GONADOTROPIN-RELEASING HORMONE AGONIST: A NEW APPROACH TO REVERSIBLE CONTRACEPTION IN FEMALE DEER

Authors: Baker, Dan L., Wild, Margaret A., Connor, Mary M., Ravivarapu, Harish B., Dunn, Richard L., et al.

Source: Journal of Wildlife Diseases, 40(4) : 713-724

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-40.4.713

Journal of Wildlife Diseases, 40(4), 2004, pp. 713–724

GONADOTROPIN-RELEASING HORMONE AGONIST: A NEW APPROACH TO REVERSIBLE CONTRACEPTION IN FEMALE DEER

Dan L. Baker,[1,7] Margaret A. Wild,[2] Mary M. Connor,[3] Harish B. Ravivarapu,[4] Richard L. Dunn,[5] and Terry M. Nett[6]

[1] Colorado Division of Wildlife, Research Center, 317 West Prospect, Fort Collins, Colorado 80526, USA
[2] National Park Service, Biological Resource Management Division, 1201 Oak Ridge Drive, Suite 200, Fort Collins, Colorado 80525, USA
[3] Department of Forestry, Range, and Wildlife Science, Utah State University, Logan, Utah 84322, USA
[4] SuperGen, Inc., 1059 Serpentine Lane, Pleasanton, California 94566, USA
[5] Atrix Laboratories, Inc., 2579 Midpoint Drive, Fort Collins, Colorado 80525, USA
[6] Animal Reproduction and Biotechnology Laboratory, Department of Physiology, Colorado State University, Fort Collins, Colorado 80523, USA
[7] Corresponding author (email: dan.baker@state.co.us)

ABSTRACT: Fertility control offers a potential alternative for controlling an abundance of wild ungulate populations where lethal methods are infeasible or unacceptable. A promising nonsteroidal, nonimmunologic approach to reversible contraception consists of agonist of gonadotropin-releasing hormone (GnRH). We evaluated the effects of the GnRH agonist, leuprolide, on reproduction, the suppression of luteinizing hormone (LH) and progesterone, blood parameters, and reproductive behavior in captive female mule deer (*Odocoileus hemionus)* during December 1999 through June 2001. Leuprolide, administered as a controlled release formulation (ATRIGEL®), was 100% effective in preventing pregnancy for one breeding season. Infertility was achieved by suppressing LH levels, which prevented ovulation and the formation of corpus luteum. Treated females regained normal ovarian function and conceived the following breeding season. Leuprolide had no adverse effects on blood chemistry and hematology, body weight dynamics, or the general health of treated females. In contrast to our predictions, leuprolide did not suppress estrous behavior in female deer during the "normal" breeding period, nor did treated females return to normal ovarian function and exhibit reproductive behaviors during the post-breeding period. This prolonged-release leuprolide formulation offers an alternative approach to reversible contraception in female deer that overcomes some of the problems associated with existing technology.

Key words: Contraception, GnRH agonist, leuprolide, luteinizing hormone, mule deer, reproductive behavior.

INTRODUCTION

Fertility control has been widely advocated as an innovative alternative to traditional methods for limiting the growth of some wild ungulate populations where lethal control is infeasible or unacceptable. Extensive research has been devoted to developing antifertility agents and delivery systems that are safe, effective, and economical (Fagerstone et al., 2002). To date, however, only modest successes have been achieved (Naugle et al., 2002; Shideler et al., 2002; Rutberg et al., 2004), and a practical and acceptable method for controlling reproduction in free-ranging wildlife populations has not yet been attained. Clearly, significant advancements with regard to treatment duration, application, and adverse effects are needed if fertility control is to become a useful tool for managing overabundant wildlife populations.

A promising new approach to contraception in wild ungulates involves using agonists of gonadotropin-releasing hormone (GnRH). GnRH is an endogenous neuropeptide that has an obligatory role in reproduction. It is naturally secreted in a pulsatile pattern from neurons in the hypothalamus and specifically directs gonadotropes in the anterior pituitary gland to synthesize and release luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These latter two hormones, in turn, control the proper functioning of the ovaries in females and testes in males (Hazum and Conn, 1988).

The chemical structure of endogenous GnRH has been determined (Matsuo et

al., 1971), and alterations in the molecule have led to the synthesis of potent GnRH agonistic analogs (Karten and Rivier, 1986). Long-term treatment with GnRH agonists has been shown to prevent ovulation by decreasing GnRH receptors on gonadotropes, receptor sensitivity to GnRH (Nett et al., 1981), pituitary LH content (Aspden et al., 1996), and by suppressing pulsatile secretion of LH and FSH (D'Occhio et al., 1996). These conditions persist as long as the agonist is present, but, once treatments are terminated, normal ovarian function is restored (Bergfeld et al., 1996). Agonists of GnRH have been used in domestic ungulates as fertility agents for controlling ovarian activity, gonadal steroidogenesis, and reproduction (McNeilly and Fraser, 1987; Montovan et al., 1990; D'Occhio et al., 2002). However, the use of a GnRH agonist to suppress ovulation and reproduction in wild ungulates is limited (Becker and Katz, 1995; Baker et al., 2002, 2003), due, in part, to the need for continuous delivery of a therapeutic dose for the duration of the breeding season. Recently, the impracticality of this approach for wildlife applications has been largely overcome by the development of long-acting biodegradable implants that can deliver a sustained release of GnRH agonist over a predetermined time period (Ravivarapu et al., 2000; Trigg et al., 2001).

In previous research, the GnRH agonist, leuprolide, was administered to female elk (*Cervus elaphus*) in a controlled-release formulation (ATRIGEL®), and 100% contraception was achieved for one breeding season, without significant behavioral or physiologic side effects (Baker et al., 2002). Concurrent with that experiment, this companion study was conducted with female mule deer (*Odocoileus hemionus*). Our specific objectives were to determine, in mule deer, 1) the effectiveness of a GnRH agonist in preventing pregnancy, 2) the duration of GnRH agonist suppression of LH and progesterone secretion, 3) the short-term behavioral and physiologic side effects (if any) of GnRH agonist treatment, and 4) the reversibility of GnRH agonist–induced infertility, if achieved.

MATERIALS AND METHODS

Animals

Mule deer, like other cervids, are seasonally polyestrus and exhibit an endogenous circannual breeding cycle of reproductive neuroendocrine activity that is influenced by photoperiod (Asher et al., 1998). In temperate North America, the breeding season is characterized by decreasing day length, with peak breeding activity occurring during mid-November to mid-December (Anderson, 1981). Parturition generally occurs in June, after a gestation period of about 200 days (Golly, 1957). Unbred females may undergo five to six recurrent estrous cycles of 18–30 days in length, extending through March (Wong and Parker, 1988). Coincidental with increasing day length, reproductive cycles cease and females remain anestrus from April until November (Plotka et al., 1977).

The captive mule deer used in this experiment were permanently maintained at the Colorado Division of Wildlife's Foothills Wildlife Research Facility (Fort Collins, Colorado, USA). Experimental animals were trained to repeated handling, weighing, blood sampling techniques, and holding pens. When not involved in the intensive sampling procedures of this study, deer were maintained in a fenced pasture (5.0 ha) of native vegetation and fed a diet consisting of ad libitum quantities of leafy alfalfa hay, grain supplement, trace mineral blocks, and water.

Experimental design

During December 1999 through June 2001, we evaluated the effects of leuprolide on pregnancy, LH and progesterone secretion, blood parameters, and the reproductive behavior of captive female mule deer. Thirteen adult female deer (2–6 yr old; weight, 65–75 kg) and two adult intact male deer (2–4 yr old; weight, 75–90 kg) were included in the experiment. Female deer were assigned to one of three experimental groups on the basis of their tractability for handling and blood sampling. Five deer (group A) received 10 mg leuprolide (D-Leu^6-GnRH-Pro^9-ethylamide) in a 90-day sustained release formulation using the ATRIGEL® drug delivery system (Atrix Laboratories, Inc., Fort Collins, Colorado; Dunn et al., 1994). This dose was derived from a similar experiment with female elk in which a 32.5-mg implant (8.3 mg kg^{-1} body weight [BW]) sup-

pressed ovulation and pregnancy for one breeding season (Baker et al., 2002). This dose was adjusted for deer on a body mass basis, and the average dose was delivered to all deer (7.0 mg kg $^{-1}$ BW). Five deer (group B) served as controls for comparing the effects of leuprolide on pregnancy rates and reproductive behavior. These groups of deer were maintained together in the same pasture with two intact adult male mule deer from 17 December 1999 until 31 March 2001. The remaining three deer (group C) served as nonpregnant controls and were placed in a separate pasture (2.1 ha) without direct contact with male mule deer. We compared LH and progesterone secretion, blood chemistry and hematology, and BW dynamics of these females (group C) with those treated with leuprolide (group A). Nonpregnant control females (group C) provided a more representative comparison to treated deer for these measurements than potentially pregnant deer, thus the need for two control groups.

Treatments were applied as follows. On the day of application (12 December 1999), deer (group A) were moved from 5-ha pastures to individual isolation pens, weighed (±0.5 kg), and sedated intramuscularly with ketamine (200 mg) + xylazine hydrochloride (100 mg of Rompun; Bayer, Leverkusen, Germany). A patch of hair (approximately 3 cm in diameter) was shaved from the shoulder region of each female, and the leuprolide formulation was injected subcutaneously, using an 18-gauge needle and a 1-ml syringe. Sedation was reversed with yohimbine (0.125 mg/kg Antagonil®; Wildlife Laboratories, Fort Collins, Colorado) administered intravenously. Once recovered, deer were returned to 5-ha pastures. Control deer (groups B and C) did not receive a placebo formulation.

Measurements

Reproduction: The effect of leuprolide on reproduction in treated (group A) and untreated (group B) deer was evaluated in two ways: by determining 1) pregnancy rates, using the presence or absence of pregnancy specific protein B (PSPB; BioTracking, Moscow, Idaho, USA) in serum collected at approximately 85 and 150 days of gestation (Wood et al., 1986), and 2) the incidence of fawning. Fawning data included parturition dates and the number and birth weights of fawns in control and treatment groups born during June–July 2000. Similar measurements were made during June–July 2001, to evaluate the reversibility of leuprolide treatment.

Hormones: The effects of leuprolide on the duration of suppression of LH were evaluated by periodically conducting pituitary stimulation trials. These challenge trials were conducted during 1 December 1999 to 11 November 2000, to determine the capability of LH cells to respond to stimulation with an exogenous dose GnRH analog. Four deer were randomly selected from group A as being representative of leuprolide-treated deer. All three untreated deer in group C were included in these comparative trials. GnRH analog (D-Ala^6-GnRH-Pro^9-ethylamide; Sigma Chemical Co., St. Louis, Missouri, USA) was administered to treated (group A) and control (group C) deer 12 days before leuprolide treatment (pretreatment) and at 45, 85, 120, 150, and 334 days after treatment. Serum samples for progesterone levels were also collected for each deer on each of these trial days. The final GnRH challenge trial (11 November 2000) provided hormonal evidence of the reversibility of LH and progesterone levels after leuprolide treatment.

Pituitary stimulation trials were conducted according to the following procedures. On the day of testing, selected deer from groups A and C were moved from 5-ha pastures to individual isolation pens, weighed, sedated (as previously described), and fitted nonsurgically with indwelling jugular catheters. GnRH analog (1 μg/50 kg BW) was administered through the catheter, and blood samples (5 ml) were collected 0, 120, 180, 240, 300, 360, 480, and 600 min after injection. After collections, blood was stored at 4 C for 24 hrs until serum was obtained by centrifugation (1,500 × G for 15 min). Serum was then stored at −20 C until it was analyzed for LH and progesterone levels. After the last blood collection, catheters were removed, and animals were returned to their respective pastures.

Blood parameters: The physiologic side effects of leuprolide were evaluated by comparing serum chemistry and hematology, seasonal BW dynamics, and the general health of treated (group A) and control (group C) deer. Blood sample collections and body mass measurements were made in conjunction with pituitary stimulation trials. Blood samples for hematology and serum chemistry assays were collected 120 days after treatment and submitted for analysis (Colorado State University Veterinary Teaching Hospital, Clinical Pathology Laboratory, Fort Collins, Colorado).

Serum chemistry profiles were obtained using a Hatachi 917 autoanalyzer (Roche/Boehringer Mannheim, Indianapolis, Indiana, USA) for the following parameters: glucose, creatinine, phosphorus, calcium, magnesium, total protein, albumin, globulin, albumin:globulin ratio, bilirubin, creatinine kinase (CK), aspartate, aminotransferase, γ-glutamyltransferase, sorbi-

TABLE 1. Description of mule deer reproductive behaviors and associated behavior categories.

Behavior categories	Reproductive behavior
Male precopulatory	Male courtship behavior directed toward an individual female to induce or detect estrus (i.e., tending, herding, rushing, low head stretch, flehmen, tongue flick, lick vulva, chivy, or rub body)
Female precopulatory	Female courtship behavior directed toward the dominant male to arouse copulatory behavior (i.e., bolt, circle male, self-urination, rub male, mount male, or lordosis)
Copulatory	Male behavior directed toward a receptive female in estrus (i.e., precopulatory mounts, intromission, or pelvic thrust)

tol dehydrogenase, sodium, potassium, chloride, and bicarbonate.

Values for the following hematologic parameters were determined using an ADVIA 120 autoanalyzer (Bayer Corp., Tarrytown, New York, USA): nucleated cells, neutrophils, lymphocytes, monocytes, eosinophils, plasma protein, erythrocytes, hemoglobin, packed cell volume, mean corpuscular volume, mean corpuscular hemoglobin concentration, platelets, and fibrinogen.

Reproductive behavior: We tested two hypotheses relative to the effects of leuprolide on the reproductive behavior of mule deer. First, because leuprolide was expected to suppress gonadotropin secretion and ovulation, we predicted that estrous behavior during the normal breeding season would be reduced in leuprolide-treated females (group A), compared with control deer (group B). Second, because depletion of the leuprolide implant (90 days) was formulated to occur before seasonal anestrus, we predicted that ovulation and associated behavioral estrus would resume in treated females (group A) before the end of the breeding period, and, as a result, the rate of reproductive interactions in these deer would be greater than that for untreated (group B) deer. These two hypotheses were tested by examining effects of the leuprolide formulation on reproductive interactions of male and female mule deer during two time periods: 1) the normal breeding season (17 December 1999–30 January 2000) and 2) post-breeding season (21 February–31 March, 2000).

On 12 December 1999, all deer in group A were treated with the leuprolide formulation and released, together with control deer (group B), into a 5-ha pasture. Five days later, two adult, intact male mule deer were placed with these females, and behavioral observations were initiated. All female deer were individually identified with color-numeric neck bands, and male deer were identified with different colored ear tags. Behavioral measurements were made from a distance of 50–300 m, from an elevated tower (10 m) situated at one end of the pasture, using binoculars and a spotting scope. As a result of equipment failure, nighttime behavioral observations were not conducted. Selected behaviors were recorded using a laptop computer and software (Ottoni, 2000; EthoLog 2.25).

Focal animal sampling procedures were used to sample reproductive behaviors over a 12-hr period (Lehner, 1996). Preliminary observations indicated that deer were most active at dawn (5:00–8:00 AM), evening (2:00–5:00 PM), and night (8:00–12:00 AM). Because we could not adequately view deer at night, we sampled at midday. Thus, time-of-day sampling periods were randomly assigned each wk using a randomized block design. Each sampling period consisted of at least 1–3 hr of continuous observations. Twenty-seven reproductive interactions were identified and recorded (Geist, 1981), but our sample size for each of these individual behaviors was small, so behaviors were grouped into three general categories: male precopulatory, female precopulatory, and copulatory (Table 1). Our experimental unit for analysis was the individual female in each experimental group. Behavioral interactions were generally of short duration (<30 sec) relative to the sampling interval; therefore, the number of occurrences of each event was recorded rather than the length of time, and calculated rates of sexual interactions were recorded as behaviors per animal per hour and then multiplied by 24, for a daily rate.

Hormone radioimmunoassay: Serum concentrations of LH were quantified by means of an ovine (o) LH radioimmunoassay (Nett et al., 1975). Mule deer serum was demonstrated to inhibit binding of ^{125}I-labeled oLH to LH antiserum in a parallel manner. Similarly, when different quantities of oLH standard (NIH-OLH-S24) were added to mule deer serum and samples were subjected to radioimmunoassay, the values obtained were increased by the quantity of oLH added (r^2=0.98, β_1=0.93, SE=0.18, P=0.002). These results indicated

that the radioimmunoassay provided a quantitative assessment of LH in mule deer serum. The limit of sensitivity of the LH assay was 0.04 ng ml^{-1}. Serum concentrations of progesterone were also determined using radioimmunoassay methods (Niswender, 1973). The sensitivity of the progesterone assay was 0.12 ng ml^{-1}. Intra- and interassay coefficients of variation for each of these assays were <10%.

Statistical analysis: Hormone concentrations were reported as untransformed arithmetic means±SE. The responsiveness of the pituitary to a pharmacologic dose of GnRH analog was determined in two ways: 1) maximum response (highest concentration of LH [ng ml^{-1}] achieved after injection, minus baseline) and 2) total amount of LH secreted (ng ml^{-1} min^{-1}), which was estimated by calculating the area under the LH response curve (Abramowitz and Stegun, 1968).

Differences among hormone concentrations were tested using least-squares analysis of variance (ANOVA) for general linear models (SAS Institute, 1997). Responses to treatment were analyzed with one-way ANOVA for a randomized complete block design with repeated measures. Treatment effects were determined using the total animal-within-treatment variance as the error term. Time was treated as a within-subject effect, using a multivariate approach to repeated measures (Morrison et al., 1976). A protected least-significant-difference test (Milliken and Johnson, 1984) was used to separate means when the overall *F* test indicated significant treatment effects ($P<0.05$).

Specific null-reproductive-behavior hypotheses that mean behavior rates were not different between treatment and control groups for both the breeding and postbreeding seasons were tested using an ANOVA model with a repeated-measures structure. In a similar manner to the hormonal analysis, time was treated as a within-subject effect using a multivariate approach to repeated measures. The covariance structure of daily behavior rates observed for each female were included in the model with a first-order autoregression structure for unequally spaced repeated measurements. Time of day, date effects, and their interactions were included in the model as fixed effects, to account for other sources of variation. We used PROC MIXED software to estimate and test for differences in mean behavior rate by treatment, time of day, and date (Littell et al., 1996). Means and SEs were estimated using least-squares analysis, and hypothesis tests were based on type III generalized estimating equations that accounted for correlations in repeated measures.

RESULTS

The prolonged release leuprolide formulation prevented pregnancy in all treated female mule deer (group A), whereas the pregnancy and fawning rates of control females (group B) were 100%. All leuprolide-treated deer were negative and controls were positive for PSPB at 85 and 150 days of gestation. No fawns were born to treated females, whereas the fawning rate of control females was 1.61±0.25 fawns per doe. Treated females regained normal ovarian function the following breeding season, conceived (100%) and gave birth to normal fawns in June 2001. Parturition dates, fawning rates, and birth weights of fawns born to leuprolide-treated and control females were similar ($F_{1,5}=1.03$, $P=0.453$) in 2001, which confirmed reversibility of leuprolide treatments.

Leuprolide prevented pregnancy in treated deer by suppressing LH levels for the duration of the breeding period (Fig. 1). In treated deer (group A), leuprolide reduced the mean maximum serum LH response ($F_{1,5}=5.34$, $P=0.035$) from pretreatment (9.1±2.2 ng ml^{-1}) to baseline levels (0.24±0.04 ng ml^{-1}) by day 45, and the suppressive effects of the agonist continued for at least 120 days after treatment. Two leuprolide-treated females showed slightly elevated serum LH levels between 120 and 150 days after treatment; however, the magnitude of the increase was not significant ($F_{1,5}=0.52$, $P<0.482$). Mean maximum serum concentrations of LH were lower ($F_{1,5}=4.98$, $P=0.018$) between treated (group A) and untreated (group C) females 45, 85, and 120 days after treatment. The suppression of LH in treated females was followed by a return to pretreatment levels, similar to those of control females ($F_{1,5}=0.86$, $P=0.647$), before the subsequent breeding season (Fig. 1). For untreated females (group C), mean maximum levels of LH declined in a linear fashion over time ($F_{1,5}=33.17$, $P=0.001$, $r^2=0.82$) from pretreatment (10.6±4.9 ng ml^{-1}) to baseline levels (0.39±0.13 ng ml^{-1}) by 150 days after treatment.

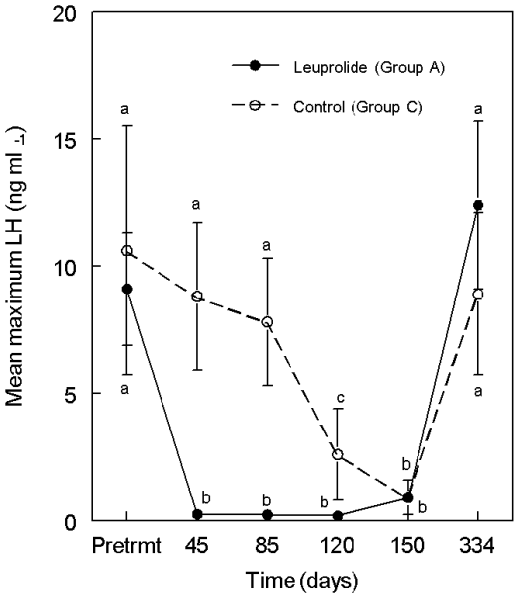

FIGURE 1. Longitudinal profiles of mean maximum serum concentrations of LH (ng ml^{-1}) in control female mule deer (group C) and females treated with a sustained release formulation containing 10 mg of leuprolide (group A) after challenge with GnRH analog. Results are shown as means±SE. Different lowercase letters indicate significant differences between means among treatment time intervals ($P \leq 0.05$).

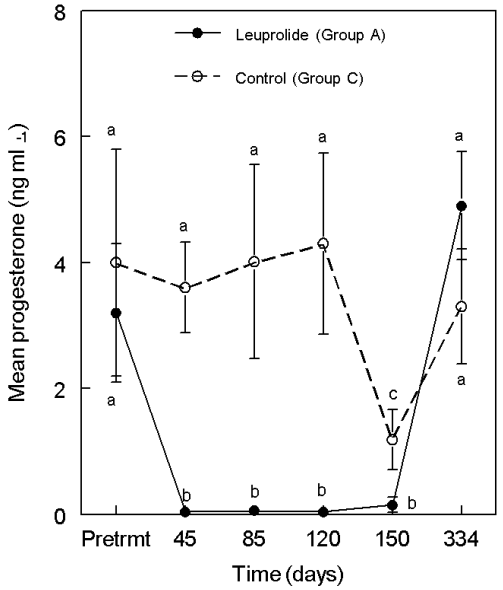

FIGURE 2. Longitudinal profiles of mean progesterone concentrations (ng ml^{-1}) in control female mule deer (group C) and females treated with a sustained release formulation containing 10 mg of leuprolide (group A). Results are shown as means±SE. Different lowercase letters indicate significant differences between means among treatment time intervals ($P \leq 0.05$).

The suppressive effects of leuprolide on corpus luteum formation and steroidogenesis were evidenced by its effects on serum progesterone levels in treated, compared with control, deer (Fig. 2). Serum progesterone concentrations of treated deer declined ($F_{1,5}=8.66$, $P=0.023$) to nondetectable levels by 45 days after treatment and remained at those levels for the duration of the breeding period, which indicated that additional ovulations did not occur. For untreated deer, serum progesterone was more variable and consistently higher ($F_{1,5}=6.58$, $P \leq 0.026$) than for treated deer 45, 85, 120, and 150 days after treatment. The increased serum progesterone concentration during this period probably reflected regular estrous cycles until after day 150, when the effects of seasonal anestrus reduced progesterone levels to basal concentrations. As evidence of contraceptive reversibility, progesterone concentrations returned to pretreatment levels during the fall 2000 breeding season.

Leuprolide had no effect ($F_{1,5}=1.34$, $P>0.685$) on serum chemistry and hematologic parameters or seasonal changes in body mass in treated deer (group A), compared with control deer (group C). With the exception of CK, a muscle-derived enzyme, all individuals were clinically similar. Treated females showed elevated CK levels compared with untreated deer ($F_{1,5}=8.74$, $P=0.037$). No health-related problems were observed for either leuprolide-treated or untreated deer during the course of the study.

Reproductive behaviors and dominance interactions were observed as soon as male deer were released into pastures with treated and control female deer. However, because the adult males were unequal in age and body size, male-to-male interactions were generally of short duration and low intensity. Dominance was established

in 3–4 days, after which the subdominant male retreated to the most-distant location of the pasture and rarely interacted with females or the dominant male for the remainder of the study.

During the normal breeding season, we observed courtship behavior between the dominant male and 10 female deer between 17 December 1999 and 30 January 2000 (34 days). Data were analyzed from 51 sampling periods (125.2 hr): 20 periods at dawn (36.2 hr), 16 at midday (36.0 hr), and 15 during evening (25 hr). The average duration of the sampling period was 1.9±0.1 hr. Behavioral observations during the postbreeding season were recorded for 7 days from 21 February 2000 to 18 March 2000. Data were collected and analyzed from seven 4-hr sampling periods (28 hr): four periods at dawn and three periods during evening. No reproductive behaviors were recorded for the midday sampling period during the postbreeding season. Observation periods averaged 4.0±0.1 hr in length. Because of equipment failure, nighttime observations were not conducted.

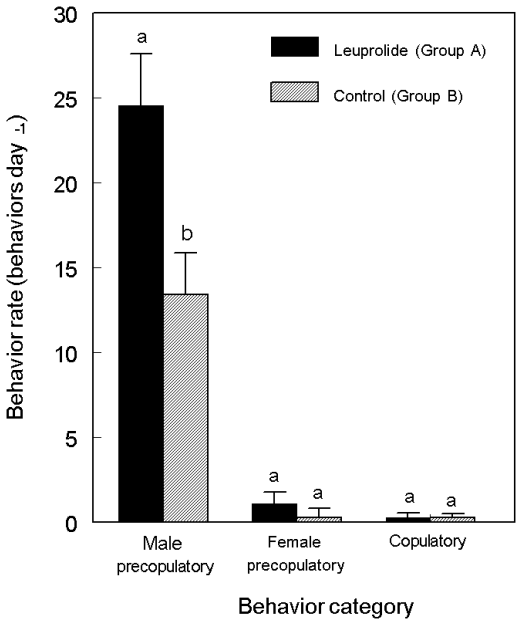

FIGURE 3. Mean (±SE) reproductive behavior rates during the breeding season for control female mule deer (group B) and females treated with a sustained release formulation containing 10 mg of leuprolide (group A). Columns with different lowercase letters indicate significant differences between means ($P \leq 0.05$).

Contrary to our first hypothesis, reproductive interactions during the normal breeding season were not reduced in leuprolide-treated females compared with controls (Fig. 3). Instead, breeding behavior rates were similar in all deer for female precopulatory ($F_{1,8}=2.52$, $P=0.151$) and copulatory behavior ($F_{1,8}=0.003$, $P=0.952$), whereas male precopulatory behavior rate was almost twice as high toward treated females compared with controls ($F_{1,8}=8.09$, $P=0.022$) (Figs. 3, 4a). Daily behavior rates were generally episodic and highly variable for all three behavior categories (Figs. 4 a, b, c). We did not detect a trend in female precopulatory behavior rate ($F_{1,498}=1.53$, $P=0.217$) or copulatory behavior rate ($F_{1,498}=2.07$, $P=0.190$), but there was a slight declining trend in male precopulatory behavior rate ($F_{1,498}=4.07$, $P=0.044$). There were no treatment × time interactions for any of the behavior categories (male precopulatory, $F_{1,498}=1.57$, $P=0.211$; female precopulatory, $F_{1,498}=0.80$, $P=0.372$; and copulatory, $F_{1,498}=0.02$, $P=0.877$).

We also rejected our second hypothesis, because the duration of leuprolide efficacy was longer than we had predicted. Leuprolide-treated females did not resume normal estrous cycles during the postbreeding season, and reproductive behavior rates did not increase compared with controls. Almost no sexual interactions were observed between the dominant male and either leuprolide-treated or control females during the postbreeding season (Figs. 4 a, b, and c).

DISCUSSION

Leuprolide, administered as a sustained release formulation, effectively suppressed secretion of LH and progesterone and prevented conception in female mule deer for one breeding season. Treated females regained normal ovarian function the following breeding season, subsequently be-

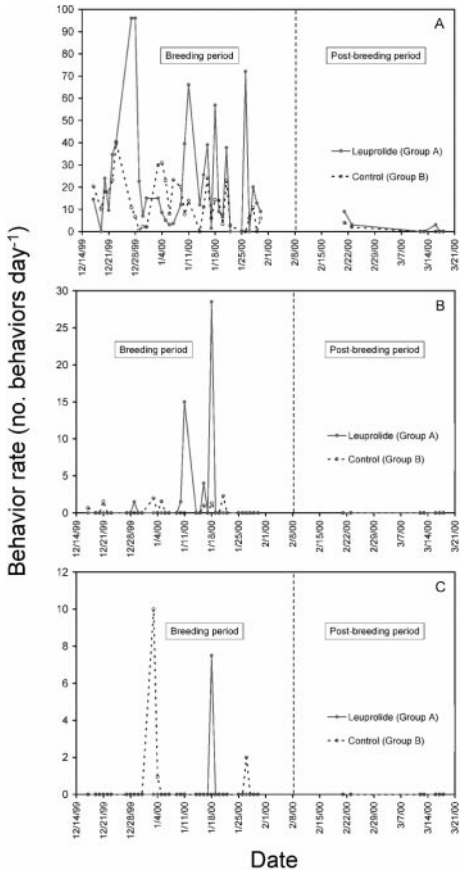

FIGURE 4. Male precopulatory (A), female precopulatory (B), and copulatory (C) behavior rates for control (white circles) and leuprolide-treated (black circles) female mule deer during the breeding period. Treated females were given a sustained release formulation containing 10 mg leuprolide. Note: *Y*-axis scale is different for each graph.

came pregnant, and gave birth to fawns that were similar in body mass and general health to those born to untreated deer. The leuprolide-induced reduction in concentrations of LH and progesterone in this study are consistent with results for females of other species treated with GnRH agonist. Likewise, the return to normal ovarian function after the cessation of agonist treatment is similar to observations reported for marmoset monkeys (*Callithrix jacchus*; Lunn et al., 1992); cattle (D'Occhio et al., 2000), dogs (Trigg et al., 2001), and elk (Baker et al., 2002).

Leuprolide suppressed the secretion of LH and progesterone and prevented ovulation for a minimum of 120 days in deer, which was 33% longer than the formulated 90-day delivery period. In elk, a similar formulation of leuprolide reduced hormone levels for an average of 190 days, or 111% longer than predicted (Baker et al., 2002). The prolonged suppression of reproductive hormones after agonist treatment has also been reported for other species, including male and female cattle (Bergfeld et al., 1996), men (Hall et al., 1999), and women (Broekmans et al., 1996). The explanation for extended hormonal suppression by leuprolide is speculative but may be due to either residual formulation being released from the implant for more than 90 days or, more likely, to pituitary dysfunction that continues for a protracted period of time subsequent to treatment (Aspden et al., 2003). Regardless of the mechanism, the delay (beyond the calculated duration of the implant) to normal ovarian function, is central to the effectiveness of GnRH agonist as a contraceptive agent in deer and elk.

Leuprolide did not suppress estrous behaviors in female deer during the normal breeding season, nor did treated females exhibit reproductive behaviors during the postbreeding season. During the normal breeding season, reproductive behavior rates for treated females were equal to or greater than those for untreated females for all behavior categories. In particular, male precopulatory behaviors, which dominated our observations, were almost twice as high toward leuprolide-treated females, compared with controls. Although these behaviors were sporadic and intermittent, when they did occur, the rate was relatively similar throughout the sampling period. In contrast, the rate of male precopulatory behaviors toward control females gradually declined during the course of the normal breeding season. This decline in behavior rate was expected and was presumably due to control females becoming pregnant and the dominant male continuing to court nonpregnant females that were exhibiting

periodic estrous behavior. The absence of reproductive behaviors during the postbreeding season can be attributed to the prolonged effectiveness of the leuprolide formulation.

Why did leuprolide-treated females exhibit estrous behavior during the normal breeding season but not become pregnant? This result was unexpected, and we can only speculate on the apparent contradiction. Female deer in this experiment were treated with leuprolide at various stages of the estrous cycle. Although leuprolide apparently prevented the development of new preovulatory follicles, existing follicles capable of secreting low levels of estradiol could have persisted for several weeks after treatment (Gong et al., 1996). In addition, preexposure to progesterone stimulates estrus when it is coupled with low progesterone levels (Robinson, 1954). Therefore, if deer ovulated during a previous estrous cycle or during a "silent estrus," as has been previously reported for deer (Plotka et al., 1977; Harder and Moorhead, 1980), and they were exposed to progesterone, estrous behavior could have been initiated by low amounts of estradiol. However, because ovulation and corpus luteum formation were blocked by the effects of leuprolide, female deer would continue to show estrous behavior without becoming pregnant.

Leuprolide does not appear to affect blood chemistry, hematology, or BW dynamics of treated females. Although CK levels were elevated in leuprolide-treated deer, we attribute those effects to disparate handling procedures between treated and control deer before blood sampling. Handling procedures for treated females (group A) were often more physically rigorous than those for controls (group C), because of the need to separate them from males. CK levels can increase in unconditioned animals after vigorous exercise and can remain elevated for 4–6 hr (Lefebvre et al., 1994). Thus, the elevated CK levels in treated deer, compared with controls, likely reflect a bias due to a difference in animal handling procedures rather than a treatment-induced response. Body mass changes were similar for both treatment and control deer and followed seasonal patterns previously reported for *Odocoileus* (Wood et al., 1962).

The reproductive behaviors of female deer in this experiment and of elk in a previous investigation (Baker et al., 2002) appear to have followed a similar, hormonally induced pattern of behavior in which leuprolide-treated females are actively engaged in estrus behavior for 4–6 wk after agonist treatment and then display a lack of sexual receptivity for the remainder of the breeding period. However, our observations offer only a limited view of the effects of leuprolide on seasonal reproductive behavior in deer and no insight into potential effects on daily or seasonal activity patterns. Clearly, population-level research is needed to complement and verify our observations on confined deer and to further assess the effects of leuprolide on social organization, energy expenditure, and survival in free-ranging animals.

In conclusion, leuprolide, administered as a controlled-release formulation offers an alternative approach to contraception in female deer and fulfills most of the previously established criteria for an acceptable contraceptive agent for wildlife applications (Kirkpatrick and Turner, 1991). First, leuprolide is highly effective (100%) in preventing pregnancy and is reversible. A single dose prevented pregnancy in all females for one breeding season, with a return to normal fertility the following year. Second, leuprolide does not require an immunologic response to achieve efficacy (Muller et al., 1997); therefore, effective contraception is accomplished without an immunogenic adjuvant, which can cause local tissue reactions (Stills and Bailey, 1991). This attribute also enhances its practical application over immunologic approaches, because only one capture and treatment is required the first year for contraception and for subsequent years, rather than relocating and retreating the same

individual annually to maintain infertility by boosting vaccines—any female in the population can be targeted for treatment. Third, leuprolide is a neuropeptide and, as a proteinaceous agent, does not pose a threat to nontarget species (including humans) or the environment. Fourth, no detrimental short-term physiologic side effects have been attributed to leuprolide treatments, and short-term behavioral effects appear minimal. In contrast, immunologic agents that impede fertilization have been shown to prolong the breeding season in males and females, thus potentially predisposing deer to the effects of increased energy expenditure in winter and unpredictable long-term behavioral consequences (McShea et al., 1997). Fifth, the small volume required for effective contraception facilitates administration by syringe dart, which has been demonstrated, in elk, to be equally as effective as subcutaneous delivery (Baker et al., 2003). Finally, leuprolide formulation is currently approved by the US Food and Drug Administration (FDA) for therapeutic use in humans. A similar approval process could be expected for its use as a fertility control agent in deer. If successful, leuprolide formulation could become commercially available for wildlife management applications in the near future.

ACKNOWLEDGMENTS

We sincerely thank L. Whittemore, J. Ritchie, D. Finley, E. Wheeler, A. Ewing, and E. Jones for technical assistance with animal handling, sample collections, and behavioral observations. We thank D. Bowden for statistical consultation and analysis. Our research was supported by a grant from the US Fish and Wildlife Service, Rocky Mountain Arsenal National Wildlife Refuge, and the Colorado Division of Wildlife (Federal Aid to Wildlife Restoration, Project 153-R4). We offer special thanks to Atrix Laboratories, Inc., Fort Collins, Colorado for their generous donation of the leuprolide formulations used in this investigation. D. J. Freddy provided a particularly constructive review of an early draft of this article. These experiments were conducted with the approval of the Colorado Division of Wildlife Animal Care and Use Committee (1–1999 and 1–2000) and in compliance with US Federal Animal Welfare Act Regulations.

LITERATURE CITED

ABRAMOWITZ, M., AND I. A. STEGUN. 1968. Handbook of mathematical functions. Dover Publishing, Inc., New York, New York, 343 pp.

ANDERSON, A. E. 1981. Morphological and physiological characteristics. *In* Mule and black-tailed deer of North America, O. C. Wallmo (ed.). University of Nebraska Press, Lincoln, Nebraska, pp. 27–97.

ASHER, G. W., M. W. FISHER, S. L. MONFORT, AND G. E. MYLREA. 1998. Endocrine control of reproduction in cervids: The enigma of temperate vs. tropical species. *In* Recent developments in deer biology, J. A. Milne (ed.). Macaulay Land Use Research Institute, Craigiebuckler, Aberdeen, UK, pp. 126–140.

ASPDEN, W. J., A. RAO, P. T. SCOTT, I. J. CLARK, T. E. TRIGG, J. WALSH, AND M. J. D'OCCHIO. 1996. Direct actions of the luteinizing hormone-releasing hormone agonist, deslorelin, on anterior pituitary contents of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), LH and FSH subunit messenger ribonucleic acid, and plasma concentrations of LH and FSH in castrated male cattle. Biology of Reproduction 55: 386–392.

———, A. JACKSON, T. E. TRIGG, AND M. J. D'OCCHIO. 2003. Pituitary expression of LHB- and FSHβ-subunit mRNA, cellular distribution of LHB-subunit mRNA and LH and FSH synthesis during and after treatment with a gonadotrophin-releasing hormone agonist in heifers. Reproduction, Fertility and Development 15: 149–156.

BAKER, D. L., M. A. WILD, M. M. CONNER, H. B. RAVIVARAPU, R. L. DUNN, AND T. M. NETT. 2002. Effects of GnRH agonist (leuprolide) on reproduction and behavior in female wapiti (*Cervus elaphus nelsoni*). Reproduction (Suppl.) 60: 155–167.

———, M. D. HUSSAIN, R. L. DUNN, AND T. M. NETT. 2003. Evaluation of remotely delivered leuprolide formulation as a contraceptive agent in captive female elk. Technical support for elk and vegetation management for Rocky Mountain National Park, Wildlife Research Report, Colorado Division of Wildlife, Fort Collins, Colorado, 125 pp.

BECKER, S. E., AND L. S. KATZ. 1995. Effects of gonadotropin-releasing hormone agonist on serum LH concentrations in female white-tailed deer. Small Ruminant Research 18: 145–150.

BERGFELD, E. M., J. D'OCCHIO, AND J. E. KINDER. 1996. Pituitary function, ovarian follicular growth, and plasma concentrations of 17 α-oestradiol and progesterone in prepubertal heifers

during and after treatment with the luteinizing hormone-releasing hormone agonist deslorelin. Biology of Reproduction 54: 776–782.

BROEKMANS, F. J., P. G. HOMPES, C. B. LAMBALK, E. BROEDERS, AND J. SCHOEMAKER. 1996. Short-term desensitization: Effects of different doses of gonadotropin-releasing hormone agonist triptorelin. Human Reproduction 11: 55–60.

D'OCCHIO, M. J., W. J. ASPDEN, AND T. R. WHYTE. 1996. Controlled, reversible suppression of oestrous cycles in beef heifers and cows using agonist of luteinizing hormone-releasing hormone. Journal of Animal Science 74: 218–225.

———, G. FORDYCE, T. R. WHYTE, W. J. ASPDEN, AND T. E. TRIGG. 2000. Reproductive responses of cattle to GnRH agonist. Animal Reproduction Science 60–61: 433–442.

———, ———, ———, T. F. JUBB, L. A. FITZPATRICK, N. J. COOPER, W. J. ASPDEN, M. J. BOLAM, AND T. E. TRIGG. 2002. Use of GnRH agonist implants for long-term suppression of fertility in extensively managed heifers and cows. Animal Reproduction Science 74: 151–162.

DUNN, R. L., J. P. ENGLISH, D. R. COWAN, AND D. P. VANDERBILT. 1994. Biodegradable in situ forming implants and methods of producing the same. US patent no. 5 278 201.

FAGERSTONE, K. A., M. A. COFFEY, P. D. CURTIS, R. A. DOLBEER, G. J. KILLIAN, L. A. MILLER, AND L. WILMOT. 2002. Wildlife fertility control. Wildlife Society Technical Review 02–2. The Wildlife Society, Bethesda, Maryland, USA, 53 pp.

GEIST, V. 1981. Behavior: Adaptive strategies in mule deer. *In* Mule and black-tailed deer of North America, O. C. Wallmo (ed.). University of Nebraska Press, Lincoln, Nebraska, pp. 157–223.

GOLLY, F. B. 1957. Gestation period, breeding and fawning behavior of Columbian black-tailed deer. Journal of Mammalogy 38: 116–120.

GONG, J. G., B. K. CAMPBELL, T. A. BRAMLEY, C. G. GUTIERREZ, A. R. PETERS, AND R. WEBB. 1996. Suppression in the secretion of follicle-stimulating hormone and luteinizing hormone, and ovarian follicle development in heifers continuously infused with gonadotropin-releasing hormone agonist. Biology of Reproduction 55: 68–74.

HALL, M. C., R. J. FRITZSCH, A. I. SAGALOWSKY, A. AHRENS, B. PETTY, AND C. G. ROEHRBORN. 1999. Prospective determination of the hormonal response after cessation of luteinizing hormone–releasing hormone agonist treatment in patients with prostate cancer. Urology 53: 898–902.

HARDER, J. D., AND D. L. MOORHEAD. 1980. Development of corpora lutea and plasma progesterone levels associated with the onset of the breeding season in white-tailed deer (*Odocoileus virginianus*). Biology of Reproduction 22: 185–191.

HAZUM, E., AND P. M. CONN. 1988. Molecular mechanism of gonadotropin releasing hormone (GnRH) action. I. The GnRH receptor. Endocrine Review 9: 379–386.

KARTEN, M. J., AND J. E. RIVIER. 1986. Gonadotropin-releasing hormone analog design. Structure-function studies toward the development of agonists and antagonists: Rationale and perspectives. Endocrine Review 7: 44–66.

KIRKPATRICK, J. F., AND J. W. TURNER. 1991. Reversible contraception in nondomestic animals. Journal of Zoo and Wildlife Medicine 22: 392–408.

LEFEBVRE, H. P., P. L. TOUTAIN, J. P. SERTHELON, V. LASSOURD, L. GARDEY, AND J. P. BRAUN. 1994. Pharmacokinetic variables and bioavailability from muscle of creatinine kinase in cattle. American Journal of Veterinary Research 55: 487–497.

LEHNER, P. N. 1996. Handbook of ethological methods, 2nd edition. Cambridge University Press, Cambridge, Massachusetts, 258 pp.

LITTELL, R. C., G. A. MILLIKEN, W. W. STROUP, AND R. D. WOLFINGES. 1996. SAS® system for mixed models. SAS Institute Incorporated, Cary, North Carolina, USA.

LUNN, S. F., G. M. COWEN, K. D. MORRIS, AND H. M. FRASER. 1992. Influence of the gonad on the degree of suppression induced by an LHRH agonist implant in the marmoset monkey. Journal of Endocrinology 132: 217–227.

MATSUO, H., Y. BABA, R. M. G. NAIR, A. ARIMURA, AND A. V. SCHALLY. 1971. Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. Biochemical and Biophysical Research Communication 43: 1334–1339.

MCNEILLY, A. S., AND H. M. FRASER. 1987. Effect of gonadotrophin-releasing hormone agonist-induced suppression of LH and FSH on follicle growth and corpus luteum function in the ewe. Journal of Endocrinology 115: 273–282.

MCSHEA, W. J., S. L. MONFORT, S. HAKIM, J. F. KIRKPATRICK, K. M. LIU, J. W. TURNER, JR., L. CHASSY, AND L. MUNSON. 1997. The effect of immunocontraception on the behavior and reproduction of white-tailed deer. Journal of Wildlife Management 61: 560–569.

MILLIKEN, G. A., AND D. E. JOHNSON. 1984. Analysis of messy data. Volume 1. Designed experiments. Lifetime Learning Publications, Belmont, California, 399 pp.

MONTOVAN, S. M., P. P. DAELS, J. RIVIER, J. P. HUGHES, G. H. STABENFELDT, AND B. L. LASLEY. 1990. The effect of potent GnRH agonist on gonadal and sexual activity in the horse. Theriogenology 33: 1305–1321.

MORRISON, J. A., C. E. TRAINER, AND P. L. WRIGHT. 1976. Multivariate statistical methods. McGraw-Hill, New York, New York, 432 pp.

MULLER, L. I., R. J. WARREN, AND D. L. EVANS. 1997. Theory and practice of immunocontracep-

tion in wild mammals. Wildlife Society Bulletin 25: 504–514.

NAUGLE, R. E., A. T. RUTBERG, H. B. UNDERWOOD, J. W. TURNER, JR., I. K. M. LIU. 2002. Field testing of immunocontraception on white-tailed deer (*Odocoileus virginianus*) on Fire Island National Seashore, New York, USA. Reproduction (Suppl.) 60: 143–153.

NETT, T. M., A. M. AKBAR, R. D. PHEMISTER, P. A. HOLST, L. E. REICHERT, JR., AND G. D. NISWENDER. 1975. Levels of luteinizing hormone, estradiol and progesterone in serum during the estrous cycle and pregnancy in the Beagle bitch. Proceedings of the Society of Experimental Biology and Medicine 148: 134–139.

———, M. E. CROWDER, G. E. MOSS, AND T. M. DUELLO. 1981. GnRH-receptor interaction. V. Down-regulation of the pituitary receptors for GnRH in ovariectomized ewes by infusion of homologous hormone. Biology of Reproduction 24: 1145–1155.

NISWENDER, G. D. 1973. Influence of the site of conjugation on the specificity of antibodies to progesterone. Steroids 22: 413–424.

OTTONI, E. B. 2000. EthoLog 2.2: A tool for the transcription and timing of behavior observation sessions. Behavior Research Methods, Instruments, & Computers 32: 446–449.

PLOTKA, E. D., U. S. SEAL, G. C. SCHMOLLER, P. D. KARNS, AND K. D. KEENLYNE. 1977. Reproductive steroids in the white-tailed deer (*Odocoileus virginianus borealis*). I. Seasonal changes in the female. Biology of Reproduction 16: 340–343.

RAVIVARAPU, H. B., K. L. MOYER, AND R. L. DUNN. 2000. Sustained activity and release of leuprolide acetate from an in situ forming polymeric implant. American Association of Pharmaceutical Scientist 1: 1–12.

ROBINSON, T. J. 1954. Relationship of oestrogen and progesterone in oestrous behavior of the ewe. Nature 173: 870–874.

RUTBERG, A. T., R. E. NAUGLE, L. A. THIELE, AND I. K. M. LIU. 2004. Effects of immunocontraception on a suburban population of white-tailed deer (*Odocoileus virginianus*). Biological Conservation 116: 243–250.

SAS INSTITUTE. 1997. SAS/STAT® user's guide 6.03 edition. SAS Institute, Inc., Cary, North Carolina, USA.

SHIDELER, S. E., M. A. STOOPS, N. A. GEE, J. A. HOWELL, AND B. L. LASLEY. 2002. Use of porcine zona pellucida (PZP) vaccine as a contraceptive agent in free-ranging tule elk (*Cervus elaphus nannodes*). Reproduction (Suppl.) 60: 169–176.

STILLS, H. F., JR., AND M. Q. BAILEY. 1991. The use of Freund's complete adjuvant. Laboratory Animal 20: 25–30.

TRIGG, T. E., P. J. WRIGHT, A. F. ARMOUR, P. E. WILLIAMSON, A. JUNAIDI, G. B. MARTIN, A. G. DOYLE, AND J. WALSH. 2001. Use of GnRH analogue implant to produce reversible long-term suppression of reproductive function in male and female domestic dogs. Journal of Reproduction and Fertility (Suppl.) 57: 255–261.

WONG, B., AND K. L. PARKER. 1988. Estrus in black-tailed deer. Journal of Mammalogy 69: 168–171.

WOOD, A. J., I MCCOWAN, AND H. C. NORDAN. 1962. Periodicity of growth in ungulates as shown by deer of the genus *Odocoileus*. Canadian Journal of Zoology 40:593–603.

WOOD, A. K., R. E. SHORT, A. E. DARLING, G. L. DUSEK, R. G. SASSER, AND C. A. RUDER. 1986. Serum assays for detecting pregnancy in mule and white-tailed deer. Journal of Wildlife Management 50: 684–687.

Received for publication 19 December 2004.