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Integrity of mating behaviors and seasonal reproduction in coyotes (*Canis latrans*) following treatment with estradiol benzoate

Debra A. Carlson [a,*], Eric M. Gese [b]

[a] *Department of Wildland Resources, Utah State University, Logan, UT 84322-5230, USA*
[b] *United States Department of Agriculture, Animal Plant Health Inspection Service, Wildlife Services, National Wildlife Research Center, Utah State University, Logan, UT 84322-5230, USA*

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ABSTRACT

Coyotes (*Canis latrans*) are seasonally monestrous and form perennial pair-bonds. Breeding is dominated by each pack's alpha male and female, and both sexes share responsibility for territory defense and pup-rearing. They are also opportunistic predators on domestic livestock and pets. But while dominant adults have been implicated as primary killers, depredation is reduced when coyotes are without pups. Contraception, therefore, may represent a non-lethal solution for conflicts between coyotes and humans. Steroid hormones successfully control fertility in some species, but have been considered contraindicated in wildlife and canids in particular; specific concerns include possible induction of aberrant behavior, or uterine and hematopoietic pathologies. Herein we describe a study examining the physiological effectiveness, health safety, and behavioral consequences following treatment of estrous coyotes with exogenous estrogen. We treated captive adult female coyotes in estrus with 0.01 mg/kg estradiol benzoate (EB), either before ($n = 5$) or immediately after ovulation ($n = 6$), then documented reproductive outcome, physiological variables and behavioral responses, during and after treatment. Pregnancy was averted in six females treated after ovulation, suggesting that appropriate timing of treatment proved crucial. A transient suppression of sexual behavior was observed, and in some cases, estrus appeared slightly lengthened. However, neither ovulation nor mating behavior was fully suppressed. Importantly, non-pregnant females (and their mates) displayed diestrous socio-sexual behavior similar to pregnant coyotes (behavioral pseudopregnancy). Furthermore, non-pregnant coyotes did not mate again until the next native breeding season, and we observed no deleterious physiological effects during diestrus or subsequent ovarian cycles.

1. Introduction

Coyotes (*Canis latrans*) are native North American wild canids. Their reproductive strategy includes social monogamy, territoriality (Camenzind, 1978; Andelt, 1985; Bekoff and Wells, 1986; Gese, 2001), and biparental care of offspring (Gier, 1968; Silver and Silver, 1969; Mengel, 1971; Camenzind, 1978; Andelt, 1985; Hatier, 1995). Confined to a single mating season each winter, coyote reproduction is regulated by the female's annual ovarian cycle, as well as seasonal fluctuation in male spermatogenesis (Hamlett, 1938; Gier, 1968; Kennelly, 1978; Sacks, 2005). Ovulation is spontaneous, synchronous, and bilateral (Hamlett, 1938; Kennelly and Johns, 1976). Gier (1968) considered early embryonic development in the coyote to be similar to the domestic dog (*Canis familiaris*); thus embryos likely pass into the uterus 8–10 days after ovulation, and placentation begins around day 16 (Holst and Phemister, 1971; Concannon et al., 1989; Tsutsui, 1989). Coyote litters averaging 3–7 pups are typically born March–May in most

* Corresponding author. Tel.: +1 435 458 9163; fax: +1 435 797 3796.
E-mail address: Debra.Carlson@usu.edu (D.A. Carlson).

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North American latitudes after a gestation of 60–63 days (Hamlett, 1938; Gier, 1968).

Within a coyote pack, the dominant (alpha) male and female control breeding and access to food resources (particularly carcasses) within their territory (Bekoff and Wells, 1986; Gese et al., 1989, 1996a). Although the coyote's diet includes a variety of foods (Gier, 1968; Andelt et al., 1987), predation of livestock can occur when domestic animals range within a coyote territory. Not unexpectedly, therefore, alpha coyotes have been implicated as the primary killers (Blejwas et al., 2002). Presumably parents are exploiting a nutritionally rich and easily accessible resource for young dependent pups, and if so, contraception represents a promising non-lethal solution to coyote-human conflicts. Accordingly, Bromley and Gese (2001a,b) demonstrated that fertility control (tubal ligation and vasectomy) significantly reduced livestock depredation, without disruption to territory or mate fidelity.

Exogenous estrogens have been used to terminate pregnancies in domestic dogs, but life-threatening adverse reactions may occur (Feldman and Nelson, 2004). After fertilization, alterations in the estradiol:progesterone ratio may affect zygote transport and embryonic development within the oviduct (Harper, 1994; Johnson and Everitt, 2000; Feldman and Nelson, 2004); and exogenous estrogen given while an embryo is in transit has been shown to result in embryonic death (Kennelly, 1969; Jöchle et al., 1975; Tsutsui et al., 2006). Tsutsui et al. (2006) also reported cases of spontaneous abortion, prolonged gestation, and reduced litter sizes in domestic dogs treated with estradiol benzoate (EB). Certain estrogen compounds and dosage regimens, however, have been associated with severe complications, such as pyometra and bone marrow suppression (Bowen et al., 1985; Miura et al., 1985; Feldman and Nelson, 2004).

In contrast to veterinary practice in the United States, a small dose (0.01 mg/kg) of EB is approved for use as a contragestive in domestic dogs in Europe (England, 1998). Among 358 pet dogs treated with 0.01 mg/kg EB after misalliance in the United Kingdom, Sutton et al. (1997) reported that 95.5% failed to whelp and there were no cases of anemia, leukopenia or thrombocytopenia. Thus drug induced pyometra and bone marrow aplasia appears to be associated with long-acting estrogen compounds, high dosages, or when products are given during the luteal phase (diestrus); whereas smaller doses of a short-acting product (such as EB) might not provoke the same complications.

Disruption of the estradiol:progesterone ratio may also have behavioral consequences that must be considered. The steroid hormones act in concert producing a profound affect on sexual behavior in many species (Pfaff et al., 1994; Johnson and Everitt, 2000) including domestic dogs (Concannon et al., 1979; Chakraborty et al., 1980). Estrogen and progesterone receptors have been identified in brain tissue and presumably have a relationship similar to those in the reproductive organs. Working with ovariectomized bitches, Concannon et al. (1979) found bitches exposed to estradiol first then treated with progesterone had greater sexual behavior scores; and the effect was more pronounced when estradiol was withdrawn. Also, bitches with estradiol continuing along with progesterone treatment displayed protracted estrous behavior.

Contraception of wildlife is associated with a myriad of technological and philosophical concerns, and suitable tools have been slow to develop (Fagerstone et al., 2002). In addition to being effective and safe, drug induced alterations in socio-sexual behaviors is an important consideration, particularly in species with a complex social structure. The present study, therefore, was designed to examine what influence steroid hormone treatment might have on intra-pair interactions of a wild canid species. Coyotes represent a good candidate for contraceptive or contragestive regimens because, as previously stated, breeding is confined to a single annual opportunity. Also, both alpha male and female defend their mates during the breeding season; thereby prohibiting access to their mates by same-sex competitors while suppressing mating among subordinate pack members. Keeping a pack intact would also help maintain social stability within the local population (Bromley and Gese, 2001a; Gese, 2001).

2. Materials and methods

2.1. Animals

Coyotes were captive born or wild caught as pups, and reared at the National Wildlife Research Center (NWRC) facility in Millville, UT, USA (41°68′N, 111°82′W). All animals were housed in outdoor enclosures with natural lighting. Single male–female pairs resided in 0.1 ha pens with access to sheltered den boxes. Three pens formed a clover-shaped cluster separated by double fencing and concrete barriers. Although physically separated, all pairs were within visual and audible range of other coyotes.

The animals were fed a commercially prepared carnivore diet (Fur Breeders Agricultural Cooperative, Sandy, UT, USA) once daily, and fasted 1 day per week. Water was provided *ad libitum*. Vaccinations were given annually against canine distemper, hepatitis, leptospirosis, parvovirus, parainfluenza, type 2 coronavirus, adenovirus, and rabies. Routine parasite control was administered as indicated. Animal care and research protocols were approved by the Institutional Animal Care and Use Committees at Utah State University (IACUC#1114) and the NWRC (QA944).

Within this colony, coyotes appeared to be synchronized and typically entered estrus mid-January to mid-February (Carlson, 2008). Pairs recruited into this study were either established (resided with each other during a previous breeding season) or introduced in October to allow formation of a pair-bond before the breeding season began. All study animals were sexually mature (Kennelly, 1978; Green et al., 2002; Sacks, 2005), and females were considered fertile having produced live pups in previous years. During 2002 and 2003, the female coyotes ranged from 3 to 11 years of age and weighed 10.9 ± 0.4 kg at the time of treatment.

2.2. Treatment protocols and reference controls

2.2.1. Protocol 1

In 2002, 10 coyote pairs were randomly assigned to either the treatment group ($n = 5$) or control group ($n = 5$). Three days after the first observed copulatory tie the female began treatment, and injections were repeated on day 5 and day 7 thereafter. Each treatment consisted of an interscapular subcutaneous injection of 0.01 mg/kg EB (Mesalin®, estradiol benzoate, 0.2 mg/ml, Intervet UK Ltd., Milton Keynes, Buckinghamshire, UK). Each control female received 0.5 ml of 0.9% sterile normal saline (NS) by interscapular subcutaneous injection.

2.2.2. Protocol 2

As described below (Section 3.1), four EB treated females from Protocol 1 became pregnant and delivered healthy full-term litters. These females, therefore, were retained in 2003 and two additional females were recruited into the treatment group. In contrast to Protocol 1, blood sampling began the day after the first observed copulatory tie and continued on alternate days until an elevation in serum progesterone concentration suggested the female had ovulated (see Section 2.7 below). Each treatment female ($n = 6$) then received an interscapular subcutaneous injection of 0.01 mg/kg EB (Oestradiol Benzoaat, estradiol benzoate, 0.2 mg/ml, Intervet International BV, Boxmeer, NA) with all females beginning treatment within 2 days of the estimated day of ovulation (day 0). Following the initial dose, two additional post-ovulation treatments were given 2 days and 4 days after the first treatment. By the same schedule, treatment-control females ($n = 3$) received a 0.5-ml interscapular subcutaneous injection of NS.

2.2.3. Colony reference group

Socio-sexual behavior and reproductive hormone profiles during breeding within this captive colony (including sexually experienced coyotes recruited for this experiment) have been studied and are reported elsewhere (Carlson and Gese, 2008). Briefly, during four consecutive breeding seasons (2000–2003) behavioral observations of 32 pairs of coyotes were recorded and categorized according to the procedure described below (Section 2.4). In addition, peripheral blood samples were collected during late proestrus, estrus, and early diestrus from a subset of 18 females; 10 mated female coyotes and 8 sequestered females (housed near their mates but separated to prevent copulation). Quantitative serum estradiol, progesterone, and prolactin concentrations were assayed, and inter-cohort (pregnant versus pseudopregnant) comparisons analyzed (Carlson and Gese, 2008). Socio-sexual behaviors and reproductive hormones were then aligned by each individual coyote's estimated day of ovulation (back calculated from day of parturition, assuming a 62-day gestation) and combined by cohort; thus characterizing a behavioral and physiological reference profile for this population.

2.3. Medical surveillance

Each time a coyote was handled for treatment, former injection sites were examined for overt signs of infection or inflammation. In addition, peripheral blood samples were routinely collected to monitor hematopoiesis via hematocrit (HCT), total white blood cell (WBC) count, and leukocyte differential; red cell morphology and platelet count estimates were also assessed. Concurrently, rectal temperatures were recorded, and vaginal secretions were examined for evidence of pyometra or pyometritis. Visual surveillance included observations for abnormal behavior such as lethargy or anorexia.

Hematology, physical, and behavioral assessments were monitored throughout diestrus and pregnancy. All treatment coyotes were re-examined in June and July for signs of latent adverse effects. Results collected from the study animals (treatment and control groups) in this experiment were compared to data previously collected from cohorts in an associated longitudinal study (Carlson, 2008). Within this study, and under the conditions described above, no adverse effects were noted following the administration of either Mesalin® or Oestradiol Benzoaat to coyotes.

During subsequent breeding seasons, 2004 and 2005, three Protocol 2 females were permitted to breed again and delivered healthy full-term litters, thus ruling out latent or long-term treatment induced infertility in these individuals. The three other females from Protocol 2 were sequestered from their mates for unrelated colony management reasons, and the single non-pregnant female from Protocol 1 was accidentally killed before another breeding opportunity (Carlson, unpublished data).

2.4. Mating behaviors

Continuous observations of the coyote pairs were conducted daily throughout available daylight hours, 07:00–18:00, January 5–March 28. The animals were habituated to low-level human activity prior to the beginning of the study, although all enclosures could be viewed through binoculars or spotting scope from sites 100–500 m away. Observers would view a pen, document any interactive behavior occurring between the mated coyotes then scan the next pen. Because this process rarely took more than 30 s per pen, all pens were viewed at least once every 5–10 min. An observer would only record a mating behavior once even if a coyote pair continued the behavior for an extended period of time (e.g. copulatory ties might last 5–45 min); however, if the behavior was terminated then re-initiated the observer would record it as distinct events (e.g. multiple mounts often precede a copulatory tie).

Characterization of social and sexual behavior (Golani and Mendelssohn, 1971; Bekoff and Diamond, 1976; Carlson and Gese, 2008) was standardized between observers and recorded. Documented appetitive and sexually explicit coyote mating behaviors included: (a) olfactory sampling (sniff/lick of the female's anogenital region by the male, female solicitation with diverted tail, and sniff/lick of the male's inguinal area by the female); (b) pre-coital mounts or mounting attempts; and (c) copulation tie/lock. Affinitive social behaviors observed in proestrus and estrus included: (a) courtship (non-antagonistic play-wrestling and play-chases, allo-grooming such as licking the face, ears

or back, also body-bumps, hip-pushes, or sleeping curled against each other); and (b) mate-guarding (the male shadowing the female around the pen walking or trotting with his head and shoulders adjacent to her flank, or when in view of a neighbor the male would stand on the female with stiff forelegs on her back, or stand over her as she lay on the ground). During diestrus, female begging was characterized by a submissive juvenile-like posture reminiscent of pups begging food from adults (tail, neck and head held below top-line of the back, rapid tail wagging, and biting, licking at male's lower jaw and mouth), which may or may not provoke a reflexive regurgitation response from the male.

2.5. Specimen collection and handling

Peripheral blood samples were collected from the cephalic or saphenous veins by venipuncture. Samples were collected during 08:00–09:30 before the animals were fed and without sedation or anesthesia. For quantitative progesterone analysis, whole blood was collected in an evacuated tube and allowed to clot at room temperature (20–24 °C) for 30–120 min. The serum was separated from the blood cells and divided into aliquots for storage. Samples to be tested within 24 h were stored at 2–7 °C, while others were frozen at ≤−20 °C for later use.

In 2002 (Protocol 1), sampling for progesterone concentrations began 3 days after the first observed copulatory tie, and a serum sample was collected on each day of treatment prior to dose administration. In 2003 (Protocol 2), blood sampling began the day after the first observed copulatory tie and was repeated every other day until the female's increasing progesterone concentrations suggested ovulation had occurred. After the first dose of EB (or NS) was given, subsequent blood samples were collected on the day of, and immediately before, the second and third doses. Thereafter in 2003, additional serum samples were collected approximately 2 and 4 weeks after treatment for evaluation of sustained luteal hormone synthesis (see Section 2.7 below).

Presence or absence of relaxin in plasma was used to diagnose pregnancy; therefore anticoagulated (sodium heparin or lithium heparin) whole blood samples were also collected. In a prior study (Carlson and Gese, 2007), pregnant coyotes tested positive for relaxin on days 25–28 of gestation and remained relaxin-positive until parturition. Thus in the present study, heparinized samples were initially collected 3–4 weeks after ovulation, and females initially testing negative were resampled 2 weeks later. All samples were promptly centrifuged and the separated plasma was stored at ≤−20 °C until testing.

Whole blood specimens for hematology were collected in ethylenediaminetetraacetic acid (EDTA) and stored at room temperature (20–24 °C). Peripheral blood smears were made as soon as possible from the EDTA samples and promptly stained. WBC count, HCT, and a leukocyte differential were performed within 8 h of collection. In 2002, baseline samples were collected at the time of the first blood draw, and new samples were collected every 2 weeks throughout diestrus and pregnancy. In 2003, an EDTA sample was collected 4 weeks after the initiation of treatment, then again 2 weeks later.

2.6. Laboratory assays

Quantitative progesterone blood concentrations were assayed by competitive binding enzyme immunoassay (EIA) (Progesterone EIA, DSL-10-3900, Diagnostic Systems Laboratories, Inc., Webster, TX, USA) using the procedure previously validated and described for coyotes (Carlson, 2008; Carlson and Gese, 2008). Serum specimens were tested the same day they were collected, while samples obtained and tested on the previous day were included in each new run to help assess and confirm changes in periovulatory progesterone blood concentrations. All samples were tested in duplicate with an intra-assay coefficient of variation (CV) threshold ≤10%. Kits from a single reagent lot were used and the inter-assay mean CV was 7.8%.

Canine relaxin was assayed using a solid-phase enzyme-linked immunoassay (ELISA) (ReproCHEK™, Synbiotics Corporation, San Diego, CA, USA). Using the procedure previously validated and described for the coyote (Carlson and Gese, 2007), the presence of relaxin was characterized by formation of a blue color within a micro-titer well. As a female progressed through her pregnancy, color development increased in intensity; whereas pseudopregnant coyotes maintained distinctively weaker (or no) color development by comparison. All initial-negative or indeterminate results were confirmed by retesting with a new sample.

Hematology variables were determined by manual laboratory methods (Davidsohn and Nelson, 1974). WBC count was performed by diluting EDTA anticoagulated whole blood (1:100) in a buffered ammonium oxalate solution (Unopette® for Platelet/WBC, Becton Dickinson and Co., Franklin Lakes, NJ, USA) and counting the number of leukocytes in a two-chamber hemacytometer. HCT was measured using a micro-capillary tube filled with EDTA whole blood and centrifuged at approximately 5000 rcf. Both WBC count and HCT were performed in duplicate and the mean calculated. Meanwhile, the peripheral blood smear was stained with a polychromatic Wright's stain and examined microscopically under high power (1000×, oil immersion). One hundred leukocytes were categorized by cell type and abnormal characteristics (if present) were noted. Also from the smear, red cell morphology and a platelet count estimate were assessed (Carlson, 2008).

2.7. Data analysis

Coyote social and sexual behaviors were categorized, aligned by the estimated day of ovulation for each individual female then compiled by study cohort. Patterns of behavior recorded in this study were compared to data similarly collected and documented for 32 mated pairs during 2000–2003 breeding seasons (Carlson and Gese, 2008). Since the behavioral patterns of treatment-control (NS) animals were found to be consistent (ANOVA, $P > 0.05$) with the behavior observed among other colony pairs, their data was combined with data from the rest of the colony, and is represented hereafter (unless otherwise noted) with the reference cohort referred to as colony.

To estimate the day of ovulation, we similarly employed historical endocrine data for interpretation of serum pro-

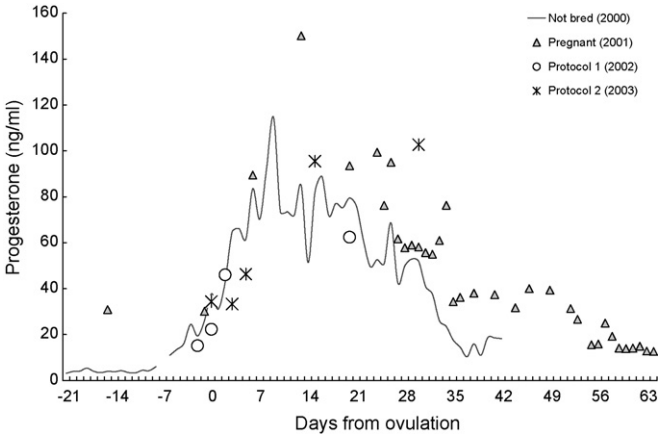

Fig. 1. Quantitative serum progesterone (ng/ml) measurements from a single female coyote during four consecutive breeding seasons, 2000–2003; aligned by the estimated day of ovulation (day 0). Reproductive history: 2000, not bred and pseudopregnant; 2001, pregnant; 2002, pregnant after treatment with 0.01 mg/kg estradiol benzoate per Protocol 1; 2003, pseudopregnant after treatment with EB per Protocol 2.

gesterone concentrations. The seven individual female coyotes treated with EB during the experimental trials (Protocols 1 and 2) were, prior to this study, enrolled in a descriptive study comparing the reproductive hormone profiles of mated and non-mated female coyotes (Carlson and Gese, 2008). The previously collected data, therefore, provided a peri-ovulatory and luteal endocrine profile against which current progesterone concentrations could be compared (see example, Fig. 1), helping predict if (and when) ovulation had occurred in each coyote. For treatment-control females, if historical data was unavailable, predictions were based on the apparent rate of change in successive progesterone concentrations. Prior experience with the quantitative progesterone EIA showed serum progesterone concentrations would nearly double around the time of ovulation (increase from day −1 to day 1; mean CV ± SE = 0.354 ± 0.055) (Carlson, 2008).

Analysis of variance (ANOVA) with repeated-measures statistical procedures (Statistical Analysis System, SAS®, version 8.2, SAS Institute Inc., Cary, NC, USA) were used to detect differences between study groups and previously collected colony data. Unless otherwise noted, we assumed a level of statistical significance <0.05.

3. Results

3.1. Reproductive outcome and hematology

In Protocol 1, female coyotes began treatment 3 days after the first observed copulatory tie, and were subsequently treated again 2 days and 4 days later. Consequently, 5 of 5 females treated with NS produced live pups, and 4 of 5 females treated with EB also became pregnant and had healthy litters. Furthermore, mean (±SE) litter size in the NS group (5.1 ± 0.4 pups) was consistent with other colony litters (5.3 ± 0.3 pups) during 2000–2003 breeding seasons, and was not statistically different ($P|t|_{0.05(2),10} \geq 0.99 = 0.346$, $F_{3,7} = 2.15$) from the mean litter size born to EB treated coyotes (6.0 ± 0.9 pups).

In Protocol 2, treatment was postponed until after the estimated day of ovulation. In this case, only 1 of 6 females treated with EB became pregnant (relaxin positive), however neither pups nor any other evidence of whelping was discovered. Meanwhile, 3 of 3 females treated with NS produced pups.

After treatment and during pregnancy, mean (±SE) peripheral WBC and HCT for NS coyotes was $11.9 \pm 0.9 \times 10^3/mm^3$ and 50 ± 1%, respectively; mean rectal temperature was 38.8 ± 0.1 °C. Similarly, mean physiological variables measured for coyotes treated with EB in Protocol 1 were: WBC, $11.5 \pm 0.5 \times 10^3/mm^3$; HCT, 49 ± 1%; and temperature, 38.9 ± 0.1 °C. At the individual level, average variation (represented as %CV from baseline pre-ovulation to prepartum) in WBC throughout pregnancy was 21.5% for NS coyotes and 21.7% for EB treated females.

Among Protocol 2 female coyotes, diestrous hematology variables were: WBC, $8.8 \pm 0.6 \times 10^3/mm^3$ and HCT, 50 ± 1%; while mean temperature was 38.6 ± 0.1 °C. It should be noted that although the WBC for NS and Protocol 1 coyotes appears slightly higher than Protocol 2, this difference was an expected normal physiological response to pregnancy. Accordingly, mean WBC among NS and Protocol 1 coyotes decreased in summer (July 2002) to $9.5 \pm 0.4 \times 10^3$ and $8.4 \pm 0.5 \times 10^3/mm^3$ respectively.

3.2. Mating behavior

We defined estrus as the phase within the ovarian cycle when the female coyote stands and accepts the male's attempts to copulate. Historically among colony coyotes, behavioral estrus ranged between 8 days before ovulation to 10 days after ovulation, with a mean length of 7.6 (±1.4SE) days (Carlson and Gese, 2008). Comparison of EB treated coyotes with colony or NS coyotes, however, yielded discrepant results. Specifically, EB treated coyotes appeared to experience an extended estrus, however, the statistical difference was only significant when compared to the colony ($P|t|_{0.05(2),27} \geq 3.12 = 0.004$, $F_{17,10} = 1.96$). When compared to the smaller group of NS animals, the treatment effect on behavioral estrus was less pronounced (Protocol 1: $P|t|_{0.05(2),11} \geq 1.61 = 0.135$, $F_{7,4} = 5.11$; Protocol

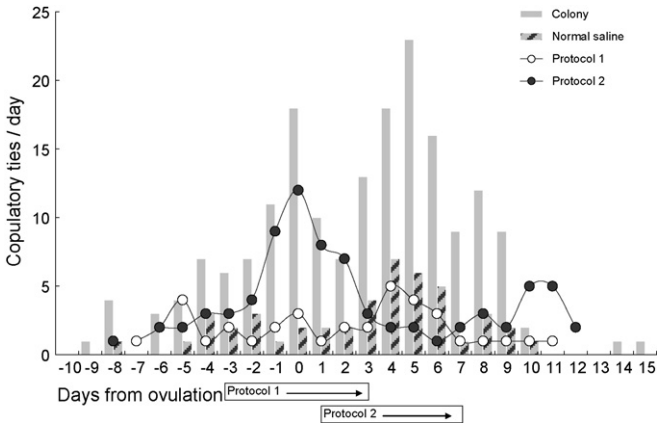

Fig. 2. Daily number of copulatory ties (aligned to the estimated day of ovulation) observed between mated pairs. Colony, 2000–2003 breeding seasons, represents generalized pattern of behavior. Treatment-controls, 2002–2003, given 0.5 ml normal saline showed no placebo effect and are grouped together. Duration of treatment (0.01 mg/kg estradiol benzoate) indicated below *x*-axis; Protocol 1 (2002) started after first observed tie; Protocol 2 (2003) started after the estimated day of ovulation (day 0).

2: $P|t|_{0.05(2),12} \geq 1.58 = 0.139$, $F_{5,7} = 1.09$). Nevertheless, one coyote pair in Protocol 1 was observed in a copulatory tie on day 11 post-ovulation, while three pairs in Protocol 2 copulated on day 11 and two pairs on day 12 (Fig. 2). By comparison, only 2 of 182 copulatory ties were observed after day 10 among colony pairs in previous seasons (2000–2003).

Similarly, once treatments began, the intensity of sexual activity in EB treatment groups deviated from expected trends. Coyotes within this colony typically experienced an increase in copulations near the day of ovulation (Fig. 2). In Protocol 1, however, the number of copulations within the EB cohort did not sustain the expected pre-ovulatory surge. Instead, activity during the treatment period abruptly declined (Fig. 2) with a significant change detected following start of treatment (day −5 to day −4; $F_{1,21} = 8.35$, $P = 0.009$). Visual comparison of treatment animals between the two experimental seasons also illustrates how active these particular pairs typically were prior to ovulation, and the suppressive influence of treatment (Fig. 2).

During treatment in Protocol 2, the coyotes again exhibited a suppression of sexual behavior (Fig. 2). Initially, this cohort's activity pattern declined in accordance with a lull historically seen during day 1–2 post-ovulation (Fig. 2), but the coyotes in Protocol 2 subsequently displayed a decrease in copulations (day 2 to day 3; $F_{1,21} = 5.81$, $P = 0.025$) accompanied by a trend reversal on day 3. While the mean number of daily copulations within the colony rose 85% between day 2 and day 3 (from 0.22 to 0.41 copulations per pair), copulations among Protocol 2 pairs decreased 57% from 1.17 to 0.50 copulations per pair.

Nevertheless, 4 of 5 pairs in Protocol 1 and 4 of 6 pairs in Protocol 2 did copulate at least once during treatment. Furthermore, after the treatments were concluded, there was a rebound in activity synchronous with the colony's established pattern (Fig. 2). This rebound, however, appeared to be better timed for Protocol 1 because it coincided with the peak sexual activity generally seen during day 3–6 post-ovulation (a period we hypothesize to be of optimal fertility); and 4 of 5 females in this cohort became pregnant.

In contrast, 5 of 6 pairs in Protocol 2 resumed copulating after treatments were terminated (day 5 and day 6), but only 1 of 6 females became pregnant. This cohort was also distinctive because of atypical activity after day 10 post-ovulation. As previously mentioned, three pairs in Protocol 2 copulated on day 11; however one pair did not tie between the day following the first treatment (day 2) and day 11 (which lasted <1 min). Also, a second pair was never observed in a copulatory tie between the initiation of treatment (day 2) and day 10, when they did tie; although they copulated again on day 11 and day 12.

Other mating behaviors, meanwhile, appeared to be less affected by treatment with EB (Fig. 3). Patterns of courtship and mate-guarding among Protocol 2 coyote pairs were similar to behavior previously documented for other mated coyotes ($F_{41,41} = 1.27$, $P = 0.443$; and $F_{27,24} = 1.15$, $P = 0.734$, respectively). Also, while the pattern of copulatory ties was significantly different between Protocol 2 treatment pairs and other coyotes ($F_{20,19} = 4.48$, $P = 0.002$), other appetitive behaviors such as olfactory sampling ($F_{38,37} = 1.69$, $P = 0.115$) and mounting attempts ($F_{29,27} = 1.95$, $P = 0.084$) were not (although mounting attempts showed the same precipitous decline during treatment as copulations).

Meanwhile, a begging behavior unique to diestrus emerged, and was performed by both pregnant and non-pregnant females ($F_{13,9} = 1.81$, $P = 0.378$). On several occasions observers confirmed that a male had regurgitated. In other cases, because of an animal's orientation, it was not possible to see food expelled but the female abruptly stopped begging and appeared to consume something (presumably regurgitate) off the ground where the male had been. Sometimes the males moved away, attempting to evade their mate's mouth-licking, but reprimands were rare. The earliest event of begging was observed on day 6 post-ovulation between a treatment pair that copulated but did not become pregnant. Begging was subsequently witnessed among other coyote pairs (pregnant and non-pregnant) throughout the following weeks,

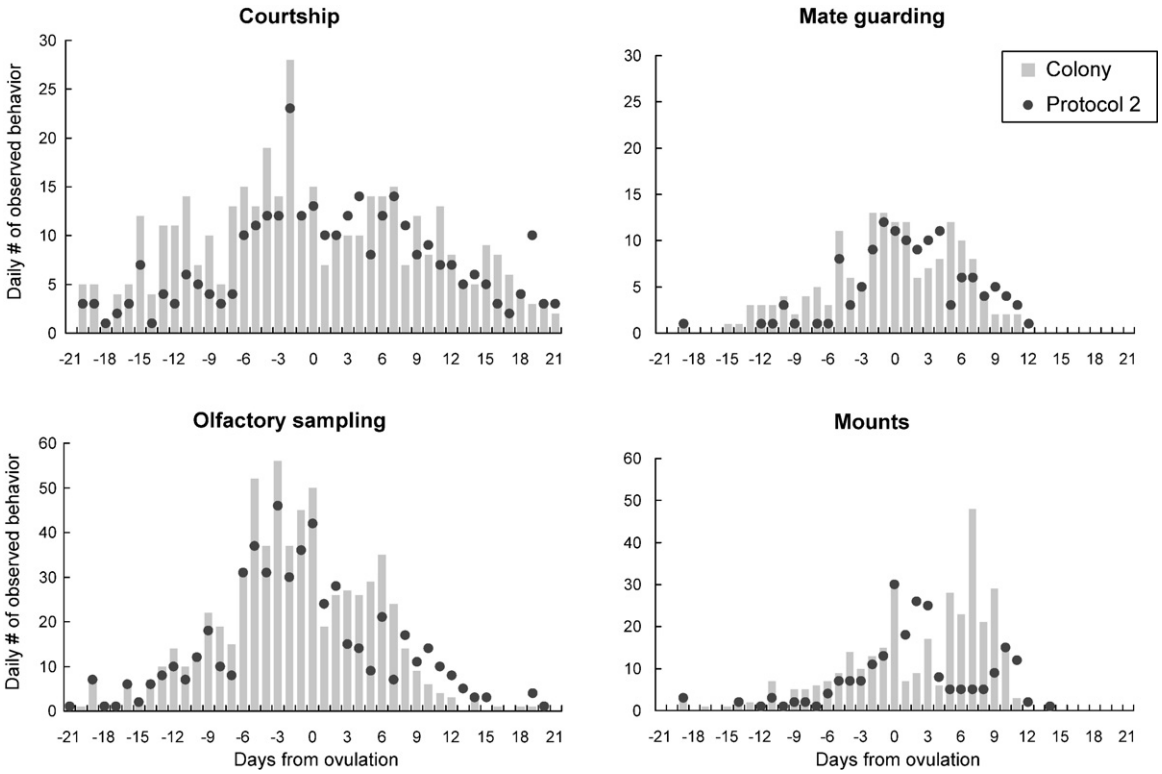

Fig. 3. Number of affinitive (courtship and mate-guarding) and appetitive (olfactory sampling and pre-coital mounts) behavior events recorded for Protocol 2 coyote pairs during 2003 breeding season. Colony represents typical pattern of coyote mating behavior aligned to the estimated day of ovulation (day 0) during 2000–2003 breeding seasons. Treatment with 0.01 mg/kg estradiol benzoate ranged from day 0 to day 6.

but termination of regular data collection precluded documentation after week 8.

4. Discussion

In the present experiment, pregnancy was averted through administration of 0.01 mg/kg estradiol benzoate; however, timing was found to be critical. When the initiation of treatment was based on the first copulatory tie (the start of estrus) reproduction was unimpaired. Four of five females in Protocol 1 ovulated during the treatment period, and resumed normal sexual activity within adequate time for successful fertilization. Meanwhile, perturbation of sexual behavior appeared transient, and there was no adverse affect on hematopoiesis or subsequent fertility.

The exception in Protocol 1 was unusual not only because this female failed to become pregnant, but also because a retrospective analysis of serum progesterone concentrations revealed she was the only female who ovulated before treatment began. In this particular case, treatments spanned day 1–5 post-ovulation; and although this coyote pair was observed in a copulatory tie during treatment, the result was very different. Interestingly, this was also the only pair in this group seen in a tie after day 10.

We assumed the coyotes had ovulated when a significant and sustained increase in individual serum progesterone was detected and hormone concentrations appeared consistent with data previously recorded (for example, Fig. 1). Also, there was a presumed opportunity for fertilization because all females copulated after ovulation, although the pattern of sexual activity varied. For example, during Protocol 1, one female experienced a split-estrus, copulating only on day 1 and day 6 post-ovulation, but had a healthy litter of four pups. Ironically, this same female failed to become pregnant in Protocol 2 although she copulated almost daily (day 0 through day 8, except day 5) during and after treatment.

While it was beyond the scope of the present study to address the specific mechanism by which EB prevented pregnancy, observations suggest fertilization could have occurred, and embryonic development, or nidation, may have been impaired. For example, plasma samples from the sole pregnant female in Protocol 2 yielded unusual relaxin results. In previous years, >90% of pregnant coyotes tested positive for relaxin by day 27 post-ovulation, yet the first sample taken from this female on day 28 was negative. The next sample collected on day 42 (although positive) had a weaker response pattern than expected and more typical of results observed on day 32. We speculate that in this particular case blastocyst(s) development may have been retarded; either because of adverse changes in oviducal fluid, or through a more direct embryotoxic effect of estradiol (Kennelly, 1969; Jöchle et al., 1975; Tsutsui et al., 2006). But with embryonic demise incomplete, a placenta (the site of relaxin synthesis in canines) could, nevertheless, have been established (Tsutsui et al., 2006). Subsequent normal development of the placenta and/or fetus, however, was

compromised because the pregnancy ultimately failed and pups were never seen.

This case was also distinctive for the greater number of copulations recorded in late estrus, seven times during day 10–12 post-ovulation; and these ties were unusually short, only 1–3 min each. Interruption of sexual activity (split-estrus) may be attributed to the iatrogenic estradiol surge (because most of the females had a suppression of mounts and ties). But the greater incidence of extended estrus (which occurred in 3 of 6 non-pregnant females as well as the failed pregnancy case described above) cannot be adequately explained in the present study; although it would be consistent with the domestic dog model to presume that re-emergence of sexual activity was linked to estradiol withdrawal and normalization of the estradiol:progesterone ratio (Concannon et al., 1979; Chakraborty et al., 1980). Such a rebound effect, however, would only be outstanding if outside the established range for a normal estrus (i.e., >10 days post-ovulation). Otherwise, a rebound occurring during the expected estrus time-frame might go unnoticed. Unfortunately, the frequency, duration, and resolution of the hormone data collected were inadequate for a retrospective analysis of such a rebound effect.

After estrus, sexually explicit behavior waned, however, mutually attentive and tactile behaviors associated with courtship continued. Overt mate-guarding also diminished, but the coyotes would play-chase and travel together around the pen; while allo-grooming, hip-pushes, and body-bumps occurred randomly throughout the day. Females also begged and received regurgitated food from their mates. Begging by non-pregnant coyotes is particularly interesting because it represents a behavioral component to covert (physiological) pseudopregnancy previously described for coyotes (Carlson and Gese, 2008).

All non-pregnant coyotes remained with their mates and were periodically observed for recrudescent sexual behavior suggesting aseasonal or premature relapse of estrus, but none was ever seen. Intra-pair interactions become increasingly quiescent as summer approached, and the behavior of pairs treated with EB appeared consistent with other colony pairs throughout the fall.

To our knowledge this is the first successful use of estradiol benzoate as a contragestive in a mated wild canid. Timing, however, was critical and post-ovulation administration appeared to be imperative for an effective outcome. It remains unknown how late in estrus EB might be effectively and safely administered. Superimposing exogenous estrogen (even in small doses) on endogenous progesterone may have deleterious physiological consequences; therefore treatment with EB should only be attempted when the female is in a stage of her ovarian cycle that can be precisely assessed and she is under close medical supervision.

Behaviorally the pair-bond appeared to be durable and resistant to the transient perturbation induced by treatment with EB. Importantly, treatment pairs not only resumed normal sexual behavior but they also proceeded to display behaviors characteristic of a pregnant diestrus (behavioral pseudopregnancy). We hypothesize that such behavioral consistency and longevity might serve as reinforcement for the pair's long-term social bond. Even without pups, perpetuation of the pair-bond would benefit the reproductive fitness of free-roaming coyotes. Territory maintenance and defense requires both the male and female's vigilance year-round, and because residents have the advantage, a coyote pair working cooperatively through the summer and fall will maintain an optimal position for successful reproduction in the next breeding season.

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