at single time points the first year after vaccination during the early ovulatory (mid-September), late ovulatory (early January), transitional (late February), and anovulatory (late April) seasons using transrectal ultrasound imaging (5-MHz linear array transducer; Televet 1000; Classic Universal Ultrasound, Tequestra, FL) [14–16]. January imaging occurred coincident with pregnancy diagnosis; at all other time points females were not pregnant. Images were saved on a laptop computer for later evaluation. We measured the total number of antral follicles, follicular diameter (mm), and the presence or absence of ovarian structures consistent with luteal tissue. We then grouped follicles into small (<4 mm), medium (4 to <7 mm), and large (≥7 mm) classes, and calculated total follicular volume combining data from both ovaries for each individual.

Analysis of Hormone Concentrations and Antibody Titer

Blood samples (10–30 ml) were collected via jugular venipuncture using a 20-gauge blood collection needle, tube holder, and 10-ml blood tubes without anticoagulant (BD Vacutainer SST; Becton Dickinson and Co., Franklin Lakes, NJ). Blood was allowed to clot at room temperature and was centrifuged for 10 min at 1500 × *g*, and serum was decanted to polypropylene tubes and stored at −80°C until assays were performed. Monthly (January–May 2006) serum concentrations of progesterone were measured by radioimmunoassay [32], a technique previously validated in elk [33]. All samples were run in duplicate in a single batch. The range of the standard curve was 0.39 ng/ml (80% ligand-labeled progesterone) to 15.0 ng/ml (20% ligand-labeled progesterone). Intraassay coefficients of variation were 14.1% for the low reference sample and 7.1% for the high reference sample.

Serum GnRH antibodies were measured prior to vaccination, monthly until calving, and prior to introducing males into female pens each year (September 2006–2009). The concentration of unbound GnRH antibodies was estimated by measuring ^{125}I-GnRH binding capacity in peripheral serum [34, 35]. Serum samples were diluted between 1:2 and 1:100 000 using 0.05 M ethylenediaminetetraacetic acid (EDTA) in 0.01 M PBS with 0.1% gelatin (EDTA-PBS gel). A total of 200 μl of diluted test sera, 100 μl of EDTA-PBS gel, and 100 μl of ^{125}I-GnRH (specific activity ~1850 Ci/mmol; ~60 000 dpm) were added to 5-ml glass tubes. Solutions were vortexed and incubated for 24 h at 4°C. One milliliter of 20% polyethylene glycol solution (6000 mw; diluted with PBS) was added, and tubes were vortexed. Tubes were incubated for 20 min at 4°C, then centrifuged for 30 min at 980 × *g*. Supernatants were decanted and tubes placed in a gamma counter (efficiency ~80%; Micromedic Systems, Horsham, PA) to record radioactivity (cpm). Total radioactivity was measured in 100 μl of ^{125}I-GnRH. Nonspecific binding was measured by adding 100 μl of ^{125}I-GnRH to 300 μl of EDTA-PBS gel and handling similarly to test samples. High antibody titer-positive control sera from rabbits [35] and elk previously vaccinated against GnRH (data not shown) as well as negative control elk sera were included with each batch. All samples were analyzed in duplicate. To provide a similar comparison between animals, we selected the 1:1000 dilution to estimate antibody concentration (pmol/ml) and reanalyzed all samples in a single batch. The intraassay coefficient of variation was 3.5%. Gonadotropin-releasing hormone antibody response is reported as picomoles of ^{125}I-GnRH bound per milliliter of serum.

Pathological Side Effects

Mean serum chemistry and hematology parameters, *M. avium* antibody status, and prevalence of injection site reactions were compared between groups. Blood was collected as described above but with the addition of a 10-ml blood tube with EDTA anticoagulant for hematology. Samples were taken prior to vaccination for each assay and then at 30, 90, and 340 days after vaccination for hematology, chemistry, and *M. avium* assays, respectively. Whole blood and serum were submitted to the Colorado State University Veterinary Diagnostic Laboratory (Fort Collins, CO) for analysis. Hematology parameters measured included: total nucleated cells, segmented neutrophils, lymphocytes, monocytes, eosinophils, platelets (all $\times 10^3$/μl), plasma protein (g/dl), red blood cells ($\times 10^6$/μl), hemoglobin (g/dl), packed cell volume (%), mean corpuscular volume (fl), mean corpuscular hemoglobin concentration (g/dl), and fibrinogen (mg/dl). Chemistry profile parameters included: glucose, creatinine, phosphorus, calcium, magnesium, total protein, albumin, globulin, and total bilirubin (all mg/dl); enzymes, including creatinine kinase, aspartate aminotransferase, gamma-glutamyltransferase, sorbitol dehydrogenase (each IU/L); and electrolytes, including sodium, potassium, chloride, and bicarbonate (each mEq/L). *Mycobacterium avium* antibody concentrations were measured using ELISA (HerdChek ELISA; IDEXX Laboratories, Westbrook, ME) and reported as optical density of the sample.

We used palpation and ultrasonography to evaluate injection sites for evidence of localized inflammation prior to vaccination, at monthly intervals until calving (February–May 2006), and prior to each breeding season (September 2006–2009). Injection sites and surrounding skin were shaved as described above. Qualitative changes in skin temperature, erythema, and swelling were assessed by comparing the injection site with adjacent skin. We used ultrasonography to evaluate muscle tissue for subcutaneous and intramuscular signs of inflammation and injury, including lesions such as hematoma, abscess, scar tissue, cellulitis, and myositis, which are associated with characteristic changes in muscle echogenicity and architecture [36–38]. Longitudinal and transverse ultrasound images of skin, fat, fascia, and muscle at the site of injection were collected using a 5-MHz linear array transducer to a depth of 80 mm with a focal zone at 40 mm and were stored on a computer for future analysis. Ultrasound images were described to indicate qualitative degree of change between prevaccination and postvaccination sampling dates in both muscle echogenicity (e.g., hyperechogenic, hypoechogenic) and architecture (e.g., fiber length, fiber pattern). All ultrasound evaluations were performed and read by a single technician. When images were consistent with a well-defined abscess accessible by percutaneous needle aspiration or if a soft, fluid-filled swelling was palpated, it was aspirated, and aerobic, including mycobacterial, cultures and anaerobic cultures were performed. Two abscesses were lanced, drained, and flushed because the welfare of the animal was a concern.

Each animal was observed for the presence or absence of lameness (e.g., limping, gait alterations, reluctance to stand or bear weight on a limb) each time it was moved from pens to the handling chute. Additionally, caretakers observed each female daily during feeding and noted if swelling or discharge was evident at the site of injection and if there were overt signs of lameness.

During the course of the experiment, three GnRH-vaccinated females died because of handling difficulties (n = 1; February 2006) or chronic wasting disease (n = 2; August 2007). Chronic wasting disease is endemic within the facility and was diagnosed in study animals using immunohistochemistry of rectal mucosa-associated lymphoid tissue, retropharyngeal lymph nodes, and brainstem at the level of the obex [39, 40]. Complete necropsies were performed by a veterinary pathologist, and injection sites along with standard tissues (e.g., representative tissues of each organ system) were removed and preserved in 10% neutral-buffered formalin for histopathological evaluation.

Reproductive Behaviors

We compared rates of reproductive behavior interactions (behavior events per hour) of male and female elk from 25 September–12 November 2006 as previously described [33]. Individually identifiable numeric/color-coded collars were placed on each female in both treatment groups. Behaviors were observed during the morning (0400–0600 h), evening (1600–2000 h), and at night (2200–2400 h) from a tower that provided good visibility of both pens. An infrared night vision monocular was used to observe behaviors during dark hours, and a spotlight was used to confirm collar identification. Four technicians similarly trained in behavior identification and blinded to treatment group performed observations. Based on previously reported elk reproductive behaviors, we identified and recorded 13 different interactions associated with harem tending, proceptivity, receptivity, and mating (Table 1) [21, 33, 41, 42]. Only male-female interactions were recorded. All behaviors were attributed to the individual female displaying or receiving the behavior. If the male displayed behavior directed at more than one female (e.g., herd tending) it was recorded as a separate behavior for each female. Because of small sample size, we grouped these specific behaviors into four general behavior categories: general breeding, male precopulatory, female precopulatory, and copulatory (Table 1). A total of 112 two-hour sampling periods were recorded in 47 days during 38 morning (74.1 h; 35%), 43 evening (81.4 h; 38%), and 31 night (58.5 h; 27%) sampling periods. Length of behavioral interactions was typically short compared with sampling interval, so each interaction was recorded as an event. Behavior rates were calculated as events per female per hour, and estimated mean weekly behavior rates were compared between treatment groups.

Statistical Analysis

Yearly pregnancy data are reported as the proportion of pregnant female elk in each treatment group (number of pregnant females in each treatment group/number of females exposed to bulls from late September to late November each year). Pregnancy proportions were compared with one-tailed Fisher exact tests. The difference in proportions was used to estimate treatment effect. We used normal approximations to binomial distributions to compute confidence limits for the differences between proportions.

To evaluate the probability of pregnancy for a given GnRH antibody concentration, we used simple logistic regression with pregnancy status, breeding season, and treatment group as classification variables, and GnRH antibody concentration as the continuous variable (PROC PROBIT; SAS 9.1; SAS Institute Inc., Cary, NC). The probability of pregnancy after treatment with the GnRH vaccine was calculated for a theoretical sample population with an intrinsic pregnancy rate of 1.0 calf per female per year, and for this study's sham-vaccinated control population, which had an intrinsic pregnancy rate of 0.96 during the four experimental years. Using a cutoff value of $\geq$20 pmol GnRH antibody per milliliter of serum to indicate infertility, we estimated the type 1 (false-positive) and type 2 (false-negative) error rates by calculating the specificity (proportion of correctly classified nonpregnant females) and sensitivity (proportion of correctly classified pregnant females) of using antibody concentration as a diagnostic indicator of pregnancy status. Specificity was calculated as the number of true nonpregnant animals indicated by the antibody concentration divided by the total number of nonpregnant females. Sensitivity was calculated as the number of truly pregnant females indicated by the antibody concentration divided by the number of total number of pregnant females. Pregnancy data were combined over 4 yr for specificity and sensitivity calculations.

TABLE 1. Thirteen individual reproductive behaviors and associated behavior categories observed and recorded 8 to 10 mo after immunization in GnRH-vaccinated and sham-vaccinated elk (*Cervus elaphus nelsoni*).

Behavior category	Reproductive behaviors
General breeding	Male behavior related to establishing, maintaining, and defending a group or harem of female elk (i.e., herding, guarding).
Male precopulatory	Male courtship behavior directed toward an individual female to induce or detect estrus (i.e., Flehmen for urine testing, chivy, sniff and/or lick body, rub body, precopulatory mount).
Female precopulatory	Female courtship behavior directed toward dominant male to arouse copulatory behavior (i.e., sniff and/or lick body, rub body, circle, mount, lordosis).
Copulatory	Male behavior directed toward a receptive female (i.e., intromission).

We tested the hypothesis that mean behavior rates between treatment groups were different using a mixed linear ANOVA model to account for both random (individual animal) and fixed (treatment, date, male, and time of day) effects in a repeated-measures structure (PROC MIXED; SAS 9.1). For each of the four behavior categories, a global model was constructed which included treatment group, time of day, and male, along with all of their first-order interactions. Date was included as an additive trend factor. Next, variance structures, heterogeneous versus homogeneous both within and between individual females, were added to the model, and Akaike Information Criterion adjusted for small sample size (AICc) was used to select the best fit model. Similarly, three covariance structures—none, compound symmetry, and spatial power—were analyzed, and AICc was used for model selection. Finally, after fitting variance and covariance structures, six reduced models were run to find the most parsimonious model to estimate treatment effects. Using the best model, mean behavior rates (±SEM) were estimated using least squares analysis, and hypothesis tests were based on type III generalized estimating equations, which account for sample size imbalance.

Similar methods were used to compare mean (±SEM) concentrations of progesterone, as well as ovarian follicle number, size, and volume. Fixed effects in the analyses were treatment status and date, and individual females were evaluated as a random effect. Differences in calf weight during the first 3 mo of life were analyzed in the same way, with dam treatment status, sire, sex, and age as fixed effects. Differences in proportions of small-, medium-, and large-size follicles were analyzed using a chi-square test with treatment status and date as classification variables (PROC CATMOD; SAS 9.1). A paired Student *t*-test (PROC TTEST; SAS 9.1) was used to evaluate differences in mean optical density readings from *M. avium* ELISA. Descriptive statistics were used to explain the similarity of hematology profiles, presence or absence of luteal tissue during early ovulatory and transitional periods, and occurrence of lesions at the site of injection.

RESULTS

Pregnancy and Calving

Gonadotropin-releasing hormone vaccination did not affect calving success in female elk treated at approximately 80–100 days of pregnancy. Serum progesterone concentrations during the second half of gestation did not differ between treatment groups ($P = 0.849$; Fig. 1). Progesterone concentrations varied by month ($P < 0.001$), but there were no treatment by month interactions ($P = 0.619$). All females, except the single 20-mo-old sham vaccinate that had midgestation pregnancy loss, delivered full-term calves. One calf born to a GnRH-vaccinated dam died during parturition due to dystocia. All calves born alive (n = 15) survived the neonatal period and were weaned at approximately 3 mo of age prior to the 2006 breeding season. Dam vaccination exposure did not affect calf weight at any time point prior to weaning ($P = 0.448$; data not shown).

GnRH Vaccine Efficacy and Duration

Pregnancy proportions in GnRH-vaccinated females were lower ($P \leq 0.05$) than in sham-vaccinated females during the first 3 yr of the experiment (Table 2). Treatment effect decreased between Year 1 and Year 4 ($P = 0.009$), from a high of 0.90 in 2007 to a low of 0.12 in 2010 (Table 2). Antibody concentrations in GnRH-vaccinated elk were detectable 1 mo after inoculation, peaked 3–8 mo after vaccination, and waned during the course of the study (Fig. 2). Gonadotropin-releasing hormone antibodies were not detectable in sham-vaccinated females (data not shown). There was a strong inverse relationship between GnRH antibody concentration and the probability of becoming pregnant ($P < 0.001$; Fig. 3). At 30 pmol/ml free GnRH antibody binding capacity in the peripheral serum, the logistic model predicted a pregnancy rate of 0.10 in a population whose intrinsic pregnancy rate approached 1.0 (Fig. 3). Only one GnRH-vaccinated female with lower than 20 pmol/ml concentrations of antibody (approximately 2–17 pmol/ml) did not become pregnant during the first three breeding seasons. Conversely, one female with consistently high serum antibody concentrations (>20 pmol/ml) did become pregnant during the third and fourth breeding seasons. Using a GnRH antibody concentration of 20 pmol/ml during September as the cutoff point, the assay had a sensitivity of 0.85 and specificity of 0.87 to predict whether a female elk would be infertile during the current breeding season.

Reproductive Behaviors

General breeding and male precopulatory behaviors were the most prevalent interactions recorded. Copulatory behaviors were observed too infrequently for meaningful analysis. Although differences in mean male precopulatory behavior rates only approached significance ($P = 0.073$), those directed toward GnRH-vaccinated females (0.45 ± 0.06 behaviors per hour) were 30% greater than those directed toward sham-

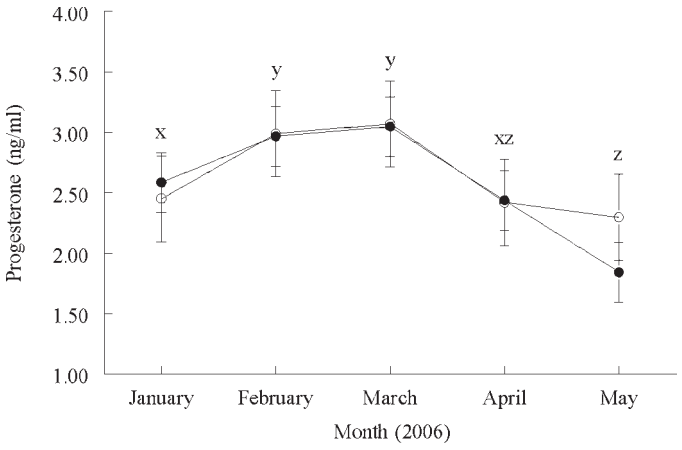

FIG. 1. Mean ± SEM monthly serum progesterone concentrations in GnRH-vaccinated (filled circles; n = 10) and sham-vaccinated (open circles; n = 7) female elk (*Cervus elaphus nelsoni*) between the time of GnRH immunization (January 2006) and parturition (May–June 2006). Means with different letters indicate differences between months ($P < 0.05$).

TABLE 2. Mean yearly pregnancy proportions (no. pregnant/no. exposed to fertile bull) and estimates of treatment effect size (difference in proportions) with 95% confidence intervals for GnRH-vaccinated and sham-vaccinated female elk (2006–2010).

	Proportion pregnant*		Treatment effect
Yr after treatment†	GnRH-vaccinated [n]	Sham-vaccinated [n]	Difference (95% CI)
0	1.0 [10]^{ax}	1.0 [8]^{ax}	0.0
1	0.10 [10]^{by}	1.0 [7]^{ax}	0.90 (0.71–1.0)
2	0.25 [8]^{byz}	1.0 [7]^{ax}	0.75 (0.50–1.0)
3	0.50 [8]^{byz}	1.0 [7]^{ax}	0.50 (0.15–0.85)
4	0.75 [8]^{az}	0.86 [7]^{ax}	0.12 (0.0–0.29)

* Proportions with different superscripts are significant ($P \leq 0.05$). Letters a and b are between treatment groups within a given year; letters x, y, and z are between years.
† Year 0, 2006 (before treatment); Year 1, 2007; Year 2, 2008; Year 3, 2009; Year 4, 2010.

vaccinated females (0.33 ± 0.06 behaviors per hour). This response became apparent after the initial 2 wk of observation (Fig. 4). Individual males were a significant covariate in male precopulatory behavior ($P = 0.002$), as were date and time of day ($P < 0.001$), and there was an interaction between bull and time of day ($P < 0.001$). Female precopulatory behaviors were not different between treatment groups ($P = 0.720$); however, precopulatory behavior of GnRH-vaccinated females persisted throughout the sampling period, whereas this behavior rate dropped to nearly zero in sham-vaccinated females after the first 3 wk of observation (Fig. 4). Date tended to be an important covariate in female precopulatory behavior ($P = 0.055$). There were no differences in mean general reproductive behavior rates between treatment groups ($P = 0.794$). Time of day and date were significant ($P < 0.001$) covariates for general reproductive behaviors.

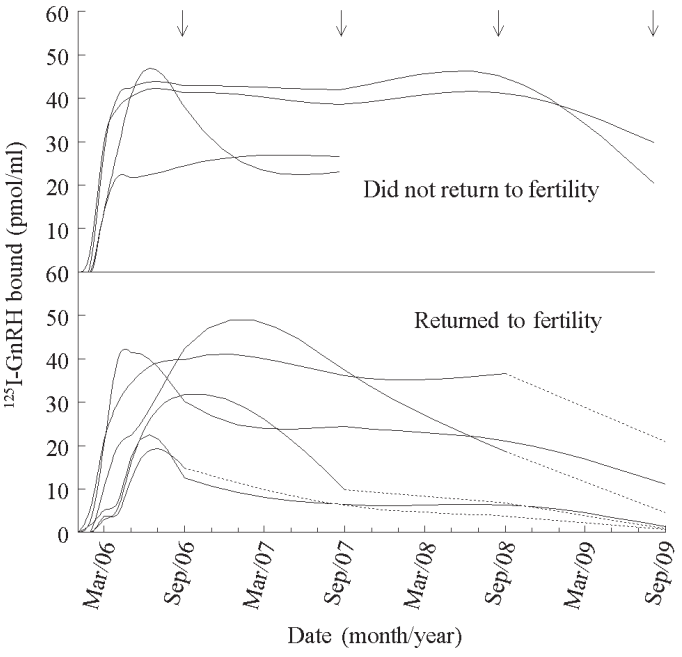

FIG. 2. Persistence of GnRH antibodies measured via ^{125}I-GnRH binding capacity in peripheral blood of GnRH-vaccinated female elk. Top panel shows antibody concentrations in females that did not become pregnant after vaccination (n = 4 of 10) throughout the 4-yr study. Two animals were lost from the study after Year 2. Lower panel shows antibody concentrations from females that either did not experience infertility (n = 1 of 10) or returned to fertility during the study (n = 5 of 10). Solid lines indicate nonpregnant females. Dotted lines indicate pregnant females. Two females became pregnant after antibodies were measured in September 2009. Arrows indicate date when males were placed in female pastures (September 2006–2009).

Pathological Side Effects

Most biochemistry and hematology parameters were within clinically normal ranges for all elk [13]. Two females, one from each treatment group, demonstrated leukocytosis (22×10^3 to 26×10^3 nucleated cells per microliter) 1 mo after injection, which was attributable to neutrophilia (9×10^3 to 12×10^3 per microliter) and lymphocytosis (9×10^3 to 11×10^3 per microliter). The GnRH-vaccinated female demonstrated mild hyperfibrinogenemia (300 mg/dl) and thrombocytopenia (57×10^3 per microliter). An additional four GnRH-vaccinated females had mild hyperfibrinogenemia (300–400 mg/dl). Although all globulin levels were within the clinically normal range (1.9–4.3 mg/dl), serum globulins increased between 0.1 and 0.6 mg/dl at 4 mo after inoculation in every female in both groups.

All animals were seronegative for Johne disease prior to vaccination. One year after injection *M. avium avium* ssp. *paratuberculosis* (MAP) antibody concentrations tended to be greater than zero ($P = 0.059$) in both treatment groups. With one exception, all females showed an increase in MAP antibody concentrations, indicated by an increase in optical density, and 3 (18%) of 17 females had sufficiently robust responses to be classified as seroconverted and Johne disease positive.

Estimated mean (±SEM) number of follicles was greater in GnRH-vaccinated females (5.6 ± 0.44) than sham-vaccinated females (2.46 ± 0.48; $P = 0.005$). Mean size of all imaged follicles in GnRH vaccinates (2.35 ± 0.29 mm; range, 0.3–12.6 mm) was smaller than that of sham vaccinates (4.73 ± 0.84 mm; range, 1.2–16.9 mm; $P = 0.014$). There was no difference in total follicular volume between groups ($P = 0.240$). Date was not a significant covariate ($P \geq 0.05$) in any of the analyses, and there were no date by treatment interactions. The GnRH-vaccinated females had more small follicles (<4 mm) but fewer medium (4 to <7 mm) and large (≥7 mm) follicles ($P < 0.001$) than sham vaccinates. Corpora

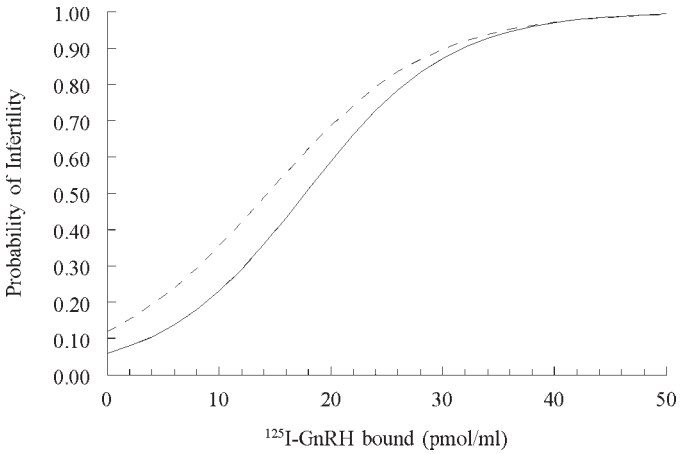

FIG. 3. Predicted relationship between ^{125}I-GnRH binding capacity (antibody concentration) and the probability of infertility in GnRH-vaccinated female elk, modeled with (dashed line; probability of pregnancy if untreated = 0.96) and without (solid line; probability of pregnancy if untreated = 1.0) intrinsic infertility.

lutea were observed in 6 (27%) of 22 images collected from sham-vaccinated females during early ovulatory and transitional season periods. In contrast, corpora lutea were never observed in GnRH-vaccinated females during the same time periods. Luteal tissue was not observed in any study animal during anestrus.

Between 15 and 52 mo after injection, 6 (35%) of 17 females (n = 4 GnRH vaccinated and n = 2 sham vaccinated) developed clinically apparent abscesses (e.g., large soft swelling with purulent material) at the site of injection. No aerobic, including mycobacterial, or anaerobic bacterial growth was detected in cultures (n = 4). Two of the four GnRH-vaccinated animals described above died of causes unrelated to vaccination (i.e., chronic wasting disease) 19 mo after vaccination. Both possessed large (~500 ml), purulent, multiloculate, encapsulated abscesses at the site of injection. Histopathology revealed pyogranulomatous inflammation and multiple acid-fast bacilli within the capsule and surrounding muscle tissue. The acid-fast bacilli were consistent with mycobacteria. We did not observe lameness at any time point in study animals.

Muscle architecture and/or echogenicity was altered in 10 (~60%) of 17 females (n = 5 from each treatment group). The earliest changes in echogenicity were observed 2 mo after injection; however, the most severe changes, including images consistent with abscessation were seen 5 to 30 mo after vaccination. Regardless of treatment group, there was a wide variation in severity of change, from muscle fiber disruption and diffuse fiber hyperechogenicity to large, hypoechogenic multiloculated areas within the muscle or between fascial planes. All of the animals with clinical abscesses had evidence of muscle fiber disruption in one or more ultrasound images prior to observing external manifestations of the abscess.

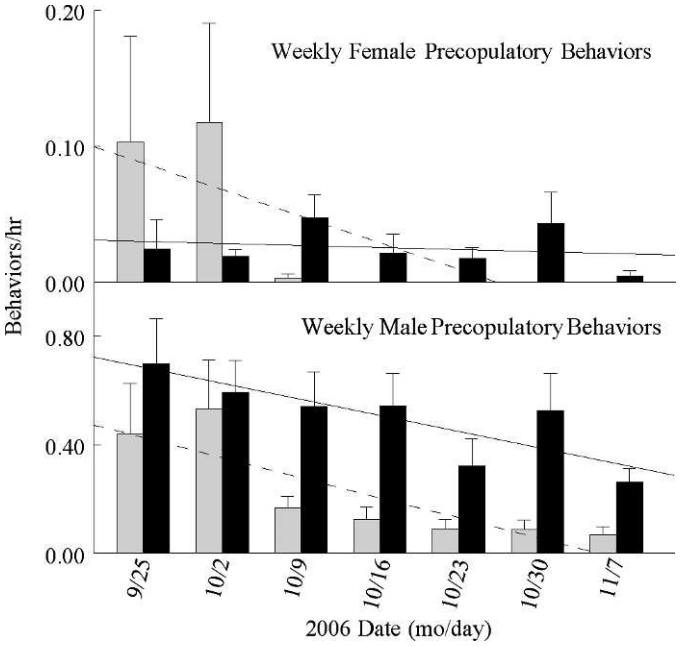

FIG. 4. Mean ± SEM weekly female precopulatory behavior rates demonstrated by GnRH-vaccinated (black bars and solid lines; n = 10) and sham-vaccinated (gray bars and dashed lines; n = 7) female elk (top panel) and mean weekly male precopulatory behavior rates received by female elk (n = 10 GnRH vaccinated; n = 7 sham vaccinated; bottom panel) during the 2006 breeding season (25 September–12 November). Regression lines illustrate persistence of precopulatory behaviors demonstrated and received by GnRH- and sham-vaccinated females.

DISCUSSION

A single vaccination against GnRH during midgestation did not disrupt pregnancy but did decrease the proportion of pregnant female elk for 3 yr after treatment. The efficacy of the GnRH vaccine decreased each year following treatment. These findings supported our predictions that this GnRH vaccine delivered to pregnant female elk reduced fertility, with decreasing efficacy over time. These findings were also consistent, in part, with those reported for nonpregnant female elk treated with GnRH vaccine using keyhole limpet hemocyanin as the carrier molecule rather than CCH [12]. However, in contrast to our measurements, Killian et al. [12] showed a weak inverse relationship between contraceptive efficacy of the vaccine and antibody levels: increasing effectiveness with decreasing antibody titers. Their result is difficult to explain biologically in light of our observations and others [24, 43] that support a strong positive association between infertility and antibody levels. Their finding may be an artifact of small sample size. Despite the association between antibody titer and contraception, we did not identify a GnRH antibody concentration cutoff that predicted contraceptive effects with high sensitivity and specificity. The most useful diagnostic assays have wide separation between negative and positive cutoff values [44, 45]. Because there was overlap in pregnancy status at given antibody concentrations, GnRH antibody concentration as a diagnostic test may be a good indicator of herd or population level of infertility, but a less reliable predictor of individual animal fertility.

The wide variation in antibody concentrations observed in our study was not surprising, given that individual humoral responses to a foreign antigen are known to be influenced by many physiological factors, including nutrition, previous exposure to the same or similar antigens, age, persistence of the antigen, current immune stimulation by other immunogens, and genetics [44]. We attempted to control for the first four potentially confounding factors; however, the latter two factors were not measured or controlled and may have influenced individual responses. Of more interest was the persistence of GnRH antibody concentrations during the course of 4 yr in GnRH-vaccinated animals. Most commercially available vaccines require an initial series of two or three vaccinations with annual revaccination to maintain significant serum antibody concentrations [44]. Previous wildlife immunocontraceptive vaccines have required similar vaccination strategies [46–48]. Only recently have single-dose applications been effective at inducing long-term antibody production and corresponding infertility [43, 49, 50]. It has been suggested that a combination of depot effect produced by a nonbiodegradable oil in water-based emulsion along with an optimized concentration of immunostimulatory killed mycobacteria is responsible for the prolonged antibody effect in GnRH-vaccinated deer [24, 51]. Our finding of extensive localized inflammation at the site of injection nearly 4.5 yr after vaccination supported this hypothesis of a depot effect, particularly given the retained and apparently dead mycobacteria within the sterile lesions. Additionally, *M. avium* antibody concentrations that were often sufficiently elevated to indicate disease status seroconversion supported the assertion that a generalized and robust humoral immune response to the vaccine was critical to its efficacy.

Both GnRH-vaccinated and sham-vaccinated groups had follicles ranging in size from less than 2 mm to more than 12 mm in diameter during the early ovulatory, transitional, and anestrous seasons. Additionally, during transitional seasons ultrasound evidence of luteal tissue was only observed in sham-

vaccinated females. Although we did not intensively measure follicular development and document ovulation, these data suggested that 12–18 mo after vaccination with GnRH, at least some females had large preovulatory-sized follicles that likely did not ovulate. The range of follicular size was consistent with earlier data from untreated elk in the transitional and anestrous periods [15, 16]. Although a full range of follicular sizes was observed in our study, mean follicular size was smaller but antral follicles were more numerous in GnRH-vaccinated compared with sham-vaccinated females. Our observations are similar, although less extreme, than those of Seekallu et al. [34]. Those authors found complete cessation of follicular waves and development of large-sized follicles in domestic sheep after a series of two vaccinations against GnRH. Differences in experimental methodology may account for this inconsistency. Our measurements were made 12–18 mo after immunization after a single vaccination, whereas they made measurements 26–66 days after booster vaccination. Their more intensive vaccination and monitoring schedule may explain the more complete hypothalamic-pituitary-gonadal axis suppression observed.

Our data indicated fewer follicles developed to the preovulatory stage, which was likely due to incomplete gonadotropin support. Attenuation of follicle development into large antral stages likely resulted in less negative feedback from dominant follicles secreting inhibin and estradiol. An increase in small follicle recruitment due to less negative feedback from dominant follicles and early regression due to incomplete gonadotropin support could account for the increase in numbers of small follicles observed in GnRH-vaccinated elk. Although follicles were on average smaller in GnRH-vaccinated females, antral follicle development persisted and suggested that gonadotropins, particularly follicle-stimulating hormone (FSH), continued to stimulate early follicular development. Although LH secretion is closely regulated by GnRH [52, 53], FSH synthesis is only partially controlled by the interaction of GnRH with its receptor [54–56]. The primary regulator of FSH synthesis is negative feedback from estradiol and inhibin, and secretion is primarily constitutive [54, 56]. It has been suggested that a component of basal LH secretion is similarly free from GnRH regulation [57]. In the current study, antral follicle development was consistent with FSH stimulation and possibly basal LH signaling, albeit at a decreased level. Absence of observed luteal tissue in GnRH-vaccinated females was consistent with a probable lack of sufficient estradiol to initiate an LH surge and ovulation. Experiments to intensively measure individual animal follicular dynamics and concurrent gonadotropin concentrations are required to test these speculative hypotheses.

Contrary to our prediction, reproductive interactions during the breeding season were not eliminated in vaccinated females. Estrous behavior in domestic ruminants is influenced not only by the presence or absence of progesterone and estradiol, but also the concentration and timing of these steroid hormones [58]. Continuous, high concentrations of progesterone (e.g., during pregnancy) usually inhibit expression of estrous behavior regardless of estradiol concentrations [58]. In contrast, sexual receptivity can be triggered by estradiol alone in most species, albeit often in the pharmacological range, as long as progesterone is not inhibitory [59, 60]. In this study, despite the apparent absence of ovulation, both female attractiveness, measured by male precopulatory behaviors, and female proceptivity, measured by female precopulatory behaviors, were maintained throughout the first breeding season after immunization in GnRH-vaccinated, and consequently nonpregnant, animals.

Interestingly, domestic male cattle will spend equal time with young nulliparous females in estrus or in the luteal phase, finding them equally attractive [61]. Male elk may similarly investigate nonpregnant females regardless of estrous status. A combination of low progesterone due to lack of ovulation, and the presence of limited estradiol due to altered follicular development, may have contributed to the continued expression of precopulatory behaviors in female elk. These mechanisms could account for pregnancy status being a stronger indicator of reproductive behavior than vaccination status. Because we observed too few copulatory behaviors to evaluate differences between treatment groups, it is unclear if vaccinated females were receptive to copulation or were only displaying proceptive behaviors. Because GnRH-vaccinated animals did not likely experience progesterone priming prior to estradiol exposure, which is important for the display of full estrous behaviors in domestic sheep [60, 62], we speculate that copulation failed to occur in nonpregnant GnRH-vaccinated animals. Regardless, it is apparent that in the relatively small captive environment of this experiment, with intensive male-female interactions, GnRH vaccination was associated with persistent breeding behaviors. Although these observations may be an artifact of captivity, the consequences in free-ranging populations are unclear but could be significant and deserve further investigation into potential ecological consequences prior to use in a management application.

In accordance with our hypothesis regarding physiological side effects, GnRH vaccination did not significantly affect blood chemistry or hematology parameters, but it did result in considerable injection site reactions. Two females showed evidence of a systemic inflammatory response, and four additional animals had mildly elevated fibrinogen, an indicator of inflammation. Every animal had increased serum globulins. These findings indicated a robust immune response to both the GnRH conjugate and sham vaccines. Abscesses were confirmed in 35% of females, which was consistent with others using a similar formulation of this vaccine in white-tailed deer [43, 63]. However, ultrasound changes indicative of myositis, trauma, and abscessation [36–38] suggested a higher incidence of injection site inflammatory lesions. It was unclear whether the pyogranulomatous inflammation at the site of injection would have resolved with time. Evaluation of the long-term physiological consequences of injection site reactions in animals treated with this vaccine will be essential for managers making choices based on animal welfare concerns.

In addition to antibodies produced in response to GnRH, antibodies were also produced to other elements of the vaccine. Mycobacteria and their cellular components are particularly immunostimulatory [64]. Antibodies produced in response to *M. avium* ssp. *avium* appear to cross-react well with commercial assays for Johne disease, a gastrointestinal disease of domestic cattle and occasionally wild ruminants, such as elk [65]. Vaccination with GonaCon-B and/or the adjuvant components increased mycobacterial antibodies in nearly every animal and induced clinical seroconversion in 18% of animals. Managers concerned with regulatory issues or Johne disease management should be aware of this finding prior to using this vaccine in free-ranging ungulates.

In conclusion, a single vaccination during midgestation with the described GnRH vaccine decreased pregnancy rates for 3 yr after treatment without compromising the existing pregnancy. This result extended potential practical use of contraception by providing multiple years of decreased fertility when applied to either pregnant or nonpregnant female elk. Hematology and serum chemistry parameters were typically normal, and vaccinated females appeared clinically healthy. Furthermore,

the vaccine appeared to be safe for the developing fetus and neonates born to vaccinated females. By contrast, the vaccine prolonged reproductive behaviors during the breeding season, a finding that has potential ecological effects that require further study. The most apparent pathological side effect of the vaccine was related to the development of sterile abscesses at the site of injection. Although lameness was never observed, most GnRH and sham-vaccinated females showed some level of tissue inflammation or abscess. Finally, we demonstrated that GnRH vaccination inhibited, but did not eliminate, follicular development despite the absence of pregnancy, possibly because of continued FSH secretion, indicating continued partial function of the hypothalamic-pituitary-gonadal axis. Our findings extend both the fundamental and practical understanding of GnRH vaccination in pregnant elk, which may assist wildlife managers in their pursuit of science-based population management.

ACKNOWLEDGMENTS

The authors thank the Colorado Division of Wildlife for providing the resident elk and handling facilities used in this investigation. We offer thanks to U.S. Department of Agriculture/Animal and Plant Health Inspection Service, National Wildlife Research Center (L. Miller, J. Rhyan, and K. Fagerstone) for the generous donation of the GnRH vaccine. We are particularly grateful to T. Davis, A. Mitchell, M. Fisher, J. Spaak, and I. LeVan for technical assistance in animal handling, sample collections, and husbandry. We are thankful for the many hours D. Germann, K. Kearl, and M. Paulek spent recording elk behaviors, for histopathological examination provided by T. Spraker, and for the endless logistical support provided by C. Broomfield and T. Powers. A special thanks to M. Allen and C. Magee, who trained multiple people in the art of radioimmunoassay, and to A. Graham and A. Latimer for laboratory assistance. Thank you to G. Sargeant, who provided statistical advice and improved our figures. This manuscript was significantly improved by the review and comments of M. Phillips and T. Shenk of the Colorado Division of Wildlife. A special thanks to M. Wild for supporting our pursuits of excellent research and professional development.

REFERENCES

1. Walter WD, Lavelle MJ, Fischer JW, Johnson TL, Hygnstrom SE, VerCauteren KC. Management of damage by elk (*Cervus elaphus*) in North America: a review. Wildl Res 2010; 37:630–646.
2. Curtis PD, Decker DJ, Stout RJ, Richmond ME, Loker CA. Human dimensions of contraception in wildlife management. In: Kreeger TJ (ed.), APHIS Technical Bulletin No. 1853. Washington, DC: USDA, Animal Plant Health Inspection Service; 1993:247–255.
3. Kirkpatrick JF, Turner JW. Chemical fertility control and wildlife management. Bioscience 1985; 35:485–491.
4. Fagerstone KA, Miller LA, Killian G, Yoder CA. Review of issues concerning the use of reproductive inhibitors, with particular emphasis on resolving human-wildlife conflicts in North America. Integr Zool 2010; 5:15–30.
5. Gray ME, Cameron EZ. Does contraceptive treatment in wildlife result in side effects? A review of quantitative and anecdotal evidence. Reproduction 2010; 139:45–55.
6. Bradford JB, Hobbs NT. Regulating overabundant ungulate populations: an example for elk in Rocky Mountain National Park, Colorado. J Environ Manage 2008; 86:520–528.
7. Counis R, Laverrière JN, Garrel G, Bleux C, Cohen-Tannoudji J, Lerrant Y, Kottler ML, Magre S. Gonadotropin-releasing hormone and the control of gonadotrope function. Reprod Nutr Dev 2005; 45:243–254.
8. Di Gregorio GB, Nett TM. Estradiol and progesterone influence the synthesis of gonadotropins in the absence of gonadotropin-releasing hormone in the ewe. Biol Reprod 1995; 53:166–172.
9. Hazum E, Conn PM. Molecular mechanism of gonadotropin releasing hormone (GnRH) action. I. The GnRH receptor.Endocr Rev 1988; 9:379–386.
10. Molenaar GJ, Lugard-Kok C, Meloen RH, Oonk RB, de Koning J, Wensing CJG. Lesions in the hypothalamus after active immunisation against GnRH in the pig. J Neuroimmunol 1993; 48:1–11.
11. Powers JG, Nash PB, Rhyan JC, Yoder CA, Miller LA. Comparison of immune and adverse effects induced by adjuvac and Freund's complete adjuvant in New Zealand white rabbits (*Oryctolagus cuniculus*). Lab Anim 2007; 36:51–58.
12. Killian G, Kreeger TJ, Rhyan J, Fagerstone K, Miller L. Observations on the use of GonaCon in captive female elk (*Cervus elaphus*). J Wildl Dis 2009; 45:184–188.
13. Haigh JC, Hudson RJ. Farming Wapiti and Red Deer. St. Louis: Mosby; 1993:15–64.
14. McCorkell R, Woodbury M, Adams GP. Ovarian follicular and luteal dynamics in wapiti during the estrous cycle. Theriogenology 2006; 65:540–556.
15. McCorkell RB, MacDougall L, Adams GP. Ovarian follicle development in wapiti (*Cervus elaphus*) during the anovulatory season. Theriogenology 2004; 61:473–483.
16. McCorkell R, Woodbury MR, Adams GP. Ovarian follicular and luteal dynamics in wapiti during seasonal transitions. Theriogenology 2007; 67:1224–1232.
17. Sargeant GA, Oehler MW. Dynamics of newly established elk populations. J Wildl Manage 2007; 71:1141–1148.
18. Cook RC, Cook JG, Mech LD. Nutritional condition of northern Yellowstone elk. J Mammal 2004; 85:714–722.
19. Cook R, Murray D, Cook J, Zager P, Monfort S. Nutritional influences on breeding dynamics in elk. Can J Zool 2001; 79:845–853.
20. Bubenik AB. Physiology. In: Thomas JW, Toweill DE (eds.), Elk of North America, Ecology and Managment. Harrisburg, PA: Stackpole Books; 1982:125–179.
21. Geist V. Adaptive behavioral stratagies. In: Thomas JW, Toweill DE (eds.), Elk of North America, Ecology and Management. Harrisburg, PA: Stackpole Books; 1982:219–277.
22. Kreeger TJ, Arnemo JM, Raath JP. Handbook of Wildlife Chemical Immobilization, 4th ed. Fort Collins, CO: Wildlife Pharmecuticals; 2002:80–104.
23. Kirkpatrick JF, Turner JW. Reversible contraception in nondomestic animals. J Zoo Wildl Med 1991; 22:392–408.
24. Miller LA, Gionfriddo JP, Fagerstone KA, Rhyan JC, Killian GJ. The single-shot GnRH immunocontraceptive vaccine (GonaCon) in white-tailed deer: comparison of several GnRH preparations. Am J Reprod Immunol 2008; 60:214–223.
25. Huang F, Cockrell DC, Stephenson TR, Noyes JH, Sasser RG. A serum pregnancy test with a specific radioimmunoassay for moose and elk pregnancy-specific protein B. J Wildl Manage 2000; 64:492–499.
26. Greer KR, Hawkins WW. Determining pregnancy in elk by rectal palpation. J Wildl Manage 1967; 31:145–149.
27. Hein RG, Musser JL, Bracken EF. Serologic, parasitic and pregnancy survey of the colockum elk herd in Washington. Northwest Sci 1991; 65:217–222.
28. Curran S, Pierson RA, Ginther OJ. Ultrasonographic appearance of the bovine conceptus from days 20 through 60. J Am Vet Med Assoc 1986; 189:1295–1302.
29. Lin J, Adams LG, Ficht TA. Characterization of the heat shock response in *Brucella abortus* and isolation of the genes encoding the GroE heat shock proteins. Infect Immun 1992; 60:2425–2431.
30. Asher GW, Fisher MW, Berg DK, Waldrup KA, Pearse AJ. Luteal support of pregnancy in red deer (*Cervus elaphus*): effect of cloprostenol, ovariectomy and lutectomy on the viability of the post-implantation embryo. Anim Reprod Sci 1996; 41:141–151.
31. Bates GN, Brooks JI, Call J. The use of prostaglandin to induce abortion in American elk (*Cervus canadensis*). J Zoo Anim Med 1982; 13:125–126.
32. Niswender GD. Influence of the site of conjugation on the specificity of antibodies to progesterone. Steroids 1973; 22:413–424.
33. Baker DL, Wild MA, Conner MM, Ravivarapu HB, Dunn RL, Nett TM. Effects of GnRH agonist (leuprolide) on reproduction and behaviour in female wapiti (*Cervus elaphus nelsoni*). Reprod Suppl 2002; 60:155–167.
34. Seekallu SV, Toosi BM, Ziegler A, Reeves JJ, Rawlings NC. Pulsed GnRH secretion and the FSH secretory peaks that initiate ovarian antral follicular wave emergence in anestrous ewes. Anim Reprod Sci 2010; 120:56–64.
35. Nett TM, Akbar AM, Niswender GD, Hedlund MT, White WF. A radioimmunoassay for gonadotropin-releasing hormone (Gn-RH) in serum. J Clin Endocrinol Metab 1973; 36:880–885.
36. Reimers CD, Fleckenstein JL, Witt TN, Müller-Felber W, Pongratz DE. Muscular ultrasound in idiopathic inflammatory myopathies of adults. J Neurol Sci 1993; 116:82–92.
37. Hashimoto BE, Kramer DJ, Wiitala L. Applications of musculoskeletal sonography. J Clin Ultrasound 1999; 27:293–318.
38. Chau CLF, Griffith JF. Musculoskeletal infections: ultrasound appearances. Clin Radiol 2005; 60:149–159.

39. Miller MW, Wild MA, Williams ES. Epidemiology of chronic wasting disease in captive Rocky Mountain elk. J Wildl Dis 1998; 34:532–538.
40. Spraker TR, Gidlewski TL, Balachandran A, VerCauteren KC, Creekmore L, Munger RD. Detection of PrP^{CWD} in postmortem rectal lymphoid tissues in Rocky Mountain elk (*Cervus elaphus nelsoni*) infected with chronic wasting disease. J Vet Diagn Invest 2006; 18:553–557.
41. de Vos A, Brokx P, Geist V. A review of social behavior of the North American cervids during the reproductive period. Am Midl Nat 1967; 77:390–417.
42. Morrison JA, Trainer CE, Wright PL. Breeding season in elk as determined from known-age embryos. J Wildl Manage 1959; 23:27–34.
43. Gionfriddo JP, Eisemann JD, Sullivan KJ, Healey RS, Miller LA, Fagerstone KA, Engeman RM, Yoder CA. Field test of a single-injection gonadotrophin-releasing hormone immunocontraceptive vaccine in female white-tailed deer. Wildl Res 2009; 36:177–184.
44. Tizard I. An Introduction to Veterinary Immunology, 1st ed. Philadelphia: W.B. Saunders Co.; 1982:119–208.
45. Thrusfield M. Veterinary Epidemiology, 3rd ed. Oxford: Blackwell Publishing; 2005:152–167.
46. Naugle RE, Rutberg AT, Underwood HB, Turner JW, Liu IKM. Field testing of immunocontraception on white-tailed deer (*Odocoileus virginianus*) on Fire Island National Seashore, New York, USA. Reprod Suppl 2002; 60:143–153.
47. Kirkpatrick JF, Turner JW, Liu IKM, Fayrer-Hosken R, Rutberg AT. Case studies in wildlife immunocontraception: wild and feral equids and white-tailed deer. Reprod Fertil Dev 1997; 9:105–110.
48. Curtis PD, Pooler RL, Richmond ME, Miller LA, Mattfeld GF, Quimby FW. Comparative effects of GnRH and porcine zona pellucida (PZP) immunocontraceptive vaccines for controlling reproduction in white-tailed deer (*Odocoileus virginianus*). Reprod Suppl 2002; 60:131–141.
49. Turner JW, Liu IKM, Flanagan DR, Rutberg AT, Kirkpatrick JF. Immunocontraception in wild horses: one inoculation provides two years of infertility. J Wildl Manage 2007; 71:662–667.
50. Fraker MA, Brown RG, Gaunt GE, Kerr JA, Pohajdak B. Long-lasting, single-dose immunocontraception of feral fallow deer in British Columbia. J Wildl Manage 2002; 66:1141–1147.
51. Miller LA, Fagerstone KA, Wagner DC, Killian GJ. Factors contributing to the success of a single-shot, multiyear PZP immunocontraceptive vaccine for white-tailed deer. Hum Wildl Confl 2009; 3:103–115.
52. Clarke IJ, Cummins JT. The temporal relationship between gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) secretion in ovariectomized ewes. Endocrinology 1982; 111:1737–1739.
53. Clarke IJ, Thomas GB, Yao B, Cummins JT. GnRH secretion throughout the ovine estrous cycle. Neuroendocrinology 1987; 46:82–88.
54. McNeilly AS, Crawford JL, Taragnat C, Nicol L, McNeilly JR. The differential secretion of FSH and LH: regulation through genes, feedback and packaging. Reprod Suppl 2003; 61:463–476.
55. Padmanabhan V, McNeilly AS. Is there an FSH-releasing factor? Reproduction 2001; 121:21–30.
56. Nett TM, Turzillo AM, Baratta M, Rispoli LA. Pituitary effects of steriod hormones on secretion of follicle-stimulating hormone and luteinizing hormone. Domest Anim Endocrin 2002; 23:33–42.
57. Brown P, McNeilly AS. Transcriptional regulation of pituitary gonadotrophin subunit genes. Rev Reprod 1999; 4:117–124.
58. Katz LS. Sexual behavior of domesticated ruminants. Horm Behav 2007; 52:56–63.
59. Fabre-Nys C, Gelez H. Sexual behavior in ewes and other domestic ruminants. Horm Behav 2007; 52:18–25.
60. Fabre-Nys C, Martin GB. Hormonal control of proceptive and receptive sexual behavior and the preovulatory LH surge in the ewe: reassessment of the respective roles of estradiol, testosterone, and progesterone. Horm Behav 1991; 25:295–312.
61. Geary TW, deAvila DM, Westberg HH, Senger PL, Reeves JJ. Bulls show no preference for a heifer in estrus in preference tests. J Anim Sci 1991; 69:3999–4006.
62. Robinson T. Relationship of oestrogen and progesterone in oestrous behaviour of the ewe. Nature 1954; 173:878.
63. Curtis PD, Richmond ME, Miller LA, Quimby FW. Physiological effects of gonadotropin-releasing hormone immunocontraception on white-tailed deer. Hum Wildl Confl 2008; 2:68–79.
64. Stills HF. Adjuvants and antibody production: dispelling the myths associated with Freund's complete and other adjuvants. ILAR J 2005; 46:280–293.
65. Manning EJ, Kucera TE, Gates NB, Woods LM, Fallon-McKnight M. Testing for mycobacterium avium subsp. paratuberculosis infection in asymptomatic free-ranging tule elk from an infected herd. J Wildl Dis 2003; 39:323–328.