

# GnRH-agonist induction of fertile estrus with either natural mating or artificial insemination, followed by birth of pups in gray wolves (*Canis lupus*)

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## Abstract

Although captive populations of endangered species such as the Mexican gray wolf (*Canis lupus baileyi*) can benefit from artificial insemination to accomplish genetic exchange, reliable techniques for timing insemination are lacking. We used the generic gray wolf (*C. lupus*) to test the efficacy of a short-acting GnRH-agonist implant, deslorelin, for inducing estrus. Of five females receiving implants on 17 or 18 January 2003, two mated naturally 10–17 days later, and the others were artificially inseminated using fresh semen, one on day 7 and all three on day 11. Relaxin tests revealed that one artificially inseminated female and both naturally mated females were pregnant on 1 March, and all three gave birth to healthy puppies on 4–6 April. Of the artificially inseminated females, only the one who subsequently conceived and gave birth was judged to be in cytologic estrus at the time of insemination. Two females were treated again with deslorelin on 12 January 2004, followed by collection of fecal samples for hormone analysis. One female, who was housed with a male, copulated on day 17 but did not conceive; the other was not with an adult male. Fecal progesterin and estrogen profiles suggested that estrus, but not ovulation, was induced. These results indicated that deslorelin could induce fertile estrus in the gray wolf, although individual response varied. Further investigation is needed to better define and control the interval between implant insertion and ovulation for optimal timing of insemination.

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## 1. Introduction

Genetic management of the captive population of the endangered Mexican wolf (*Canis lupus baileyi*), a subspecies of the gray wolf (*C. lupus*), can be problematic. Because of their monogamous mating

system, separation of pairs can result in considerable stress. Thus, achieving desired heterozygosity through transfer of individuals to accomplish recommended genetic pairings, presents difficulties beyond those faced with many other species. Our commitment to the U.S. Fish and Wildlife Service's Mexican Wolf Recovery Program is to develop minimally, or non-invasive, techniques for genetic exchange, e.g., artificial insemination (AI). However, the timing of inseminations in Mexican wolves is more difficult than in domestic dogs. Ovulation detection protocols for dogs,

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such as blood sampling for hormone analysis and vaginal cytology, require regular handling, which is not possible under the management plan of the Mexican wolf. Collection of fecal samples is non-invasive, but fecal steroid assays take longer to complete than those for serum, and wolves are often housed in groups in large outdoor enclosures, which makes collection of daily fecal samples from selected individuals very labor intensive, if not impossible, under some management systems. An alternative to ovulation detection for AI is induction of estrus or ovulation, followed by timed insemination.

There has been considerable effort to develop a method to reliably induce estrus and ovulation in dogs [1–5]. Encouraging results have been obtained with GnRH administered as either injections or continuous or pulsatile infusions [6–9]. A new short-acting GnRH-agonist implant represents a potentially simplified delivery method (Volkman et al., this issue). We tested the efficacy of this implant for inducing fertile estrus and ovulation in the generic gray wolf, as a model for the endangered Mexican wolf.

## 2. Materials and methods

The wolves were maintained at the Wildlife Science Center in Forest Lake, MN, USA, in outdoor enclosures and fed carcasses of white-tailed deer, supplemented with commercial dog food. Five females were anesthetized (ketamine HCl: Ketaset, Boehringer Ingelheim Vetmedica, St. Joseph, MO, USA; and xylazine: Rompun, Bayer, Shawnee Mission, KS, USA) on either 17 or 18 January 2003 (Table 1) for insertion of a deslorelin implant (2.1 mg, Ovuplant™: Peptech Animal Health, Australia) underneath the vulvar mucosa.

Two of the females (A and C) were housed with males throughout the study and allowed to mate naturally. The three other females (B, D and E), housed away from males, were anesthetized on 24 and 28 January (7 and 11 days, respectively, after implant insertion) for collection of blood samples and vaginal smears and for artificial insemination. Serum was

separated by centrifugation and frozen until analysis at the Endocrinology Laboratory at the Saint Louis Zoo. Serum progesterone and fecal progestins and estrogens were measured by radioimmunoassay, as described in Valdespino et al. [10]. Vaginal smears were examined as described by Asa et al. [11]. Three male wolves were anesthetized (ketamine and xylazine) for semen collection by electroejaculation. Fresh semen was deposited either vaginally or cervically using a modification of the Norway catheter (Farstad and Thomassen, personal communications). All five females were anesthetized again on 1 March to obtain blood samples which were collected into heparinized tubes, centrifuged, and plasma frozen until relaxin was analyzed by enzyme-immunoassay (ReproCHEK: Synbiotics Corp., San Diego, CA, USA) to determine pregnancy status.

On 12 January 2004, deslorelin implants were inserted again into two females (B and C) as described above. One (B) was housed with an adult male and the other with an adolescent male. Treated females were fed ground meat containing colored plastic beads (3 mm in diameter, Cabela's Inc., Sidney, NB) to distinguish their feces from those of the males. Fecal samples were collected daily until 2 February and weekly thereafter until 10 April, and frozen until analysis at the Zoo.

Fecal steroids were solubilized using a modification of the method of Shideler et al. [12] by Bauman and Hardin [13]. Approximately 0.5 g of fecal material was shaken overnight in 5 mL modified phosphate-saline buffer containing 50% methanol, 0.1% bovine serum albumin and 5% Tween 20 (polyoxyethylene sorbitan monolaurate, a surfactant). Following centrifugation, supernatants were decanted and stored at  $-70^{\circ}\text{C}$  until assayed. Solid matter remaining in the extraction vials was weighed after drying overnight at  $100^{\circ}\text{C}$ .

Fecal estrogens were quantified using the Ultra-Sensitive Estradiol DSL-4800 kit (Diagnostic Systems Lab, Webster, TX, USA). The antibody has a high affinity for estradiol and low cross-reactivity with other naturally occurring estrogens (2.4%, estrone; 0.20%, estrone-D-glucuronide; 0.01%, estrone-3-sulfate; 0.34%, equilin; 3.40%, D-equilenin; 0.21%, 17-estra-

Table 1  
Dates of deslorelin implant insertion, artificial insemination or natural mating and parturition for five female gray wolves

Female	Ovuplant inserted	Artificial insemination	Natural mating	Parturition
A	18 January	–	28 January to 4 February	5 April
B	17 January	24 and 28 January	–	–
C	18 January	–	28 January to 4 February	4 April
D	17 January	24 and 28 January	–	–
E	17 January	24 and 28 January	–	5 or 6 April

diol; 0.21%, 16-ketoestradiol; 2.56%, 17-estradiol-3-glucuronide; 0.17%, estradiol-3-SO<sub>4</sub> and 0.64%, estriol). The assay has a sensitivity of 2.2 pg/mL, with the lowest standard used being 3 pg/mL. The intra-assay coefficient of variation was 7.48% and the inter-assay coefficients of variation for four separate internal controls were 16.5, 14.2, 14.0 and 15.9% for serum, and 20.27, 17.95, 16.58 and 21.76 for fecal assays.

Serum progesterone and fecal progestins were measured using an Active Progesterone DSL-3900 kit (Diagnostic Systems Laboratories Inc.). The antibody cross-reactivities are, progesterone, 100%; 5 $\mu$ -pregnane-3,20-dione, 6%; 11-deoxycorticosterone, 2.5%; 17 $\mu$ -hydroxyprogesterone, 1.2%; 5-pregnane-3,20-dione, 0.80%; 11-deoxycortisol, 0.48% and 20-dihydroprogesterone, 0.10%. The assay has a sensitivity of 0.12 ng/mL, with 0.4 ng/mL being the lowest standard used. Intra-assay coefficient of variation was 4.5%, and the inter-assay coefficients of variation for three internal controls were 9.8, 6.1 and 7.2 for serum, and 14.29, 9.31 and 10.24 for feces.

Dates of mating and artificial insemination for deslorelin-treated females were compared to previous mating dates in Wildlife Science Center records ( $N = 26$ ) to test the hypothesis that estrus was induced at an earlier date than would have occurred spontaneously.

### 3. Results

At least three of the five wolves treated in 2003 showed fertile estrus after deslorelin treatment. Relaxin concentrations on 1 March 2003 indicated that one artificially inseminated (E) and both naturally mated females were pregnant. The two naturally mated females (A and C) gave birth on 4 and 5 April, and the artificially inseminated female E on the morning of 6 April (Table 1).

Only one female (E) was judged to be in cytological estrus (98% superficial cells) on 24 January, 7 days after implant insertion (Table 2). She was inseminated, using a modification of the Norway catheter, with fresh semen (2.5 mL) containing approximately 750 million spermatozoa, of which 90% were progressively motile. Because the catheter could not be passed through the cervix, semen was deposited deep in the vagina. Vaginal smears of females B and D contained 78 and 51% superficial epithelial cells, respectively, almost all of which were nucleated (Table 2), indicating proestrus, but not estrus. Attempts to pass the insemination catheter were unsuccessful in both of these females. Smears from all three females contained numerous red blood cells, but no leukocytes.

On 28 January, 11 days after implant insertion, the vaginal smear of female B contained 90% superficial cells. However, because the catheter could not be passed through her cervix, fresh semen (3 mL containing approximately 900 million spermatozoa, of which 80% were progressively motile) was deposited deep into her vagina. Three millilitres fresh semen containing approximately 2000 million spermatozoa, 90% of which were progressively motile, was deposited into the cervical opening of female D, who had 71% superficial cells. Female E, with the highest percentage of anuclear superficial cells (Table 2), also was inseminated cervically with 300 million spermatozoa, 90% of which were progressively motile.

The deslorelin-treated females housed with males also mated on 28 January. However, because they subsequently continued to mate until 4 February, 10–17 days after implant insertion, their exact dates of conception could not be calculated. Relaxin concentrations on 1 March 2003 indicated that both were pregnant. Parturition occurred on 4 and 5 April (Table 1), resulting in possible gestation lengths of 60–68 days, counting from the first and the last mating

Table 2

Serum concentrations of progesterone (and percentage of nucleated and anucleated superficial epithelial cells in vaginal smears) on dates of deslorelin implant insertion, artificial insemination (AI) and at mid-gestation in three wolves subjected to AI and two naturally mated between January 29 and February 4

Date	Female A	Female B	Female C	Female D	Female E
17/18 January <sup>a</sup> implant	1.1 <sup>b</sup>	1.5	2.1	2.3	1.7
24 January <sup>c</sup> AI		2.2 (70%/8%) <sup>d</sup>		2.7 (50%/1%)	3.3 (64%/35%)
28 January <sup>c</sup> AI		5.2 (73%/17%)		11.4 (50%/21%)	11.9 (36%/62%)
1 March <sup>e</sup>	16.8	15.4	9.4	33	24.8

<sup>a</sup> Deslorelin implant insertion.

<sup>b</sup> Serum progesterone concentration (ng/mL).

<sup>c</sup> Artificial insemination.

<sup>d</sup> Percentage of nucleated/anucleated superficial cells in vaginal smears.

<sup>e</sup> Mid-gestation estimated date in pregnant females (A, C and E).

date. In previous years, each female had given birth to pups on 21 April and 5 May. The only AI female (E) diagnosed to be pregnant on 1 March, gave birth to pups at 67–68 days after the second insemination (Table 1), within 1–2 days of the naturally bred females.

Historic mating dates ranged from 1 February to 4 March (mode: 16 February, mean: 19 February, standard deviation (S.D.): 7.1 days). A Shipiro–Wilk test showed the historic mating dates to be normally distributed. Thus, the second AI date on 28 January was not only earlier than any previously observed mating, but was 3 S.D. earlier than the mean mating date for the colony.

Surges in fecal progesterin concentrations were detected on days 16 and 18 days, respectively, for both females following deslorelin insertion on 12 January 2004 (Fig. 1). The female housed with an adult male (B) mated the day before the surge (17 days after implant insertion), but after progesterins had begun to increase. Then, fecal progesterins declined, increased again, and remained elevated until late March. For female C (who was not housed with an adult male), despite a fecal progesterin surge at day 16, progesterin concentrations did not subsequently remain elevated, but increased almost

2 months later, in late March. Samples were not collected beyond 10 April, so the duration of the rise was not determined.

#### 4. Discussion

To our knowledge, this is the first report of successful induction of estrus and ovulation followed by the birth of live pups using either natural mating or artificial insemination in a wild canid. Typical mating dates for wolves in this colony are mid- to late February, and the average length of proestrus, judged by erythrocytes in vaginal smears, has been documented to be 6 weeks [14]. Thus, in mid-January when the implants were inserted, the females may have been in early proestrus. More likely, however, estrus in these females was induced and not naturally occurring. Colony records of the last 25 years revealed the earliest mating to be 1 February for only one pair and the mode was 16 February. Because all previous matings occurred considerably later than the dates recorded for the deslorelin-treated females in the present study, we feel confident that estrus and the subsequent ovulations were indeed induced by the implants.

The intervals between implant insertion and first mating was 11 days for the two females in 2003, and 17 days for the one female in 2004 with access to a mate, emphasizing variability in response. However, implants were inserted a week earlier in 2003, which may have affected the time to response. Proestrus may have been more advanced in the females implanted later, so that stimulation evoked a more rapid response.

During 2003, the onset of mating activity in the two naturally bred females coincided with the last day of artificial inseminations in the remaining three females, which suggests that the inseminations were performed too early. This suggestion is further supported by the fact that the three females that conceived all gave birth within 1–2 days of each other. Gestation lengths calculated for the naturally mated and artificially inseminated females ranged from 60 to 68 days, depending on whether the first or last day of observed mating was used. Gestation in the wolf has been reported to range from 60 to 66 days [15], based on observed matings and first detection of puppies. However, neither those observations nor those in the present study were continuous, so the true range has not been established. Gestation in the domestic dog, calculated from first day of mating until parturition, is 57–72 days, with a mean of 65.3 days [16]. Although it is not known how many days spermatozoa can survive and remain fertile in the wolf's reproductive tract, the

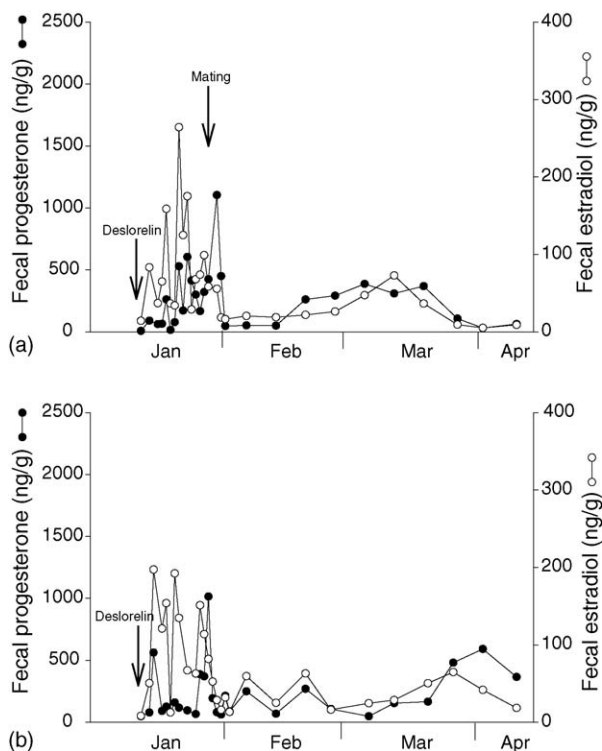


Fig. 1. Fecal estrogen and progesterin concentrations for two gray wolf females implanted with deslorelin on 12 January 2004: (a) female wolf B and (b) female wolf C.

gestation length of 67 or 68 day for the artificially inseminated female does fall within the range for the dog.

Although the implants were allowed to remain in place, the pregnancies were not interrupted by the continued presence of the implants, which might have resulted in down-regulation of LH, as has been reported in some instances when deslorelin was used in mares [17,18]. Because samples for steroid hormone measurement could not be collected regularly following insemination, we cannot separate failure of the implants to adequately stimulate estrus and ovulation from failure to achieve fertilization in the two females that did not give birth or from possible down-regulation of LH that might have interrupted pregnancies.

Steroid hormone results from two females during the second year suggest that estrus was induced, but fecal progesterin concentration during the post-ovulatory period were low as compared to data for pregnant or pseudopregnant wolves (unpublished). Although female B showed a subsequent rise in fecal progesterins in mid-February, the typical time of estrus and ovulation, the progesterin increase in late March for female C suggests that ovulation may have been delayed until beyond the period typical for the species. Although fecal estrogen and progesterin concentrations are not directly comparable quantitatively to serum concentrations, in our experience with both gray and Mexican wolves the patterns of excretion are similar.

In domestic dogs, a common objective is to be able induce fertile estrus at any time during the year, whereas in Mexican wolves it is preferable that conceptions occur near the natural breeding season. Our goal is to control the estrous cycle for scheduling the time of artificial insemination, not out of season breeding. To that end, deslorelin implants hold promise as a management aid for the Mexican wolf captive breeding program. However, further study is necessary to better define and control the interval between implant insertion and ovulation for optimal timing of insemination.

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