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REPRODUCTIVE BIOLOGY OF THE COYOTE (*CANIS LATRANS*):  
INTEGRATION OF BEHAVIOR AND PHYSIOLOGY

by

Debra A. Carlson

A dissertation submitted in partial fulfillment  
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Wildlife Biology

Approved:

---

Eric M. Gese, PhD  
Major Professor

---

Ramona T. Skirpstunas, DVM, PhD  
Committee Member

---

Thomas D. Bunch, PhD  
Committee Member

---

Michael L. Wolfe, PhD  
Committee Member

---

Frederick F. Knowlton, PhD  
Committee Member

---

Byron R. Burnham, EdD  
Dean of Graduate Studies

UTAH STATE UNIVERSITY  
Logan, Utah

2008

## ABSTRACT

Reproductive Biology of the Coyote (*Canis latrans*):

Integration of Behavior and Physiology

by

Debra A. Carlson, Doctor of Philosophy

Utah State University, 2008

Major Professor: Dr. Eric M. Gese  
Department: Wildland Resources

Wild *Canis* species possess a unique suite of reproductive traits including social monogamy, copulatory lock/tie, and biparental care. Females are seasonally monestrous and experience an obligatory pseudopregnancy after spontaneous ovulation. While these characteristics have been ascribed to coyotes, an integrated profile of behavior and physiology has not yet been described. In this study, temporal correlations between steroid hormone levels and socio-sexual mating behaviors were documented, as were changes in vaginal epithelium. Pseudopregnancy was compared to pregnancy by contrasting hormone (progesterone, estradiol, prolactin and relaxin) profiles of unmated females to patterns obtained in alternate years when they bred. Meanwhile, social interactions between pseudopregnant females and their mates appeared similar to pregnant coyotes, suggesting a proximate role of pseudopregnancy in pair-bond enforcement. Finally, out-of-season stimulation of ovarian hormones and estrous

behaviors suggested that reproductive seasonality of the coyote may possess some degree of plasticity, providing an adaptive response mechanism to environmental change.

(214 pages)

## DEDICATION

To Poodle & Mr P, Siren, Betsy NP, Xena, Loosey & Buggy, TW & Buster, Ilean, Sybil, CJ, Rocket & Mr Rocket, Leadhead, Spawn & Bear, South40 pair, Ms M, Papa, Beavis & Mrs B, Friend, 10.2 pair; and all the other coyotes I came to know so well. Herein I describe what I thought I should learn, yet I am incapable of putting into words what they ultimately taught me, in their own way.

I will never forget.

To Gryphon's Nemesis and Tru Blue Heather, divas both, who went with me anywhere, anytime, tails aloft.

No one has ever had better counselors.

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Michael Ebinger and David Stoner thankfully indulged the periodic need for philosophical discourse that I came back to school to find; and when the universe and I collided wills, their kind condolences were welcome during ensuing personality failures.

I appreciate the time and thoughtful critiques of my committee members: Tom Bunch, Tom DeLiberto, Eric Gese, Doug Hammon, Fred Knowlton, Ramona Skirpstunas, and Mike Wolfe. Even my comprehensive exams and defense were fun. I am particularly grateful to Fred Knowlton who generously shared his experiences and time with me long before I coerced him onto my committee. His expansive interest in my project affirmed and bolstered my own enthusiasm. And to Eric Gese whose advice, encouragement, and support have been invaluable to his foster-student.

My ultimate gratitude, of course, belongs to Frances B. and Dorothea A. Carlson who forgot to tell their daughter there might be limitations other than the sky.

Debra A. Carlson

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## CHAPTER I

### INTRODUCTION

The coyote (*Canis latrans*) is one of eight extant species of the genus *Canis*, with three native to North America: coyote, gray wolf (*C. lupus*) and red wolf (*C. rufus*). Coyotes are adaptable omnivorous predators that continue to survive despite eradication campaigns and recreational hunting; however other canid species are considered threatened or in danger of extinction (e.g., *C. l. baileyi*, *C. rufus*, and *C. simensis*).

Conservation efforts are underway for threatened canines and some programs include captive breeding and reintroduction. Knowledge gained through the study of coyote reproductive physiology and behavior may provide insight into reproductive strategies and tactics of other *Canis* congeners, suggesting solutions to difficulties encountered in captive breeding. Alternatively, fertility control may be required for expanding populations of reintroduced wild canines, particularly if they disperse into areas where their presence is unwanted. But more specifically, investigation into the reproductive characteristics of coyotes helps illuminate some adaptive strategies of a resilient species.

It should be noted that Chapters III-VI within this dissertation were written as individual manuscripts for submission to scientific publications. The content and format of these chapters, therefore, vary slightly conforming to the style of journal (specified in a footnote at the beginning of each chapter) in which the data might best fit. Since the introduction of each manuscript includes a literature review pertinent to the data presented, the following review (Chapter II) of reproductive physiology and behavior is a bit redundant. However, certain supporting information about canine reproduction, too

burdensome for a publication manuscript, is nevertheless included here. While this information may be tedious for some readers, others may find a review helpful to better understand the nuance of information proffered from this research.

As cited in Chapter II, previous work in coyote reproduction investigated gross anatomy, ovarian histology, estrous steroids, gestation length and litter size; depending on the technology and resources available at the time of study. This current project extends our knowledge of coyotes by contributing descriptions of additional reproductive hormones (prolactin and relaxin), as well as, comparative profiles of pregnant and non-pregnant females. The endocrine profiles, described primarily in Chapter IV, also present estradiol and progesterone secretion patterns yet with finer resolution and over a broader period of the estrous cycle than previously reported.

It is important to recall that hormone synthesis is dynamic during the ovarian cycle, fluctuating accordingly within and between the various phases. Periodic pulses may be spontaneous or in concert with other secretory products, stimulating or suppressing physiological and behavioral systems (e.g., transmigration of vaginal epithelium or evocation of proceptive and receptive behaviors). There can also be notable individual variability between females, even on the same day of the cycle. Thus mean values must be judiciously considered, with awareness of phase and timescale for which they are reported. To control for individual variability, I worked with the same set of females through each year of the study. I used daily sampling, aligned to the day of ovulation, to calculate weekly mean values (reported in Chapter IV); meanwhile use of a contemporary steroid enzyme-immunoassay (EIA) methodology provided enhanced



sensitivity. The resulting data differentiating pregnancy from pseudopregnancy served thereafter as physiological reference parameters for subsequent experiments.

Socio-sexual mating behaviors were also temporally aligned by day of ovulation; and correlations between behavior and reproductive hormones are also described in Chapter IV. Coyotes (like other wild *Canis* species) have a complex and unusual mating strategy not often found in mammals; they are socially and perennially monogamous, and males assist in pup-rearing. Yet mate fidelity and male parental care in wild canids has rarely been addressed. Hopefully, description of mating behaviors in this dissertation will stimulate further ethological work; for example: If the domestic dog abandoned monogamy and biparental care through its association with humans, how flexible (or stable) might these strategies be in wild canids? Or by extension: If coyotes (ironically) find refuge from hunters by relocating closer to humans in cities, will constant access to year-round resources render paternal care superfluous? How strong might a male coyote's fidelity be if his mate isn't pregnant; and accordingly, what is the enforcing mechanism that maintains the perennial pair-bond?

The experiments reported in Chapters V and VI barely begin to address these questions, but they were insightful nevertheless. In one experiment, females in estrus were treated with low-dose short-acting estradiol benzoate that effectively terminated pregnancy prior to embryonic implantation. Subsequent socio-sexual interactions between non-pregnant coyotes and their mates were interesting because they were indistinguishable from behavior observed when the females were pregnant. In another experiment, treatment of females in fall (during late anestrus) with a gonadotrophin-releasing hormone (GnRH) analog provoked ovarian steroid synthesis. Furthermore,

sexual behavior reminiscent of the native winter breeding season were displayed by both the treated females and their mates, but in October.

The importance of an integrated perspective when addressing reproduction can not be over-emphasized, particularly for species with unique and complex suite of features. Coyotes are closely related to domestic dogs and hybrid offspring are fertile. Nevertheless, coyotes are not dogs. Coyotes maintain an evolved mating strategy that is uncommon among mammals (and absent in dogs in particular). The following chapters describe some proximate associations between hormones and behavior, as well as, hypothetical extrapolations regarding the ultimate role of these relationships. Since it remains arguable whether certain features of coyote reproductive ecology are compulsive or facultative, hopefully this dissertation will stimulate further investigations into the adaptive potential of coyotes and other wild canids.

## CHAPTER II

### PREVIOUS WORK – LITERATURE REVIEW

#### COYOTE REPRODUCTION

Coyotes are seasonally monestrous (Gier 1968; Hamlett 1938; Kennelly and Johns 1976; Stellflug et al. 1981). Reproductive recrudescence is initialized in the late fall followed by mating in mid to late winter. After a 60-63 day gestation, litters averaging 3-7 pups (extremes of 1-12) are born March – May in most North American latitudes (Clark 1972; Gier 1968; Hamlett 1938; Kennelly et al. 1977; Knowlton 1972; Nellis and Keith 1976).

Coyotes are also socially monogamous and territorial (Andelt 1985; Bekoff and Wells 1982, 1986; Bromley 2000; Bromley and Gese 2001; Camenzind 1978; Gese 2001) with both sexes participating in establishment of a territory and defense from potential challengers. In addition, both sexes provide food to pups after weaning, and subordinate helpers (usually older offspring) assist in defense and den sitting (Andelt 1985; Bekoff and Wells 1982, 1986; Camenzind 1978; Gier 1968; Hatier 1995; Mengel 1971; Silver and Silver 1969). Typically only a pack's dominant male and female mate and produce offspring (Andelt 1985; Bekoff and Wells 1982, 1986; Gese 2001; Gese et al. 1989, 1996). Depending on several ecological and social factors, pups begin dispersing from their natal territories as early as 6-9 months of age, or they may remain as subordinate associates (Andelt 1985; Bekoff and Wells 1982, 1986). Bekoff and Wells (1982) reported 83% of helpers remaining with their parents were male, suggesting

that females are more often the sex that disperses first. In contrast, Andelt (1985) found a slightly greater juvenile emigration rate among males than females (12% versus 4%).

Juveniles enter their first reproductive cycle at approximately 10 months of age, and are capable of successfully reproducing (Hodges 1990; Kennelly 1978; Kennelly and Johns 1976) if social constraints and environmental conditions permit (Clark 1972; Gier 1968; Knowlton 1972; Nellis and Keith 1976). Yearlings (approximately 22 months old) and adults (34 months and older) however comprise the greater proportion of breeding animals (Windberg 1995), and reports on wild populations suggest greater fecundity among adult females than yearlings (Gese et al. 1989; Nellis and Keith 1976; Windberg 1995).

While environmental and social constraints may limit the reproductive success of younger animals (Andelt 1985; Bekoff and Wells 1986; Knowlton and Gese 1995), Windberg (1995) provided physiological evidence of the increased reproductive capacity of females 3 – 10 years of age. He found that a greater proportion of females in this cohort had ovulated or had fetuses *in utero* than did females from younger or older groups. Recently submitted data by Green et al. (2002) also reported greater fecundity (based on number of placental scars) among female coyotes ages 3 – 8 years.

The fertility and seasonality of the male coyote is also well documented (Bekoff and Diamond 1976; Gier 1968; Hamlett 1938; Hodges 1990; Kennelly 1972). These studies support the conclusion that juvenile male coyotes are capable of fertilizing a receptive female. However, the same ecological constraints limiting the early reproductive success of young females would similarly affect reproduction for young males.

## REPRODUCTIVE PHYSIOLOGY

Many characteristics of reproduction are presumed to be conserved among members of the genus *Canis*, with some similarities and differences between domestic dogs, wolves and coyotes previously described (Gier 1968; Kennelly and Johns 1976; Seal et al. 1979). The genetic relationship between wild and domestic canids has also been investigated (Wayne et al. 1989; Wayne and Koepfli 1996), and the existence of fecund hybrids has been well documented (Gier 1968; Gipson et al. 1975; Kennelly and Roberts 1969; Mengel 1971; Silver and Silver 1969; Wayne and Koepfli 1996). Genetic analyses of wild specimens have suggested the existence of gray wolf-coyote hybrids, red wolf-coyote hybrids, and the hybridization of domestic dog and Ethiopian wolf (*C. simensis*). In addition, the analyses demonstrated that hybrids have bred back into the wolf populations to such a significant degree that the genetic evidence was available through random sampling of the populations (Wayne and Koepfli 1996).

Gross anatomy and microscopic tissue examinations were the focus of primary research in coyote reproductive physiology conducted by Hamlett (1938) and later by Gier (1968). Their findings were confirmed and extended by Kennelly (1972) and Kennelly and Johns (1976). Stellflug et al. (1981), Hodges (1990), and Parrish (1994) have described some endocrine features of the coyote's estrous cycle. Knowledge about the wolf's reproductive cycle was advanced by Seal et al. (1979, 1987) and by Kreeger et al. (1991). However, the greater weight of knowledge about canine reproduction comes from work done with the domestic dog with compendiums published by several authors (Concannon et al. 1989; Feldman and Nelson 2004; Jeffcoate 1998; Olson et al. 1989).

*Anestrus*.—Descriptions of canine reproduction must inevitably include the different phases of the estrous cycle (anestrus, proestrus, estrus, and metestrus / diestrus). These phases are commonly defined by distinctive and observable patterns of behavior, hormone secretion, and vaginal cytology (Evans 1993). Anestrus is often described as a period of reproductive quiescence because there is an absence of overt sexual behavior and physiological changes remain unremarkable (Concannon et al. 1989; Feldman and Nelson 2004). The gonadotrophic hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), however, are secreted during anestrus and fluctuations are thought to be evidence of follicular recruitment (Feldman and Nelson 2004; Olson et al. 1982).

In both the domestic bitch (Jeffcoate 1993) and the wolf (Kreeger et al. 1991) plasma prolactin levels persisted throughout anestrus. Kreeger et al. (1991) demonstrated a circannual secretion pattern that was very consistent among male and female wolves “with peak levels occurring just prior to the summer solstice.” Patterns of hormone secretion in late anestrus / early proestrus also suggested some correlation between prolactin levels and the end of anestrus (Jeffcoate 1993; Jöchle 1997; Kreeger et al. 1991). Experimental studies in dogs with prolactin inhibitors cabergoline and bromocriptine have supported this observation (Jöchle et al. 1989; Okkens et al. 1997b; Verstegen et al. 1999). Also, hyperprolactinemia has been associated with the suppression of LH secretion - possibly through the down-regulation of gonadotrophin-releasing hormone (GnRH) secretion from the hypothalamus (Kordon et al. 1993).

Throughout anestrus, progesterone levels in blood remain low (Concannon et al. 1989; Feldman and Nelson 2004) but studies examining estradiol secretion during

anestrus are conflicting. Jeffcoate (1993) reported low estradiol levels in beagles until late anestrus / early proestrus, hypothesizing that such levels were due to low ovarian activity during this time. In contrast, Olson et al. (1982) demonstrated fluctuating concentrations of estradiol in mixed breed bitches throughout anestrus, with some peak levels reaching those usually associated with proestrus. They proposed that waves of follicular development occurred during anestrus allowing small bursts of estrogen production; however, luteinization did not follow, so progesterone synthesis was not detected before follicular regression. Both studies agreed that overt evidence of estrogen production (such as vulvular swelling, sanguineous discharge or vaginal epithelial changes) was absent during this phase.

While external indications of reproductive activity appear diminished during anestrus, this phase is the time when the uterus involutes and undergoes repair for the next cycle (Feldman and Nelson 2004) and its duration has been described as “obligatory” (Verstegen et al. 1999). Hamlett (1938) observed that the ovaries from coyotes taken five months after the pups had been born were still not fully restored and that the cells of the degenerating corpora lutea could still be seen. He assumed that any ovary with “fully developed corpora lutea” would not be able to mature new follicles in time for a female to enter a “second heat” in the same year.

Recent attempts to induce estrus in the domestic bitch have successfully used GnRH (Concannon 1989; Concannon et al. 1997), leuprolide acetate (a synthetic GnRH agonist) (Cain et al. 1990; Inaba et al. 1998) or cabergoline (Verstegen et al. 1999). In two of the studies, the later into anestrus the treatment was given, the more positive the treatment effect (Inaba et al. 1998; Verstegen et al. 1999).

*Proestrus.*—The beginning of proestrus is commonly defined as the time when a serosanguineous discharge from the vagina is first observed. This phase is dominated by estrogen and may range from 2 to 25 days (average of 9 days) in the domestic bitch (Evans 1993; Feldman and Nelson 2004). Hypertrophy and hyperplasia of the uterine endometrium and vaginal epithelium occur in response to the increased synthesis of estrogen (Priedkalns and Leiser 1998); also, cellular changes in the vulva, oviducts and mammary glands may be noted (Feldman and Nelson 2004).

Proestrus is also the phase of increased follicular activity when the oocytes are maturing and the follicles are preparing for ovulation. Evidence of up-regulation of LH is found in minor spikes in the blood levels that appear just prior to its end (Concannon et al. 1989; Feldman and Nelson 2004; Olson et al. 1989).

In contrast, proestrus in female coyotes can be as long as two to three months when defined by the presence of vaginal bleeding (Kennelly and Johns 1976; Stellflug et al. 1981). Playfulness and teasing by the female with a prospective mate is a common behavioral feature shared by the domestic bitch (Feldman and Nelson 2004) and the female coyote (Zemlicka 1995), but neither female will tolerate mounting by a male or permit copulation until estrus.

*Estrus.*—Estrus has two important components: first, is the behavioral aspect when the female permits a male to mount and copulate; second, is the physiological aspect when organic changes transform and prepare the female's reproductive tract for fertilization and pregnancy.

Decreasing estradiol in concert with increasing progesterone has been shown to influence the copulatory behavior observed in dogs (Concannon et al. 1977, 1979). This



same synergistic hormone pattern marks the start of the female coyote's fertile estrus (Hodges 1990; Stellflug et al. 1981), and enhanced male interest in the female is represented by such observable behavior as shadowing, genital sniffing or licking, and attempted mounts (Bekoff and Diamond 1976; Zemlicka 1995). Prior to entering estrus, the female will commonly rebuff the male's attempts to mount either with passive avoidance tactics (e.g., walking away or lying down) or with more aggressive admonitions (e.g., snapping or growling). However, when in estrus, the female will not only become more cooperative during the male's sexual attempts (Bekoff and Diamond 1976), she will also begin to solicit his interest with genital presentation, lordosis and tail flagging (D.E. Zemlicka, personal communication).

Vaginal smears remain a major tool for predicting estrus, both in the domestic bitch (Feldman and Nelson 2004) and in the female coyote (Kennelly and Johns 1976). Under the influence of proestrous estrogen secretion, the epithelial lining of the vagina thickens causing the cells lining the lumen to be pushed farther from their sustaining blood source (Feldman and Nelson 2004). In response, these cells become increasingly more keratinized (Priedkalns and Leiser 1998). Together, the additional layers of epithelium and the keratinization of the superficial layer help to protect the vagina from injury during copulation (Feldman and Nelson 2004; Priedkalns and Leiser 1998). The progression of cellular change in the vaginal epithelium is closely associated with the cyclical changes of the estrous cycle, and has been characterized for the domestic bitch (Christie et al. 1972; Feldman and Nelson 2004; Priedkalns and Leiser 1998). In addition to changes in the epithelial cells, the presence or absence of erythrocytes and leukocytes may also be observed on a vaginal smear. For both the domestic bitch (Feldman and

Nelson 2004) and the female coyote (Kennelly and Johns 1976) leukocytes will be present during proestrus and diestrus but absent during estrus.

*Ovulation and implantation.*—The most profound aspect of estrus is the spontaneous ovulation of an immature oocyte and the subsequent transformation of the ovarian follicle (Concannon et al. 1989; Olson et al. 1989). A decrement in estrogen secretion precedes the sudden peak surge of LH that triggers final preovulatory changes. In domestic bitches, ovulations occur approximately two days after the LH surge and appear synchronous (Concannon et al. 1977, 1989; Tsutsui 1989). Generally, most fertile matings appear to occur between 2 days before, and 4.5 days after, ovulation (Tsutsui 1989). Similarly, evidence from the coyote suggests that estrus is also approximately 10 days (Hodges 1990; Kennelly and Johns 1976) and that ovulation is bilateral (Hamlett 1938; Kennelly and Johns 1976).

After ovulation the oocyte begins its passage through the oviduct and towards the uterus. Muscular contractions within the oviduct help propel spermatozoa to the oocyte but, after fertilization, ciliary action is the primary mode of transport for the zygote (Priedkalns and Leiser 1998). Although estrogen and progesterone are crucial for normal oviduct function, high doses of estrogen after fertilization can have a negative effect on pregnancy (Johnson and Everitt 2000; Sutton et al. 1997). Research in beagle bitches (Jöchle et al. 1975; Kennelly 1969) has suggested that a high dose of estrogen administered while the embryo was still in passage through the oviduct would prolong the transport time and result in embryonic death. In the domestic bitch, estrogen levels in blood continue to decline sharply and the blastocysts pass into the uterine horns 10 days after ovulation. They will spend another 5 days in trans-migration between the uterine

horns before implanting on day 21 or 22 following the LH surge (or 19-20 days after ovulation) (Concannon et al. 2001). In coyotes, Gier (1968) and Kennelly (1978) assumed that implantation was similar to the domestic bitch; however, Kennelly and Johns (1976) were resistant to draw conclusions about the timing of ovulation and conception.

After ovulation the ruptured follicle is called the corpus luteum and becomes a critical, although temporary, endocrine organ. Although the space previously occupied by the oocyte has collapsed, hypertrophy and hyperplasia of the cells within the corpus luteum give rise to a structure larger than the parent follicle (Priedkalns and Leiser 1998). Because the corpora lutea can dominate intra-ovarian space, Hamlett (1938) predicted that it would be virtually impossible for a coyote to ovulate again any time soon after her primary estrus.

*Diestrus.*—Because it is difficult to distinguish metestrus in the domestic bitch, authors often use the terms metestrus and diestrus interchangeably when referring to the phase after estrus (Evans 1993; Feldman and Nelson 2004; Priedkalns and Leiser 1998). The most distinguishing features characterizing the initiation of this phase are the female's rejection of the male's attempts to copulate, and a dramatic increase in progesterone secretion (Feldman and Nelson 2004). After luteinization and throughout diestrus, the primary task of the corpora lutea is the synthesis of progesterone (Concannon et al. 2001; Priedkalns and Leiser 1998). In order to maintain a viable pregnancy, progesterone concentrations must be maintained above a certain threshold, and the dog is among a few other domestic animals in which the conceptus is entirely dependent on the corpus luteum for the sustaining hormone (Johnson and Everitt 2000).

In addition, prolactin is recognized as a critical luteotrophic element supporting the corpus luteum, and thereby, the production of progesterone (Concannon et al. 2001; Gobello et al. 2001b; Okkens et al. 1997a; Onclin and Verstegen 1997a).

Another unique feature of canine reproductive physiology is the presentation of a compulsory pseudopregnancy in non-mated females (Asa and Valdespino 1998; Feldman and Nelson 2004). Even in a non-gravid diestrus, the luteal phase is nearly equal in length to that of gestation, and with similar circulating levels of progesterone and estrogen (Concannon et al. 2001; Feldman and Nelson 2004; Onclin and Verstegen 1997b). Serum prolactin levels increase during diestrus of both the pregnant and non-pregnant, although synthesis tends to be greater during pregnancy (Kreeger et al. 1991; Onclin and Verstegen 1997b). “Overtly” pseudopregnant domestic bitches often secrete elevated blood prolactin concentrations more reminiscent of true pregnancy (Gobello et al. 2001b; Okkens et al. 1997a); and may present with such symptoms as, developed mammarys, lactation, nesting, digging, weight gain, or mothering of inanimate objects (Feldman and Nelson 2004; Gobello et al. 2001a). While prolactin secretion has been investigated in wolves (Kreeger et al. 1991) and coyotes (Parrish 1994), a case distinguishing overt (clinical) pseudopregnancy from covert (sub-clinical) pseudopregnancy in wild canines has not yet been described.

While blood levels of prolactin (and in some cases progesterone) may be slightly higher during pregnancy (Concannon et al. 2001; Onclin and Verstegen 1997b); the overlap of hormone concentrations between pregnant and non-pregnant animals preclude the use of prolactin (or progesterone) as a reliable diagnostic marker (Concannon et al. 2001; Feldman and Nelson 2004; Onclin and Verstegen 1997b). However, in the

domestic bitch, relaxin can be detected in the peripheral blood after the third or fourth week of pregnancy, and is not detectable in non-pregnant animals (Steinetz et al. 1987; Tsutsui and Stewart 1991). The source of relaxin production varies among species (Sherwood 1993), but its secretion in the bitch has been shown to originate in the placenta (Steinetz et al. 1989; Tsutsui and Stewart 1991). In their study, Steinetz et al. (1987) reported that plasma relaxin remained detectable for nine weeks during lactation in one breed of dog, but interestingly, the hormone disappeared soon after whelping in another; the discrepancy remains unexplained. Nonetheless, relaxin detection in canine plasma can provide an alternative to palpation or ultrasound as a diagnostic tool for the prediction of pregnancy.

As diestrus proceeds, the vaginal wall and the vaginal epithelium regress. Examination of a vaginal smear reveals the re-emergence of nucleated squamous epithelial cells and the reappearance of leukocytes (Feldman and Nelson 2004). In contrast to domestic bitches, a bloody vaginal discharge persists through both pregnant and pseudopregnant diestrus in female coyotes and erythrocytes can be seen on vaginal smears (personal observation).

Diestrus ends with parturition or evidence of corpora lutea regression (usually in the form of decreasing progesterone secretion). Luteolysis is initiated by a series of endocrine signals originating from the conceptus and the endometrium (Concannon et al. 2001; Johnson and Everitt 2000). As the corpora lutea regress, progesterone concentrations fall and parturition soon follows (Concannon et al. 1989). Anestrus is usually said to begin with whelping, however in the non-pregnant female the delineation between diestrus and anestrus is less distinct (Feldman and Nelson 2004).

## REPRODUCTIVE BEHAVIOR

Canid mating systems are outstanding among mammals because of the rare combination of certain features: monogamy, a seasonal and monestrous reproductive cycle, biparental and alloparental care of infant pups, a prolonged proestrus and diestrus (luteal phase), pseudopregnancy, copulatory lock/tie, behaviorally enforced reproductive skew, and territoriality (Asa and Valdespino 1998). Although these characteristics play a variable role in the strategies of the different species, the commonality of these features is remarkable.

The ultimate causes of seasonal breeding explain why this is a common feature of the reproductive strategy of many wild animals. The major reason is the timing of births to coincide with the most optimal season for provisioning of the young (Bronson and Heideman 1993). But some species have been shown to be more “facultative” or opportunistic than “obligatory” and may be less strict in their adherence to a specific season. The seasonality of some species with widely distributed populations will demonstrate a relationship between latitude and seasonality, while others appear responsive to variations within their local environment. An alternative influence of seasonal breeding may involve mate defense. Sillero-Zubiri et al. (1998) hypothesized that among subpopulations of Ethiopian wolf synchronization of estrus among the dominant females monopolized the males’ attention and reduced the incidence of extra-pack copulations (Sillero-Zubiri et al. 1996).

In many animals the primary cue for seasonal breeding activity is the change in day length (Turek and Van Cauter 1993). But interestingly, while a specific

photoperiodic cue may be stimulatory for one species, it fails to evoke a response in another species even when both groups give birth in the same season (Bronson and Heideman 1993). Melatonin (produced in the pineal gland) is critical in the communication of day-length to other systems and its effect is expressed in the release of GnRH from the hypothalamus, in GnRH pulse frequency, and in the sensitivity of the pituitary to gonadal steroid hormone feedback (Turek and Van Cauter 1993). However when melatonin was given to wolves in late spring, it failed to evoke reproductive activity (Kreeger et al. 1991).

Domestic dogs remain monestrous but have lost the seasonality feature that is characteristic of wild *Canis* species and they may breed in any season of the year (Feldman and Nelson 2004). Also, captive F<sub>1</sub> coyote-dog hybrid females have been reported to shift their estrus to an earlier period in the fall (Kennelly and Roberts 1969; Silver and Silver 1969). But pinealectomy failed to affect the seasonal reproduction of captive wolves (Asa et al. 1987; Kreeger et al. 1991).

Among wild canines, it is thought that the strategic role of pseudopregnancy may facilitate the alloparental care given to pups by subordinate adult females residing with the parents (Asa 1997; Asa and Valdespino 1998; Kreeger et al. 1991; Mech 1970). Helper females bring food back to the den and defend the offspring of the dominant female; but more remarkably, in some species, they also have the capacity to suckle the infant young (coyote: Camenzind 1978; dwarf mongoose: Creel 1996). These physiological and behavioral manifestations of maternal behavior are thought to be evoked by elevations of prolactin during the latter part of diestrus (Jöchle 1997; Kreeger et al. 1991).

Communication and social interactions among coyotes have been studied in the wild (Bekoff and Wells 1986; Gese 1998; Gese and Ruff 1998) and in captivity (Bekoff and Diamond 1976; Harrington et al. 1987; Lehner 1978; Zemlicka 1995). Howling and yip-howls, territorial scent marking (such as: male/female tandem urination or defecation, urination over feces), and ground scratching, are auditory and olfactory signals that increase during courtship and mating. Visual signaling can include urination posture and scratching in view of a mate or competitor, but also a myriad of body postures, tail positions, facial expressions, and intra-pair spatial orientation will be used as communication. Tactile signals between pairs during courtship and mating can facilitate communication of general health and vigor, and reproductive status; they may also help reinforce the pair-bond during pregnancy.

After weaning much of the signaling associated with the breeding season begins to subside, even though a pair will remain together and continue social interactions with each other as well as with their pups. Communication tactics necessary for the maintenance of territory and pack cohesion will persist throughout the summer and fall, eventually intensifying as proestrus and estrus again begin to approach. These changes in behavior are believed to be hormonally driven (Packard et al. 1985; Asa and Valdespino 1998), and may be useful indicators of changing estrous phases or as evaluation tools for pharmaceutical manipulations.



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## CHAPTER III

RELAXIN AS A DIAGNOSTIC MARKER OF PREGNANCY<sup>1</sup>**Abstract**

The diagnosis of pregnancy in the domestic dog (*Canis familiaris*) often employs specialized equipment, experienced staff, and the cooperation of the bitch. These procedures can be challenging when the subject is a wild canid, particularly in a field setting. In addition, reproductive hormone assays are unreliable as a diagnostic tool because the estrous profiles of pregnant and pseudopregnant canines are similar. However, research has demonstrated that the hormone relaxin can be detected in maternal blood after embryonic implantation, but remains negligible in non-pregnant females. We investigated the use of relaxin as a diagnostic marker of pregnancy in the coyote (*Canis latrans*). A commercially available canine relaxin enzyme immunoassay (ReproCHEK™) was used to test plasma collected from 124 female coyotes over four consecutive breeding seasons. Mating activities of the captive females were observed; then peripheral blood samples were collected at intervals throughout pregnancy, as well as after parturition. Results demonstrated that relaxin could be detected in the plasma of pregnant coyotes after 28 days of gestation, and in some cases as early as 23 days, while non-pregnant females and male coyotes consistently tested negative. Relaxin also remained detectable in the plasma of the majority of females tested 10-12 weeks after parturition. This qualitative assay for relaxin proved to be a reliable diagnostic tool for

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<sup>1</sup> Content and style of this chapter has been formatted for submission to the journal *Animal Reproduction Science*. Debra A. Carlson and Eric M. Gese, coauthors.

pregnancy in the coyote. In addition, blood sampling was relatively easy, could be accomplished with minimal handling, and did not require sedation or anesthesia.

## **Introduction**

The coyote (*Canis latrans*) is a medium-sized wild canid, native to North America and closely related to the gray wolf (*Canis lupus*), red wolf (*Canis rufus*), and domestic dog (*Canis familiaris*) (Roy et al., 1994). Coyotes are considered seasonally monestrous (Hamlett, 1938; Gier, 1968) and socially monogamous (Andelt, 1985; Bekoff and Wells, 1986; Gese, 2001). While there is some regional variation in the actual breeding season, coyotes generally mate in mid- to late-winter and deliver an average litter of 3-7 pups in the spring (Hamlett, 1938; Gier, 1968; Knowlton, 1972; Gese et al., 1989). Typically, only the dominant male and female within a coyote social group produce a litter (Gese et al., 1989, 1996), although subordinate associates will help defend the pups and territory (Andelt, 1985; Bekoff and Wells, 1986; Gese, 2001).

The reproductive hormone profile of the coyote's estrous cycle has been studied (Stellflug et al., 1981; Hodges, 1990) and appears to share certain features with the patterns described for the wolf (Seal et al., 1979; Kreeger et al., 1991; Walker et al., 2002) and domestic dog (reviewed in: Concannon et al., 1989, 2001). Like the wolf and dog, differentiating a pregnant coyote from a non-pregnant female can be difficult if based on serological assessment alone. Pregnant and pseudopregnant coyotes have similar patterns of serum progesterone secretion, and the absolute concentration levels vary widely among individuals (Chapter IV this text). Also, while mid-gestation prolactin levels in coyotes rise significantly above those observed in non-gravid diestrous

females, the absolute values still overlap (Chapter IV this text). Thus single blood sampling for progesterone or prolactin seems unreliable as a method of determining reproductive status in coyotes. The hormone relaxin, however, has not yet been explored in this species.

Relaxin is a polypeptide shown to affect the reproductive tissues of mammals, most commonly “cervical extensibility and uterine contractibility” (Sherwood, 1994). The source of relaxin synthesis varies among species, but the predominant sites are the corpus luteum, placenta, and uterus (Sherwood, 1994). Depending on the species, detection of relaxin in peripheral blood is not always restricted to pregnant females; however in the domestic dog it has been established as a pregnancy-specific hormone (Steinetz et al., 1987, 1989). The site of synthesis in the bitch has been elucidated (Tsutsui and Sherwood, 1991; Klonisch et al., 1999) and primarily ascribed to the placenta, although the hormone can also be traced in the ovary and uterus. These latter tissues may be areas influenced by the paracrine deposition of relaxin. A clinical study of domestic dogs using the commercially available canine relaxin enzyme-linked immunoassay (ELISA) ReproCHEK™ (Synbiotics Corporation, San Diego, CA, USA) reported detection of the hormone in maternal peripheral blood as early as 25 days after ovulation (Buff et al., 2001).

In support of a longitudinal investigation in the reproductive biology of the coyote, a diagnostic tool was needed to easily distinguish between pregnant and pseudopregnant females, while minimizing research induced disturbances. Although there are behavioral and physiological differences between the coyote and domestic dog, there are many common reproductive features (Gier, 1968; Silver and Silver, 1969;

Kennelly and Johns, 1976; Kennelly, 1978). Therefore, we tested the use of relaxin as a serological marker of pregnancy in the coyotes with the hypothesis it would be as successful in a wild canid as it has been in its domestic congener.

## **Materials and methods**

### *Animals*

During four consecutive years (2000 – 2003), 124 intact female coyotes were tested for the presence or absence of relaxin at variable times following copulation and/or parturition. The coyotes were captive born and reared at the National Wildlife Research Center (NWRC) facility near Millville, UT, USA. All animals were housed in outdoor enclosures with natural lighting. Male-female pairs resided in 0.1 hectare outdoor pens with access to den boxes sheltered within observation buildings. Multiple pens were within visual and audible range of each other but separated by fencing and concrete barriers. Some individual animals were sequestered from their mates during the breeding season and served as unmated controls. In these cases, the females were housed in sheltered outdoor kennels which also included den boxes for privacy.

The subjects ranged from 2-12 years of age and known weights ranged from 7.6-13.8 kg. They were fed a commercially prepared carnivore diet (Fur Breeders Agricultural Cooperative, Sandy, UT, USA) once daily, and fasted one 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. All

protocols were approved by the NWRC (QA799, QA944 and QA987) and Utah State University (IACUC#1114) Institutional Animal Care and Use Committees.

### *Specimen collection and handling*

During the breeding season (January – March), mated pairs were observed in their pens throughout the day. Mating behavior (Golani and Mendelsohn, 1971; Bekoff and Diamond, 1976) was recorded including mounting attempts and copulatory ties. Peripheral blood specimens were initially collected 2-3 weeks after the first observed copulatory tie. These initial samples were presumed to be before embryonic implantation or placental development (Tsutsui, 1989; Concannon et al., 2001) and were therefore expected to test negative for relaxin. Subsequent samples were then periodically collected from the females until a positive result was obtained.

Anti-coagulated blood specimens were collected by venipuncture or an indwelling venous catheter, into evacuated tubes containing either sodium heparin or lithium heparin. Samples were collected before the animals were fed, and without sedation or anesthesia. The plasma was separated from the whole blood as soon as possible and stored at  $\leq -20^{\circ}\text{C}$  until testing. Due to behavioral differences between coyote pairs, the earliest samples were ultimately collected at an estimated 11 days of gestation with additional samples randomly obtained throughout diestrus. In addition, 44 females were randomly sampled for 20 weeks after parturition to assess how long relaxin remained detectable by this assay.

### *Relaxin assay*

Presence or absence of canine relaxin was determined with a qualitative ELISA, ReproCHEK™ (Synbiotics Corporation, San Diego, CA, USA). The assay utilizes polyclonal anti-relaxin antibodies in solid phase (microtiter wells), and canine specific anti-relaxin monoclonal antibodies conjugated to horseradish peroxidase (HRP). Testing was performed on thawed samples (50 µl) according to the manufacturer's instructions using the optical density (OD) measurement option (Appendix C).

Nine intact females that were not bred or who had been paired with castrated males, and 8 intact males were included as internal controls. Because the absorbance values of these control animals sometimes exceeded the manufacturer's negative control (intra-run comparison), a range of values (<0.030 OD) representing a negative result was established. Mated females that initially tested negative were resampled 1-2 weeks later to either confirm the negative test for relaxin or obtain a positive result.

### **Results**

During the study, 83 female coyotes tested positive for relaxin prior to parturition and appearance of pups (Table 1). Concurrently, 26 females repeatedly tested negative and no pups were seen. Four females tested positive for relaxin although pups were subsequently never found, and because neither ultrasonography nor radiography was performed it remains inconclusive whether these 4 females were actually pregnant. In contrast, 2 females who tested positive were known to have experienced spontaneous abortions, as confirmed by recovery of expelled fetuses.

In the first 2 years of the study, 8 males, 7 unmated females, and 2 females with castrated mates were also sampled (Table 1). The OD measurements of these animals provided some guidelines as to how this assay would perform against plasma from non-pregnant coyotes. The measurements of these negative internal controls fell within the range of the manufacturer's negative controls throughout the study (Table 2).

Absorbance values were recorded for 82 full term pregnant females. A gestation length of 62 days was assumed (Gier, 1968; Kennelly, 1978) and the OD readings were aligned using the day of parturition as the reference point. Partitioning of the gestation period into weekly intervals showed optical density increasing by week 4 (Table 3).

When more than one sample was collected, it was possible to estimate the day of gestation based upon when a coyote first showed a positive relaxin test result (Table 4). Under these conditions, the earliest positive result was obtained at an estimated 23 days gestation; on days 24-27 results either were negative, indeterminate, or positive. However from day 28 on, all results were positive and had an  $OD \geq 0.100$  (Fig. 1). We also note that while some negative and indeterminate results were recorded, at least 90% of the samples collected on days 25-27 were interpreted as positive (Table 4).

An indeterminate threshold range of 0.030–0.050 OD was established based upon the absorbance values of the internal coyote controls and those of the manufacturer's reagents; and reinforced by the absorbance measurements recorded for the mated females with and without pups. All internal (coyote) controls were  $\leq 0.032$ , and the difference between the maximum ReproCHEK™ negative control (0.037) and the minimum ReproCHEK™ positive control (0.051) was in the range of 0.038–0.050 (Table 2). Samples ( $n = 69$ ) from the 26 mated females without pups fell in the range of -0.005-

0.072 (median 0.009 OD). The singular female with the OD measurement of 0.072 retested two weeks later at 0.033; and while 5 other samples in this group were also between 0.030–0.050, 62 samples (90%) were  $<0.030$ .

Serial blood sampling from 5 subjects revealed that within 48-72 hours a pregnant female coyote could display a rapid increase in OD measurements representing a plasma-conversion from negative to positive. For example, 1 female changed from 0.015 to 0.117 in 2 days, while another from 0.012 to 0.160 in 3 days. This latter data suggested that the absorbance readings of females who were pregnant (true-positives) would quickly increase in intensity (Fig. 1), thus differentiating them from non-pregnant cohorts within a short period of time. However, the timing of a sample collection might produce an indeterminate result (0.030-0.050 OD), and such results need to be rechecked.

After parturition, 44 females were sampled initially during the first 4 weeks post-partum, then randomly sampled from week 9 through week 20 (Table 5). All females continued to test positive (OD measurement  $>0.050$ ) until day 72 when 1 female had an OD reading of 0.001. All other post-partum subjects remained positive until the next female tested negative (0.005) at day 87. Gradually all females reverted to negative levels (Fig. 1) and the last OD measurement that was  $>0.030$  was recorded on day 127.

## Discussion

We found the hormone relaxin to be an acceptable diagnostic marker of pregnancy in the coyote. Through 4 consecutive breeding seasons all female coyotes with young tested positive prior to parturition (including spontaneous abortions). Under these conditions, a true-positive was a female that had pups and tested positive for



relaxin. Once these subjects converted from negative to positive (between 23-28 days gestation) the intensity of the OD measurements increased rapidly (Fig. 1). This marked increase in OD made a true-positive distinguishable from cohorts who were never seen with pups. Because more traditional diagnostic tools (e.g., ultrasonography or radiography) were not routinely used, the negative cohorts could not be confirmed as being not-pregnant (true-negatives); however 26/30 (86.6%) of the females without pups and 17/17 (100%) of the control coyotes tested negative for relaxin.

Another useful aspect of relaxin in the determination of a coyote's reproductive status was that it remained detectable throughout gestation, and well into the post-partum period (Fig. 1). Thus, collection of a meaningful specimen became less dependent on critical timing. All samples collected after day 28 of gestation were  $> 0.100$  OD until the end of week 10 post-partum (Fig. 1), and this level of color development (a visible blue color) is easily discernible from the ReproCHEK™ negative control (no color).

Our results indicate a marked variability in absorbance values among individuals; however, we observed a general trend of increased color intensity as the female progresses through her pregnancy (Fig. 1). In addition, there appeared to be a slight peri-partum decrement in relaxin followed by a short rebound in the first month of lactation (Fig. 1). For technical reasons the manufacturer makes no attempt to correlate color intensity or absorbance with circulating levels of relaxin, and this study did not provide any evidence that these measurements could predict either reproductive success or litter size. Follow up testing of the 2 females who experienced spontaneous abortions (neither the etiology nor the day of fetal death is known) produced very different results. Both females were positive 3 or 5 days before the fetuses were expelled; but while the first

female tested negative 4 and 10 days after, the second female remained positive up to 34 days later.

In conclusion, we found this assay easy to perform; and although a spectrophotometer was used in this study, it was actually not a requirement. Color development of unknown samples can be compared to the ReproCHEK™ negative control (per the manufacturer's instructions), but our experience suggests that in coyotes a weak-positive result should be interpreted carefully. Managers or investigators wanting to employ this method should not be discouraged by the pre-partum variability or timing of plasma-conversion (Fig. 1). These problems can be avoided if sampling and testing is postponed at least 4 weeks (28 days) from the time the most active mating behavior is observed. Also, it is recommended that initial negative results be confirmed by a new specimen 1-2 weeks later (when possible). It should be remembered that the length of gestation is rarely fixed in any mammal, and some individual variability should be expected. The coyote's gestation period is reportedly 60-63 days (Gier, 1968; Kennelly, 1978), but it should be anticipated that some females may experience a premature or delayed accouchement. With these considerations in mind, this assay should be a reliable tool in this species and possibly other wild congeners of *Canis*.

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**Table 1** Number of coyotes within each cohort that tested positive or negative for relaxin during 2000-2003 breeding seasons; NWRC facility, Millville, UT, USA

	Mated females			Control animals		
	With pups	No pups	Aborted	Males	Unmated females	Females with castrated mate
Positive	83	4	2 <sup>a</sup>	0	0	0
Negative	0	26	0	8	7	2

<sup>a</sup>Confirmed via recovery of fetuses

**Table 2** Optical density measurements of ReproCHEK™ reagent (Synbiotics Corporation, San Diego, CA, USA) and internal (coyote) controls during 2000-2003 breeding seasons; NWRC facility, Millville, UT, USA

	Positive relaxin control	Negative relaxin control	Female coyotes not-bred	Male coyotes
Minimum	0.051	-0.001	0.004	0.001
Maximum	0.487	0.037	0.032	0.018
Mean	0.249	0.009	0.015	0.009
Median	0.252	0.006	0.014	0.009
<i>n</i>	63	63	13	8

*n* = number of manufacturer's controls or coyote samples collected and tested

**Table 3** Range of weekly relaxin optical density readings measured during gestation (standardized to 62 days) for female coyotes during the 2000-2003 breeding seasons; NWRC facility, Millville, UT, USA

	Week of gestation								
	1	2	3	4	5	6	7	8	9
Minimum	<i>n/a</i>	0.010	0.000	0.009	0.125	0.855	1.066	1.060	0.825
Maximum	<i>n/a</i>	0.039	0.042	0.653	2.387	2.309	2.123	1.886	2.004
Median	<i>n/a</i>	0.034	0.008	0.154	0.750	1.627	1.735	1.581	1.594
<i>n</i>	0	4	25	42	42	21	14	13	11

*n* = number of coyote samples included in each weekly data set; *n/a* = samples were not collected in the first week

**Table 4** Range of daily relaxin optical density readings for female coyotes, days 23-28 of gestation during the 2000-2003 breeding seasons; NWRC facility, Millville, UT, USA. Days are aligned from day of parturition and assume a common 62 day gestation for all females

	Day of gestation					
	23 <sup>a</sup>	24	25	26	27	28 <sup>b</sup>
Minimum	0.020	0.009	0.049	0.097	0.024	0.100
Maximum	0.138	0.424	0.188	0.242	0.502	0.653
Median	0.043	0.056	0.107	0.140	0.275	0.497
% Pos	25	50	91	100	90	100
<i>n</i>	4	4	11	4	10	7

% Pos = percentage of samples (*n*) testing positive per day

<sup>a</sup>Earliest day of gestation when a positive sample was observed; <sup>b</sup>all samples from day 28 forward tested positive



**Table 5** Range of post-partum relaxin optical density readings for female coyotes shown in 2 week increments; 2000-2003 breeding season, NWRC facility, Millville, UT, USA

	Weeks post-partum								
	1-2	3-4	‡ 9-10	11-12	13-14	15-16	17-18	19-20	
Minimum	0.180	1.076	0.072	0.001	0.004	0.004	0.007	0.009	
Maximum	1.858	2.103	0.801	0.193	1.167	0.766	0.136	0.036	
Median	0.773	1.315	0.328	0.123	0.090	0.049	0.028	0.015	
% Neg	0	0	0	17	26	28	62	80	
<i>n</i>	37	5	4	6	19	25	13	5	

‡ indicates that females were not sampled between weeks 4 through 9 after parturition

% Neg = percentage of samples (*n*) testing negative in each 2 week data set

**Fig. 1** Individual optical density measurements of relaxin in female coyotes sampled during 2000-2003 breeding seasons, NWRC facility, Millville, UT USA. Parturium plasma samples (n = 214) were collected from 82 females between day 10 of gestation and parturition. Post-partum sampling continued after the 2000-2002 breeding seasons with 44 females (n = 114) spanning 140 days, although sampling was suspended between days 28 – 60. Data were aligned by day of parturition (indicated by the vertical dashed line) and assumed a full term gestation of 62 days. During this study, the optical density measurements for non-pregnant females consistently remained below the 0.100 OD threshold as indicated by the horizontal dashed line. Solid line represents the median observed OD measurement.

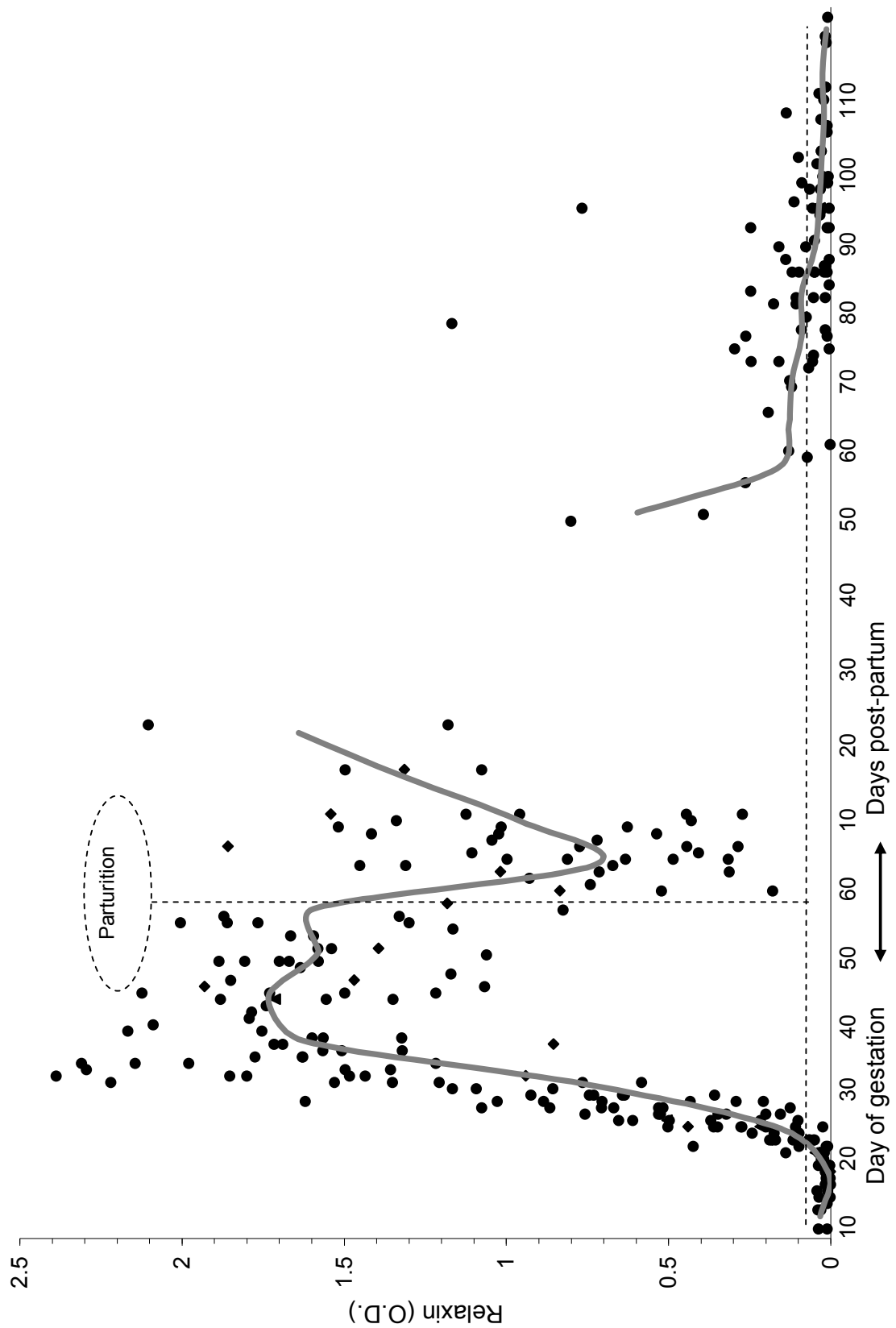


Fig. 1

CHAPTER IV  
INTEGRATION OF MATING BEHAVIOR, REPRODUCTIVE HORMONES  
AND VAGINAL CYTOLOGY<sup>2</sup>

**ABSTRACT**

The reproductive biology of wild *Canis* species is often described as unique among mammals due to an unusual combination of behavioral and physiological characteristics including a seasonally monestrous cycle, copulatory lock/tie, obligatory pseudopregnancy, social monogamy, and biparental care of the young. We investigated social behavior, endocrine profiles, and vaginal cytology of female coyotes (*Canis latrans*) during 4 breeding seasons, 2000-2003. Estradiol, progesterone, prolactin, and relaxin blood levels were measured, while mating behavior and changes in vaginal epithelium were documented. After aligning the data from each individual to her estimated day of ovulation, we compared pregnant coyotes with non-pregnant females and evaluated temporal relationships among hormone levels, behavior, and vaginal cytology. We found that patterns of proceptive and receptive behaviors correlated with the secretion of steroid hormones, as did vaginal epithelial cytomorphosis. In addition, while progesterone levels of pregnant and pseudopregnant coyotes were indistinguishable, prolactin demonstrated a discernible inter-group difference and relaxin was only detectable in pregnant females. Although this study included characteristics not previously published for this species, it also showed how key aspects of reproduction were correlated temporally, and emphasized the importance of an integrated perspective

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<sup>2</sup> Content and style of this chapter has been formatted for submission to the *Journal of Mammalogy*. Debra A. Carlson and Eric M. Gese, coauthors.

when addressing the reproductive biology of coyotes, or other wild canid species.

## INTRODUCTION

Coyotes (*Canis latrans*) are medium-sized wild canids indigenous to North America. They are seasonally monestrous (Gier 1968; Hamlett 1938; Kennelly and Johns 1976; Stellflug et al. 1981), socially monogamous, and territorial (Andelt 1985; Bekoff and Wells 1986; Bromley and Gese 2001; Camenzind 1978; Gese 2001). Once bonded, a coyote pair remains together for an indefinite number of years sharing responsibility for territory maintenance. Litters averaging 3-7 pups are typically born March – May in most North American latitudes after a gestation of 60-63 days (Gier 1968; Hamlett 1938; Kennelly et al. 1977; Knowlton 1972) and both parents participate in the care and rearing of young pups (Andelt 1985; Camenzind 1978; Gier 1968; Hatier 1995; Mengel 1971; Silver and Silver 1969).

Mature offspring may disperse or remain within their natal territories assisting in the defense of resources and infant pups, but typically only the dominant male and female breed (Andelt 1985; Bekoff and Wells 1986; Gese 2001; Gese et al. 1989, 1996).

Juvenile coyotes <12 months of age can be reproductively active in their first winter, but available evidence suggests juvenile and yearling (12-24 months) females are less fecund than adult females  $\geq 2$  years of age (Gier 1968; Green et al. 2002; Hamlett 1938; Kennelly and Johns 1976; Sacks 2005; Windberg 1995). Older females  $\geq 10$  years of age gradually pass into reproductive senescence (Green et al. 2002; Sacks 2005) while a male coyote was reported to have sired pups when  $\geq 12$  years of age (Gese 1990). Older coyotes may continue to maintain territory residency or revert to a transient lifestyle (Gese 1990;

Windberg 1995).

The reproductive tracts of adult coyotes experience extensive remodeling during the breeding season, and histological evidence suggests a female coyote is incapable of serial ovulations, even if she is not impregnated during her first estrus (Gier 1968; Hamlett 1938; Kennelly and Johns 1976). Ovulation is spontaneous, synchronous, and bilateral. The subsequent corpora lutea crowd other ovarian tissue to such a degree that the existence of additional tertiary follicles appears improbable. Furthermore, ovarian retrogression is protracted, the corpora lutea taking more than 9 months to degenerate thereby inhibiting a new wave of follicular recruitment (Hamlett 1938).

Hypertrophy of the uterus and vagina are also remarkable with gross morphological differences between sexually mature and immature females (Gier 1968; Kennelly and Johns 1976). Juvenile females, however, may experience up-regulation of reproductive hormones (specifically estradiol) and the concomitant physical signs such as vulvar edema and a serosanguineous vaginal discharge, but not ovulate (Hodges 1990; Stellflug et al. 1981;); follicular development arresting prior to, or at, the tertiary stage (Kennelly and Johns 1976). Alternatively, subordinate females may ovulate, but proestrus and estrus in these individuals appears delayed relative to the estrous phases of dominant female pack-mates, and typically the subordinate female fails to breed (Hodges 1990).

Among wild species, not dependent on human intervention, successful reproduction relies on a progression of key elements (Asa and Valdespino 1998; Kleiman and Eisenberg 1973). Mate acquisition, conception, gestation, and parental care rely upon effective synchronization of physiological processes, anatomical modifications, and

social behaviors. During 4 consecutive breeding seasons (2000-2003) we measured the levels of estradiol, progesterone, prolactin, and relaxin in coyote sera and plasma. Concurrently, samples of exfoliated vaginal epithelium were collected in proestrus, estrus, and diestrus, and examined microscopically; observations of mating behavior were also documented. The data was then aligned to each individual female's estimated day of ovulation. Females housed with their mates (and became pregnant) were compared to a non-pregnant cohort (sequestered females). Herein, we describe our observations and findings, comparing pregnant coyotes with pseudopregnant females, but also examine associations among behavior, endocrine patterns, and vaginal cytology. Our data suggest important relationships exist between these factors, and integrated examinations of complex systems can increase our understanding to an extent that might not be accrued from simple experimental constructions.

## MATERIALS AND METHODS

*Animals.*—Coyotes were captive born or wild caught as pups, and reared at the National Wildlife Research Center (NWRC) facility in Millville, Utah (41°68' N, 111°82' W). All animals were housed in outdoor enclosures with natural lighting. Male-female pairs resided in 0.1 hectare pens with access to sheltered den boxes. Three pens formed a clover shaped cluster separated by double fencing and concrete barriers; therefore, all pairs were within visual and audible range of other coyotes. Some animals were sequestered from their mates during the breeding season and served as non-pregnant controls. In these cases, the coyotes were housed individually in sheltered outdoor

kennels with attached den boxes for privacy, and mated pairs were placed in neighboring kennels during their separation.

The female coyotes ranged from 2-12 years of age, and known weights ranged from 7.6-13.8 kg. They were fed a commercially prepared carnivore diet (Fur Breeders Agricultural Cooperative, Sandy, Utah) once daily, and fasted one 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 use guidelines of the American Society of Mammalogists (Animal Care and Use Committee 1998) were followed, and research protocols were approved by the Institutional Animal Care and Use Committees at Utah State University and the NWRC.

*Specimen collection and handling.*—Peripheral blood samples were collected weekly from the cephalic or saphenous veins by venipuncture, or daily from the external jugular vein via an indwelling catheter. Samples were collected 0700-0900 before the animals were fed and without sedation or anesthesia. In some cases sampling began as early as 4 weeks before a female was receptive to her mate's attempts to copulate, or 6 weeks before a sequestered female ovulated. Blood sampling continued throughout estrus and diestrus with the latest samples collected 3 weeks after the birth of pups. To minimize investigator disturbance of mating activity, blood collections from paired females was restricted to weekly sampling until 3 weeks after the first copulatory tie was observed.

For quantitative progesterone, estradiol, and prolactin analyses whole blood was collected in an evacuated tube and allowed to clot at room temperature (20-24°C) for 30-



120 minutes. The serum was separated from the blood cells, divided into aliquots, and stored at  $\leq -20^{\circ}\text{C}$  until testing (Appendices A and B). Specimens for the qualitative relaxin assay were collected as whole blood in evacuated tubes containing sodium heparin or lithium heparin. The plasma was separated as soon as possible and stored at  $\leq -20^{\circ}\text{C}$  until testing (Appendix C).

Exfoliated epithelial cells from the vagina were collected weekly, typically the same day as the blood sample. The specimens were collected using a sterile swab pre-moistened with normal saline. The swab was gently passed into the vaginal vault, carefully avoiding the clitoral fossa, and rotated against the lining of the vaginal lumen (Feldman and Nelson 2004). Once withdrawn the swab was immediately rolled along a clean glass slide in 2-3 rows. The sample was allowed to air dry at room temperature then fixed and stained as soon as possible (Appendix E).

*Mating behavior.*—Continuous scanning observations of 32 coyote pairs were conducted daily throughout available daylight hours, January through March, 2000-2003. The animals were habituated to low level human activity prior to the beginning of the study period although all enclosures could be viewed through binoculars or spotting scope from observation sites situated 100–500 m away. The observer would view a pen and document any interactive behavior occurring between the mated coyotes, then scan the next pen. Because this process rarely took more than 30 seconds per pen, all pens were viewed at least once every 5-10 minutes.

Characterization of behavior was standardized between observers, and courtship and mating behavior was documented (Bekoff and Diamond 1976; Golani and Mendelsohn 1971). The coyote mating behaviors recorded included: (a) courtship (non-

antagonistic play-wrestling and play-chases, mate-grooming such as licking the face, ears or back, also body-bumps, hip-pushes, or sleeping curled against each other); (b) 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); (c) overt sexual activity (an attempted mount usually preceded by the male standing perpendicular to the female with his head or one bent foreleg on her shoulders or back, male mounting the female, and copulatory tie/lock); and (d) mate guarding (male shadowing the female around the pen, 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).

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., playing might last for 15 minutes and through several scanning passes), thus avoiding redundant documentation. An exception would be if the behavior was terminated then reinitiated (e.g., pre-copulatory mounts). All observed behaviors were categorized then aligned by day to the estimated day of ovulation for each individual female.

*Reproductive hormone assays.*—Quantitative measurement of progesterone in coyote sera was performed by competitive binding enzyme immunoassay (EIA) according to the manufacturer's instructions (Progesterone EIA, DSL-10-3900, Diagnostic Systems Laboratories, Inc., Webster, Texas). By this method, horseradish peroxidase (HRPO)-labeled progesterone competed with free progesterone in coyote sera for a fixed quantity of rabbit anti-progesterone. Microtiter wells coated with goat anti-rabbit IgG captured the antibody-bound progesterone. Extraneous material was rinsed

from the well, and the addition of a tetramethylbenzidine (TMB) chromogenic solution permitted photometric measurement (Benchmark microplate reader, Bio-Rad Laboratories, Hercules, California) of reagent standards, reagent controls, and unknown samples. Unknown coyote samples were then compared to a standard curve generated for each run using Microplate Manager / PC software (version 4.0, Bio-Rad Laboratories, Hercules, California); the quantity of progesterone being inversely proportional to the intensity of color development (Appendix A).

Quantitative measurement of estradiol was also performed by competitive binding EIA (3<sup>rd</sup> Generation Estradiol EIA, DSL-10-39100, Diagnostic Systems Laboratories, Inc., Webster, Texas). In this assay (Appendix B), an estradiol-biotin conjugate competed with free estradiol in coyote sera for available rabbit anti-estradiol sites fixed to microtiter wells. Streptavidin-HRPO was added, binding to the biotinylated estradiol. The addition of TMB precipitated color development in the reagent standards, reagent controls, and unknown coyote samples that was measured with a photometer (Benchmark microplate reader, Bio-Rad Laboratories, Hercules, California). Color development was inversely proportional to the quantity of estradiol captured in each well, and unknown coyote samples were compared to a standard curve generated for each run using Microplate Manager / PC software (version 4.0, Bio-Rad Laboratories, Hercules, California).

Coyote samples were not pretreated or extracted prior to testing for progesterone or estradiol. When possible, specimens from the same cohort were tested together to reduce reagent lot-to-lot variability; each sample was tested in duplicate. Validation procedures were performed including linearity and recovery assessments, and the assays

were determined to be acceptable for use in this species (Appendix F). Intra-assay coefficient of variation (*CV*) was  $\leq 10\%$  for all results included in the data set. For the progesterone assay, within-lot inter-assay mean *CV* was 9.6%, and inter-lot *CV* was 22.5%. For the estradiol assay (single lot only) inter-assay mean *CV* was 11.2%.

Serum prolactin was quantitatively measured by a canine prolactin radio-immunoassay (RIA) at the Colorado State University Endocrine Laboratory (Colorado State University, Fort Collins, Colorado). In this double-antibody assay, prolactin in coyote sera competed with  $^{125}\text{I}$  canine prolactin for a fixed amount of rabbit anti-canine prolactin antibodies. Anti-rabbit IgG was added and the level of radioactivity of the precipitated pellet was measured. Unknown coyote samples were compared to a standard curve, the amount of iodinated antibody-antigen complexes detected being inversely proportional to the amount of prolactin in the coyote sera.

A qualitative enzyme-linked immunoassay (ELISA) for canine relaxin (ReproCHEK™, Synbiotics Corporation, San Diego, California) was used for the determination of relaxin in heparinized coyote plasma (Appendix C). Free relaxin in an unknown sample was captured between polyclonal anti-relaxin antibodies in solid phase (microtiter wells) and monoclonal anti-relaxin antibodies conjugated to HRPO. Subsequent color development was directly associated with the presence or absence of relaxin in the sample. Absorbance was measured photometrically and the optical density (OD) readings were used to distinguish between pregnancy and pseudopregnancy as described elsewhere in the coyote (Chapter III this text).

*Vaginal exfoliative cytology.*—Air-dried samples of vaginal epithelial cells and uterine exudate on glass slides were fixed with methanol and stained with a modified

Wright-Giemsa stain (Diff-Quik®, Jorgensen Laboratories, Loveland, Colorado). The slides were then examined under high dry magnification (x400) with at least 5 fields per row ( $\geq 10$  fields per slide) of stained material viewed. The observed epithelial cells were characterized as: parabasal, small intermediate, large intermediate, superficial, and keratinized (anuclear) superficial (Shutte classification: Christie et al. 1972; Feldman and Nelson 2004), and their relative representation in the sample were graded on a semi-quantitative scale of 1-5 (Bradley and Benson 1974). In addition, inclusions such as white blood cells (WBC), red blood cells (RBC), mucus, amorphous debris (degenerating blood and epithelial cells), and spermatozoa were also noted (Appendix E).

*Data analysis.*—Female coyotes who resided with their mates were enrolled in the “pregnant” cohort group if they were observed with live pups after parturition. In 2001, 2 females experienced mid- and late-term spontaneous abortions. Since the etiologies were unknown, the hormone profiles of these individuals were excluded, although their estrus behavior and cytology data were retained with the pregnant cohort. Females who were sequestered from their mates during estrus were assigned to the “non-pregnant” group; behavioral data was not collected for these coyotes.

Data was aligned by the estimated day of ovulation for an individual before it was compiled by study group. This estimate was either back-calculated from the day of parturition, or based upon the change in serum progesterone levels. Data presented by Kennelly and Johns (1976) suggested that coyotes ovulate immature (primary) oocytes, similar to domestic dogs (*Canis familiaris*). If true, then fertilization probably does not occur until 2-3 days after ovulation (domestic dog: Tsutsui 1989). In this study therefore, gestation was standardized and assumed to be 62 days from fertilization (Gier 1968;

Hamlett 1938; Kennelly et al. 1977) or 64 days after ovulation. Alternatively, the estimated day of ovulation for females who did not whelp was inferred from the change in their daily progesterone levels (P.W. Concannon, personal communication); specifically, the day on which progesterone levels approximately doubled in concentration from prior samples.

Hormone and vaginal cytology data are presented herein as weekly mean values of all females within a cohort, and results obtained on the estimated day of ovulation are included in “Week 1.” In circumstances in which multiple hormone assay results were available for an individual within a given week, a weekly mean value for that individual was calculated in order to normalize the influence of each individual’s contribution to the cohort mean. Behavioral observations were also aligned by the day of ovulation for each individual female then reported as cumulative daily or weekly data for the cohort.

Multivariate analysis of variance (MANOVA) and repeated-measures statistical procedures were used to analyze endocrine hormone profiles and detect differences between study groups, and between successive weeks (Statistical Analysis System, SAS®, version 8.2, SAS Institute Inc., Cary, North Carolina). Furthermore, Pearson correlation coefficient and multiple regression procedures were used to analyze relationships between hormones and behavior, and between hormones and vaginal cytology. Unless otherwise noted, we assumed a level of statistical significance to be less than 0.05.

## RESULTS

*Behavior.*—Prior to the beginning of behavioral estrus (day -8), most intra-pair activity consisted of courtship behavior, including mate-grooming, play-wrestling, play-chases, body-bumps, and hip-pushes (Fig. 2). A few days before the first copulatory tie was witnessed, olfactory sampling (i.e., anogenital and inguinal sniff/lick) by both the male and female began to increase, as well as mate guarding. Also within this pre-copulatory period, males began mounting attempts. These attempts, however, were usually rejected by the female using gentle admonition, aggressive rebuff, or passive avoidance tactics (such as sitting, lying down, or running away).

Mounting attempts continued to increase, and the first copulatory tie was used to delineate the start of estrus. An increased frequency of mounting was expected during estrus because this was the antecedent posture to copulation; but, in contrast to the response observed in proestrus, the females were tolerant and receptive to the males' attempts. Often the female would solicit attention by positioning herself in front of the male and diverting her tail; and it was common for a male to mount/dismount several times before a mount terminated in an intromission and tie.

Physical contact such as body-bumps and hip-pushes continued to rise during pre-ovulatory estrus. Mate guarding postures such as a stand-over within view of neighbors and shadowing became more frequent. Olfactory sampling (male and female) increased almost three-fold; specifically, vaginal sniff/lick by males doubled on day -6 from the previous day, continued to increase, then peaked on the estimated day of ovulation.

Immediately following the peri-ovulatory pulse of activity there was a brief quiescence before the sexual activities of the cohort pairs peaked again on day 5, particularly copulations (Fig. 2). When coyote pairs were observed in >1 tie per day, the multiple ties most frequently occurred on days 4-6. Mate guarding also showed a post-ovulatory surge on days 5-6, with a maximum on day 5. Mounting attempts peaked on day 7 although this was associated with a decline in the number of successful copulatory ties. As estrus waned, females began to reject some (but not yet all) of the males' attempts to copulate. Although the earliest a copulatory tie was observed during this study was on day -9, and the latest on day 15, 98.4% (179/182) of all ties occurred between day -8 and day 10. Finally, while physical bodily contact remained high in late estrus, play behavior was only sporadically observed until the transition into diestrus.

On day 11 post-ovulation, sexual activities abruptly declined and observation of copulatory ties (2/182), mounting attempts (4/341), olfactory sampling (14/667), or mate guarding (2/163) became relatively rare (1.1%, 1.2%, 2.1%, and 1.2%, respectively) during the remaining 10 days (Fig. 2). In 2001 a pair was observed in a single tie 17 days after the previously recorded copulation; similarly in 2002 another coyote pair was witnessed in a tie 15 days after their previous mating. Back-calculation from parturition suggested an earlier date of fertilization in both cases, and since neither tie was associated with other sexual behaviors (and they lasted <2 minutes), these events were considered to have had some other unexplained intra-pair social function (Gier 1975; Schenkel 1967) rather than a sign of extended or split estrus.

While there was a dearth of sexual activity, the coyotes in diestrus continued to engage in physical contact such as mate-grooming, body-rubs, play-wrestling, and



chasing. In addition, a previously unseen behavior emerged, begging (Fig. 2). Beginning on day 10 and continuing through the end of the observations, females were periodically observed in a juvenile-like submissive behavior (Schenkel 1967) that successfully provoked regurgitation of food by the male. Specifically, the female would approach her mate with her tail held low and wagging, then she would lick his mouth or gently bite his lower jaw, matching his movement if he tried to turn away. Sometimes the male would admonish the female and escape. At other times, however, the female would cease the behavior and appear to be eating off the ground. In 7/55 observed cases the observer confirmed that the male regurgitated the food being consumed by the female.

*Reproductive hormones.*—During 3 consecutive years (2000-2002) serum samples were collected from 16 intact female coyotes (represented by 11 individuals). Serum progesterone levels were determined from 727 specimens, and sample subsets were also used to measure estradiol ( $n = 405$ ) and prolactin ( $n = 205$ ). In one breeding season, 8 females were sequestered from their mates in order to describe the endocrine patterns of non-pregnant (pseudopregnant) female coyotes during late-proestrus, estrus, and diestrus. These individuals, however, were included in the pregnant cohort in the alternate year immediately before or after their non-pregnant season.

Fluctuating serum estradiol levels generally increased during proestrus in both coyote study groups (Fig. 3), and an inter-week comparison, week -3 to week -2, suggested a significant incremental rise in mean values ( $F_{1,8} = 20.93$ ,  $P = 0.002$ ). Also during this time, the rate of change among females residing with their mates appeared different in contrast to the sequestered females ( $F_{1,8} = 7.84$ ,  $P = 0.023$ ) although the

between-group weekly means remained statistically indistinct (week -3,  $P = 0.917$ ; week -2,  $P = 0.245$ ). In estrus, weekly pre-ovulation (week -1) estradiol levels peaked at (mean  $\pm$  SE)  $57.1 \pm 7.3$  pg/ml ( $n = 5$ ) among mated females (pregnant cohort) and  $44.2 \pm 9.4$  pg/ml ( $n = 5$ ) in the non-mated (non-pregnant cohort), while post-ovulation (week 1) levels subsequently declined in both groups.

Estradiol levels continued to fall from late estrus to early diestrus (week 1 to week 2). Although the decrement appears greater for the non-pregnant cohort (Fig. 3), the rate of change was similar between groups ( $F_{1,8} = 0.07$ ,  $P = 0.801$ ), and comparison of the between-group mean difference was borderline (week 2,  $P|t|_{0.05(2),8} \geq 2.16 = 0.063$ ,  $F_{4,4} = 4.45$ ). From week 2 to week 3, however, the study groups demonstrated a notable divergence ( $F_{1,8} = 6.41$ ,  $P = 0.035$ ) in estradiol levels. Specifically, the pregnant cohort experienced a transient spike in week 3 ( $38.9 \pm 6.7$  pg/ml,  $n = 5$ ) that was different ( $P|t|_{0.05(2),8} \geq 2.70 = 0.027$ ,  $F_{4,4} = 1.80$ ) from the non-pregnant group ( $16.2 \pm 5.0$  pg/ml,  $n = 5$ ) (Fig. 3). Nevertheless, serum estradiol levels continued to fall in both cohort groups, and fluctuations appeared to dampen as pregnant females approached parturition and non-pregnant females entered anestrus (Fig. 3).

Although estradiol was the predominant ovarian hormone in proestrus, progesterone synthesis was detectable in female coyotes during this period (Fig. 4). Immediately prior to estrus, the incremental change in mean progesterone levels, from week -2 to week -1, was significant ( $F_{1,14} = 6.24$ ,  $P = 0.026$ ). Furthermore, while the most notable change observed was peri-ovulation (week -1 to week 1:  $F_{1,14} = 27.94$ ,  $P < 0.001$ ), successive weekly levels rose or fell significantly ( $P < 0.05$ ) from week -2

through week 7; exceptions were week 1 to week 2 ( $F_{1,14} = 3.22, P = 0.094$ ), and week 3 to week 4 ( $F_{1,14} = 0.93, P = 0.352$ ).

During estrus, progesterone levels in the mated females (pregnant cohort) rose from (weekly mean  $\pm$  SE)  $58.9 \pm 25.5$  ng/ml ( $n = 7$ ) to  $89.3 \pm 33.4$  ng/ml ( $n = 8$ ), while the non-mated females rose from  $18.5 \pm 8.2$  ng/ml ( $n = 8$ ) to  $74.1 \pm 16.0$  ng/ml ( $n = 8$ ). The mean levels, however, were not different between-groups (week -1,  $P = 0.179$ ; week 1,  $P = 0.208$ ; and week 2,  $P = 0.687$ ). In fact, no overall effect of status was detected throughout the study period (week -2 to week 7:  $F_{9,6} = 2.34, P = 0.157$ ), possibly due to the degree of individual variability observed among the coyotes. For example; among females in estrus and residing with their mates (pregnant cohort), the progesterone minimum, maximum, and CV, respectively, were: week -1 = 2.8 ng/ml, 181.4 ng/ml, 1.1; week 1 = 6.7 ng/ml, 266.5 ng/ml, 1.0; and week 2 = 10.3 ng/ml, 305.5 ng/ml, 1.1. Among sequestered females in estrus (non-pregnant cohort) the same hormone parameters were: week -1: 5.4 ng/ml, 75.1 ng/ml, 1.3; week 1: 13.3 ng/ml, 108.9 ng/ml, 0.7; and week 2: 15.8 ng/ml, 147.5 ng/ml, 0.6.

Regardless of the variation, the secretion pattern of progesterone was generally consistent between study groups. Progesterone levels (mean  $\pm$  SE) peaked between week 3 (pregnant,  $104.6 \pm 37.0$  ng/ml,  $n = 7$ ) and week 4 (non-pregnant,  $85.0 \pm 20.4$  ng/ml,  $n = 8$ ) in pregnancy and diestrus. Subsequently, levels declined in both groups. The pregnant cohort appeared to experience a transient surge in week 7, but the groups remained statistically indistinct until parturition and the end of sample collection (Fig. 4).

In contrast to progesterone, there was a distinctive overall effect of status ( $F_{6,6} = 6.03, P = 0.023$ ) on coyote prolactin blood levels. During early pregnancy and diestrus,

week 2 through week 4, mean prolactin levels did not markedly change within either cohort group; however, subsequent weeks showed a pronounced elevation among pregnant females (Fig. 5). Specifically, a significant change occurred between week 4 and 5, both in mean prolactin levels ( $F_{1,11} = 41.26, P < 0.001$ ) and inter-cohort rate of change ( $F_{1,11} = 13.34, P = 0.004$ ). Levels in pregnant coyotes rose from  $24.8 \pm 2.0$  ng/ml (week 4,  $n = 5$ ) to  $33.0 \pm 3.5$  ng/ml (week 5,  $n = 5$ ), while among non-pregnant coyotes prolactin increased from  $19.6 \pm 2.0$  ng/ml (week 4,  $n = 8$ ) to  $21.8 \pm 2.2$  ng/ml (week 5,  $n = 8$ ). Thereafter, prolactin levels remained elevated throughout pregnancy, parturition, and the first week of lactation in those coyotes observed with live pups. Non-pregnant females, meanwhile, also continued to synthesize prolactin but at lower levels ( $P \leq 0.006$ ) (Fig. 5).

Relaxin was previously confirmed as a reliable pregnancy-specific marker in the coyote (Chapter III this text), and data from that tangent project were added to the assay results of this study for the comparative analysis presented herein (Fig. 5). Relaxin was detectable in pregnant coyotes within 4 weeks after ovulation; specifically, 10/11 pregnant coyotes tested positive ( $OD > 0.050$ ) on day 27 post-ovulation, and 20/21 females with pups were positive on days 28-30. By comparison, relaxin was not detected ( $OD < 0.033$ ) in samples collected from 2 females residing with castrated mates, 7 non-mated females, or 8 male coyotes. In addition, from week 5 through parturition, relaxin remained detectable in all samples collected from pregnant females; and although absorbance intensity weakened, post-partum females did not revert to negative until several weeks after whelping.

*Vaginal exfoliative cytology.*—A serosanguineous discharge from the vagina was not always apparent upon gross examination of a female coyote in proestrus, but RBC were typically observed microscopically when the vaginal smear was examined (Fig. 6). Epithelial cells on these smears were primarily of parabasal and intermediate cell types but gradually, as the female progressed through proestrus, the exfoliated epithelial cells appeared larger and samples presented as admixtures of parabasal, small and large intermediate, and superficial epithelial cells (Fig. 7). RBC, amorphous debris, and mucus remained grossly obvious throughout proestrus (Fig. 8). Leukocytes however, were only occasionally seen on smears from this stage, and their occurrence was likely due to secondary passage with the high number of RBC rather than by diapedesis.

Superficial epithelial cells became predominant in pre-ovulation samples from the vaginal lining and their peak emergence coincided with the pre-ovulatory estradiol peak on day -4. These cells were either keratinized (anuclear) or retained pyknotic nuclei, and continued to represent the majority cell type through ovulation and the week following (Fig. 7). Concurrently, the appearance of RBC, mucus, and amorphous debris were diminished and WBC were rarely seen (Fig. 8). Thus, the appearance of superficial cells (nuclear and anuclear) against a clear background was the characteristic vaginal smear during estrus in the coyotes (Fig. 9), particularly after ovulation.

Spermatozoa were sometimes viewed in the vaginal smears, confirming that mating had occurred (Fig. 9) but they were an unpredictable and erratic element during estrus. In several circumstances spermatozoa were not recovered although the females were known to be actively breeding. We assumed that sperm deposition in the coyote was trans-cervical, as in the domestic dog (Feldman and Nelson 2004), and if true, would

thus explain the inconsistent appearances. Nevertheless, among those samples that did contain spermatozoa, most were collected during the period of frequent copulations, days 3–6 post-ovulation.

The relative frequency of intermediate epithelial cells began to increase again in week 2 as diestrus began and estrus ended; and as diestrus progressed, superficial cells disappeared and parabasal cells reemerged as the predominant epithelial cell type (Fig. 7). Also during diestrus, RBC, amorphous debris, and mucus became more abundant again (Fig. 8). But, most notable in this phase was the reappearance and disproportionate number of leukocytes (Fig. 10), particularly in relationship to the number of RBC (Fig. 8). Thereafter, a fluctuating mix of blood cells and cellular debris persisted into anestrus. We observed no discernable difference between vaginal smears from pregnant coyotes and those from pseudopregnant females.

## DISCUSSION

Reproduction in the domestic dog (*Canis familiaris*) has been extensively studied (for a review see Concannon et al. 1989, 2001), and although differences between dogs and coyotes exist, there are also similarities. Both species spontaneously ovulate; but in general, the estrous cycle of the domestic dog is aseasonal, while the coyote is obligated to a single breeding season each year. Also, the extended recovery period after ovulation and pregnancy (anestrus) is foreshortened in the domestic canine. Proestrus is only a couple of weeks among domestic bitches, while vaginal bleeding appears 2-3 months before a coyote enters estrus; and unlike domestic dogs, coyotes form long-term and socially monogamous pair-bonds.

In the domestic bitch, the steroid hormones (estradiol, progesterone) have been linked to modifications in sexual behavior (Concannon et al. 1979) and remodeling of the reproductive tract for the rigors of mating and pregnancy (Feldman and Nelson 2004). Meanwhile, the peptide hormones (relaxin, prolactin) play critical physiological roles as well. Relaxin is synthesized in the placenta affecting the tensile action of smooth muscles in the reproductive tract (Sherwood 1994), and has been shown to be pregnancy specific in dogs (Steinetz et al. 1987) and coyotes (Chapter III this text).

Prolactin is luteotrophic, extending the synthesis of progesterone by postponing atresia of the corpora lutea (Onclin and Verstegen 1997). It also facilitates milk production (Jöchle 1997). Female canids show a marked elevation in progesterone prior to the end of behavioral estrus, and diestrus in this group is further characterized by the sustained synthesis of progesterone and prolactin, even in non-pregnant females (pseudopregnancy). Pseudopregnancy has also been described in the gray wolf (*Canis lupus*: Seal et al. 1979), and appears to be a conserved physiological feature that may be associated with parental behavior in wild canids (Asa 1997; Kreeger et al. 1991). The possible adaptive benefits and social implications of pseudopregnancy are compelling when examining the life history strategy of coyotes, wolves, or other wild canids (Asa 1997; Asa and Valdespino 1998; Kleimen and Eisenberg 1973).

In this study, coyote reproductive recrudescence was evident in the nascent endocrine levels of estradiol and progesterone, the distinctive changes in social behavior, and the response of the vaginal epithelium to fluctuating hormones. Furthermore, we found reproductive features such as hormone concentrations, behavioral manifestations, and cellular modifications to be closely related.

*Proestrus.*—Proestrus is a crucial period of preparation and staging, both physiologically and behaviorally. Serum levels of estradiol and progesterone suggested up-regulation of steroid synthesis (Figs. 11A and 11B) while social interactions increased in comparison to behavior commonly observed in the summer and early fall (Fig. 12). Intra-pair physical contact such as play-wrestling, allogrooming, hip-pushes, body-bumps, and sleeping together were frequently seen. Also agonistic and defensive displays increased, such as reproaching a mate in close proximity to border fencing, or exchanging antagonistic threats with neighboring pairs.

There was a strong positive relationship between mate-guarding and olfactory sampling during proestrus ( $r = 0.739$ ,  $P = 0.0039$ ). Olfactory sampling may inform both the male and female of their mate's physiological status, and/or stimulate other behavior. We also observed a relationship between olfactory sampling and mounting attempts ( $r = 0.581$ ,  $P = 0.0374$ ), as well as between olfactory sampling and courtship behaviors ( $r = 0.567$ ,  $P = 0.0432$ ). Eventually, proceptive behavior yielded to receptive behavior as estrus approached, and the females became more tolerant and cooperative with the males' attempts to mount and copulate.

Meanwhile, the appearance of numerous red cells in vaginal exudate, and transformation of vaginal epithelial cells from parabasal to intermediate (Fig. 13), suggested progressive remodeling of the reproductive tract. Gross hypertrophy of the vaginal wall and increased vascularization around the reproductive tract were also notable physical characteristics. We observed no apparent differences between adult females who became pregnant, and those who were sequestered. But by comparison, smears collected in winter from free-ranging juvenile females and captive spayed females



were distinctly different from the smears of the study animals presented here; specifically, transmogrification of parabasal to superficial cell types was never observed (Carlson, unpublished data). Thus, anticipation of mating appeared to evoke alterations in coyote physiology, behavior, and anatomy.

*Estrus.*—Females in this captive population were naturally synchronized; each individual began estrus within a 4-week winter period, mid-January to mid-February. We defined estrus as the period within the reproductive cycle when coyote pairs were observed in a copulatory tie. During a tie, the male and female are mechanically locked by engorgement of the bulbus glandis within the vaginal vestibule. Among the coyote pairs observed in this study, the copulatory tie lasted 5-45 minutes, with ties occurring early in estrus lasting longer than those observed towards the end. During the breeding seasons 2000-2003, 98% (179/182) of observed ties occurred within a span of 19 consecutive days (day -8 to day 10); while at the individual level, estrus lasted 7.6 ( $\pm 1.4$  SE) days.

Twenty-three percent (42/182) of all copulatory ties occurred between day -8 and day -1; a time when progesterone synthesis was increasing and estradiol was reaching its pre-ovulation peak on day -4. We found, however, that in estrus copulatory ties appeared to have a significant relationship with progesterone (Fig. 14A) but a poor correlation with estradiol ( $r = -0.084$ ,  $P = 0.7324$ ). Furthermore, an initial spike in the daily number of ties (18 events) was observed on the estimated day of ovulation, a day when the mean progesterone levels experienced the greatest incremental change ( $CV_{(\text{day}-1 \text{ to day } 0)} = 0.275$ ); and another major peak in copulations (23 events on day 5) immediately followed a secondary surge in progesterone ( $CV_{(\text{day } 3 \text{ to day } 4)} = 0.247$ ) (Fig. 12). Thus, since 60%

(203/341) of mounts and 65% (119/182) of ties occurred during post-ovulation estrus, the coyotes may have concentrated their reproductive effort to coincide with the optimal time for viable sperm to encounter mature ova; while pre-ovulation sexual behavior may serve as a reinforcement of the pair bond.

Eighty-four percent (558/667) of olfactory investigations occurred during estrus with peak activity on day -3 (56 events), reflecting an increase in vulval sniff/lick but also a spike in female solicitations (Fig. 12). Meanwhile, 88% (143/163) of mate-guarding events also occurred during estrus, and as in proestrus, mate-guarding and olfactory sampling maintained a positive relationship ( $r = 0.550$ ,  $P = 0.0146$ ).

We noted peaks in mate-guarding (11-13 events per day) on day -5, day -2 through day 1, and day 5; days immediately adjacent to peaks in steroid hormone activity and sexual behavior (Fig. 12). However, while mate-guarding and copulatory ties appeared to be positively correlated ( $r = 0.521$ ,  $P = 0.0221$ ), mate-guarding did not appear to be statistically related to estradiol ( $r = 0.248$ ,  $P = 0.3054$ ) or progesterone ( $r = -0.032$ ,  $P = 0.8964$ ) levels, either singly or as covariables ( $_{Adj}R^2 = 0.047$ ,  $P = 0.5629$ ). This lack of correlation was not unexpected. Male coyotes played a significant role in the observed mating behavior and the influence of testosterone during the breeding season must be an important factor. Nonetheless, the association between rising progesterone levels and overt sexual behavior that was detected may reflect the female coyote's sexual determination. A male may shadow a female, investigate her anogenital scent, play, groom her, etcetera, but copulation will not occur without her permission and explicit cooperation.

In estrus, a coyote's vulva felt turgid but relaxed and we found passing a swab into the vagina for collection of the exudate to be easier than in proestrus. Serial vaginal smears demonstrated that cytomorphosis of vaginal epithelium, from parabasal cells to superficial cells, was transient and occurred in concert with the pre-ovulation rise in estradiol levels (Fig. 13). As estradiol levels diminished superficial cells disappeared, and a strong association was detected between estradiol blood levels and the appearance of superficial cells (Fig. 14B). While this evidence suggests vaginal smears might be used as a surrogate measure of breeding condition, defining the specific day of ovulation was difficult; changes in cytology did not appear to be correlated with progesterone levels ( $r = -0.340$ ,  $P = 0.3358$ ).

Nonetheless, vaginal smears can serve as a qualitative assessment of estradiol synthesis, and might be used to predict a range of days during which a female might be expected to ovulate (if not predicting the precise day). Between the pre-ovulation estradiol peak on day -4 and post-ovulation day 7 there were moderate to many (grade 4 to 5) superficial cells, divided equally between nuclear and anuclear (keratinized). Other classes of epithelial cells were rare; RBC, amorphous debris, and mucus were conspicuously diminished, and WBC were absent. Thus in cases when mating behavior is difficult to document or is protracted, vaginal smears might help determine when a female coyote is entering a period of optimal fertility, or when she is passing out of the period of receptivity.

*Diestrus.*—Demarcation of diestrus from estrus was defined as the point when the females refused to stand for copulations with their mates. Serum progesterone levels, however, continued to rise (Fig. 12) and were sustained for several weeks after ovulation

in non-pregnant females as well as pregnant coyotes. As progesterone began to decline there appeared to be a transient rebound in pregnant coyotes (Fig. 11B); however, the downward trend generally persisted as the coyotes approached parturition and the pattern appeared synchronous with the non-pregnant cohort. Furthermore, although progesterone levels in the non-pregnant females appeared to have reached a nadir earlier than in the pregnant coyotes, we were not able to distinguish a statistical difference between study groups at any time within diestrus.

Estradiol continued to dampen, and as it was withdrawn vaginal epithelium reverted back to a normal state (Fig. 13). RBC, amorphous debris, and mucus reappeared in the vaginal smears, but WBC became more prevalent than previously observed in either estrus or proestrus. In addition, as sexual behavior waned food-begging immediately (day 10) appeared as a newly acquired feature (Fig. 2). Episodes of begging, and sometimes regurgitation, were seen throughout pregnancy with greater intensity occurring during weeks 4 and 5. Interestingly, begging was not restricted to pregnant females. In a tangent study, mated females who were not pregnant were also observed begging food (and receiving regurgitate) from their mates in diestrus (Chapter V this text).

In conjunction with sustained levels of progesterone, prolactin and relaxin emerged as distinctive hormones, distinguishing pregnancy from pseudo-pregnancy. Relaxin became detectable in week 4 (post-ovulation) and was followed by an increase in prolactin in week 5; furthermore, it was during week 5 that the prolactin levels of pregnant females differentiated from those of the non-pregnant cohort. Prolactin levels thereafter remain elevated in pregnant females as compared to non-pregnant

(pseudopregnant) females. It has been hypothesized (but not yet established) that relaxin might serve as a signal between embryo and mother, possibly stimulating prolactin synthesis, which in turn facilitates the persistence of progesterone (required for the maintenance of pregnancy in canines) (Concannon et al. 2001). Although there appeared to be a reversal of the downward trend in progesterone levels among pregnant females in week 7, the inter-group and inter-week differences in mean hormone levels were statistically non-significant. Therefore, while an affect between relaxin, prolactin, and progesterone may exist, we were unable to establish a definitive link in the coyote even though there appeared to be a positive association between prolactin and relaxin during pregnancy (Fig. 14C).

Through intensive examination of certain critical components of the coyote's social behavior, endocrine profiles, and vaginal cytomorphosis, we were able to discern a few key elements in the reproductive biology of this species. In particular, it might be useful to biologists studying wild or captive populations to know what tools would be available for assessing a breeding population. The value of seeing a pair in a copulatory tie, viewing spermatozoa on a vaginal smear, or detecting pups in a den are obvious. But in the absence of such indicators, investigators might also depend upon the observation of female receptive behavior, mate guarding (such as shadowing), frequency of olfactory investigations, and begging between adults. Serial measurement of progesterone and prolactin blood levels, even short term, can provide evidence of ovulation or suggest pregnancy even though individual levels are various. Detection of relaxin can be a reliable diagnostic tool for pregnancy. The relative prevalence of exfoliated epithelial cells, red cells, leukocytes, and other inclusions on a vaginal smear can help predict the

breeding status or reproductive potential of an animal. But importantly, the use of several of these elements together will increase the overall confidence in determining the reproductive status of an individual, or population of coyotes.

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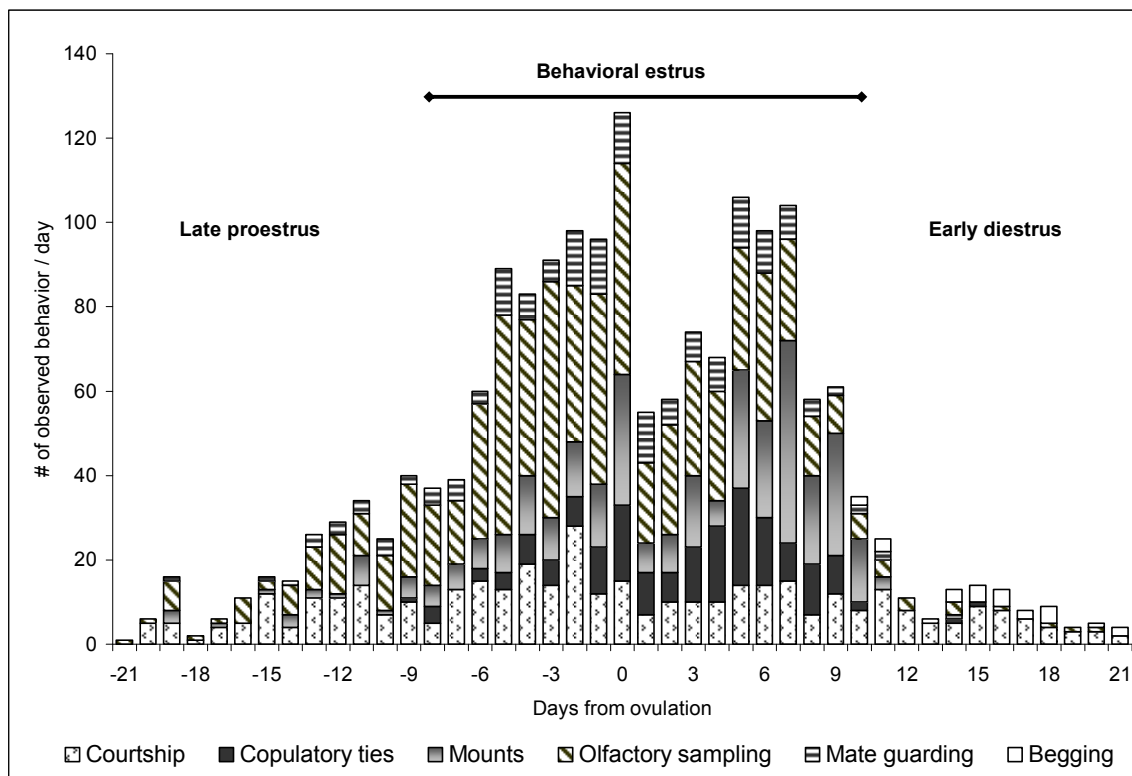
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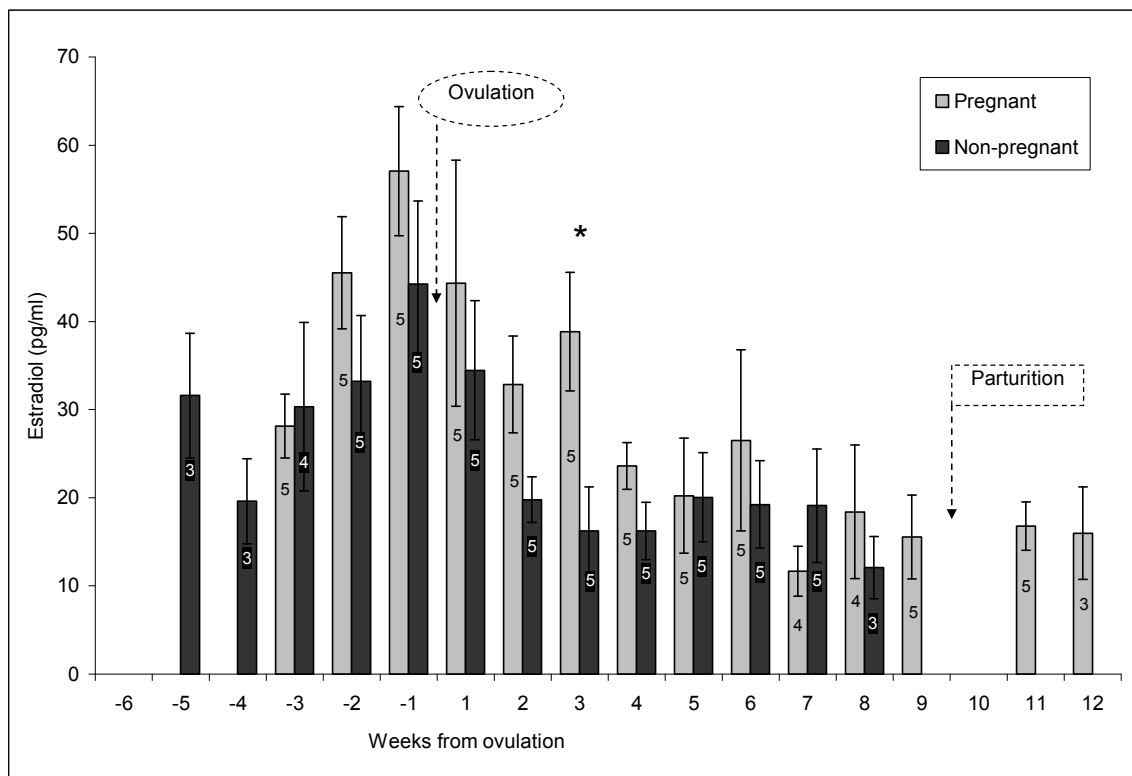
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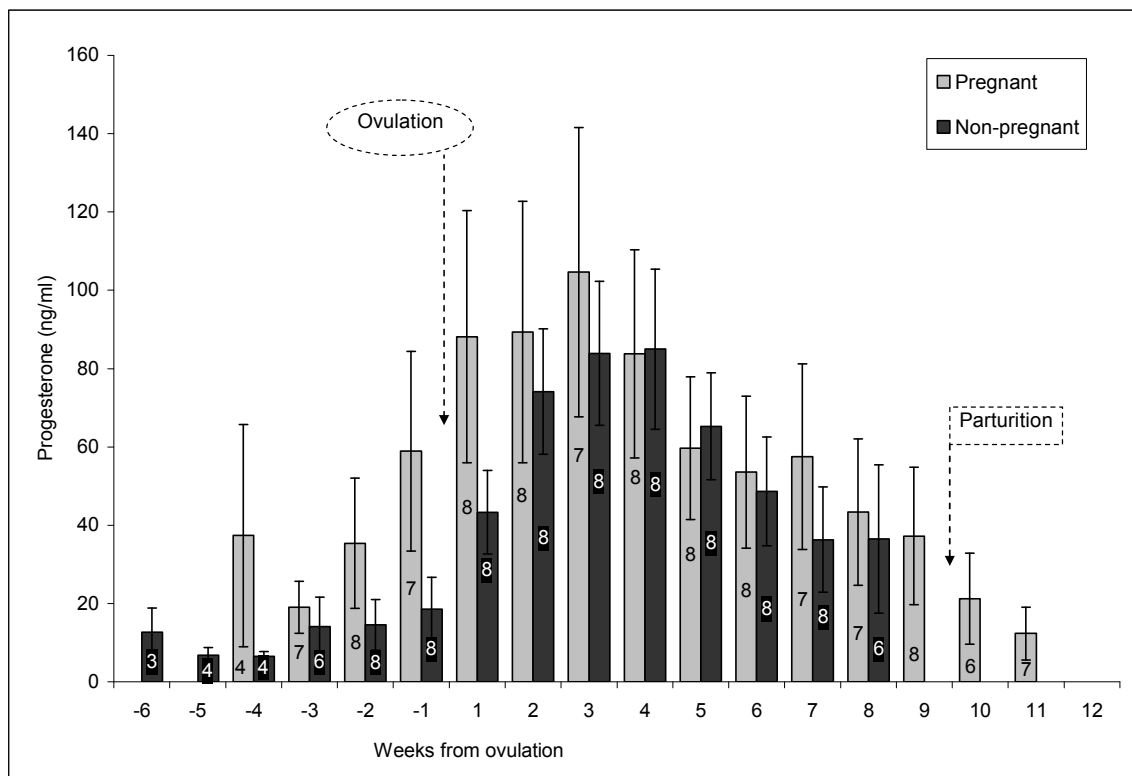
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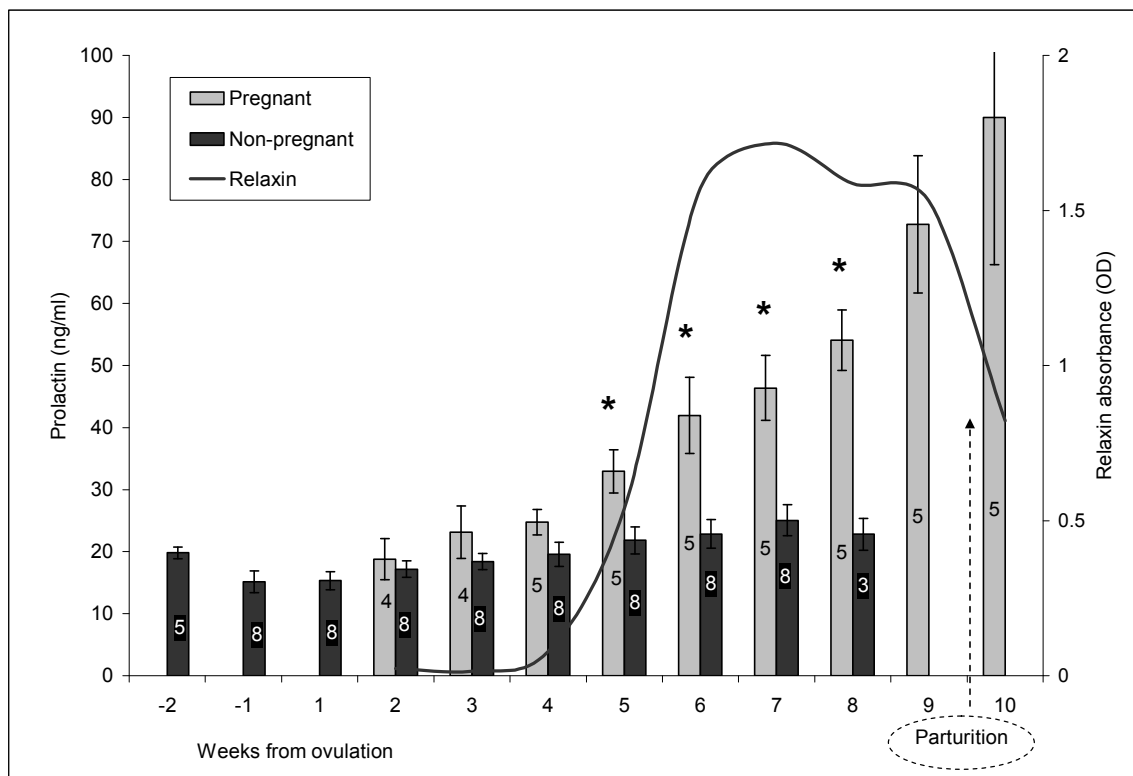
**FIG. 2.**—Coyote social and mating behaviors shown as daily cumulative data ( $n = 1757$ ) from 32 mated pairs during 2000-2003 breeding seasons. Observations represent 21 days before and after the estimated day of ovulation (day 0 on the chart). Behavioral estrus ranged from day -8 to day 10 (as shown by the solid bar).



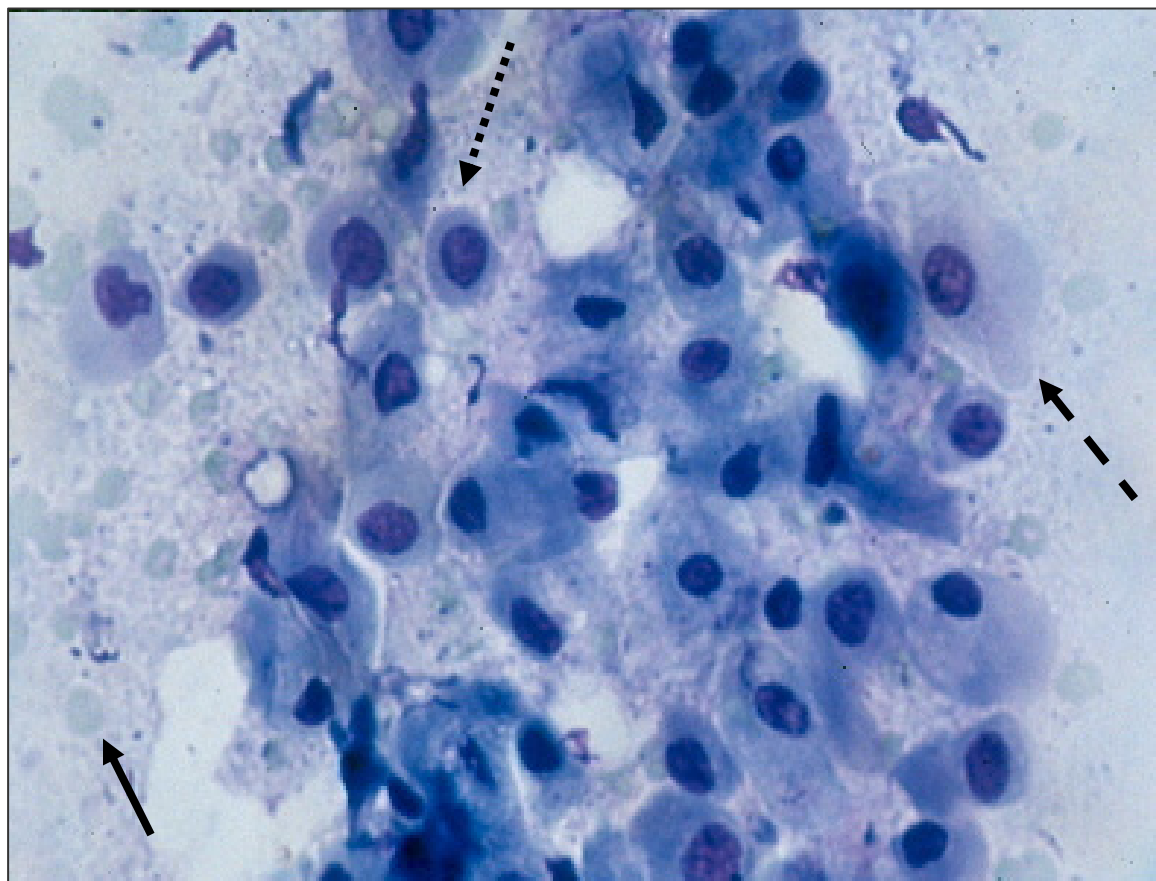
**FIG. 3.**—Weekly mean ( $\pm$  SE) serum levels of estradiol (pg/ml) from 5 pregnant ( $n = 117$ ) and 5 non-pregnant ( $n = 288$ ) coyotes during 2000-2002 breeding seasons; aligned to the estimated day of ovulation (day 0). Asterisk (\*) indicates statistical difference detected between study groups. Number within column bar indicates the number of individuals included in a specific weekly data set. Missing columns are weeks for which there were insufficient data available.



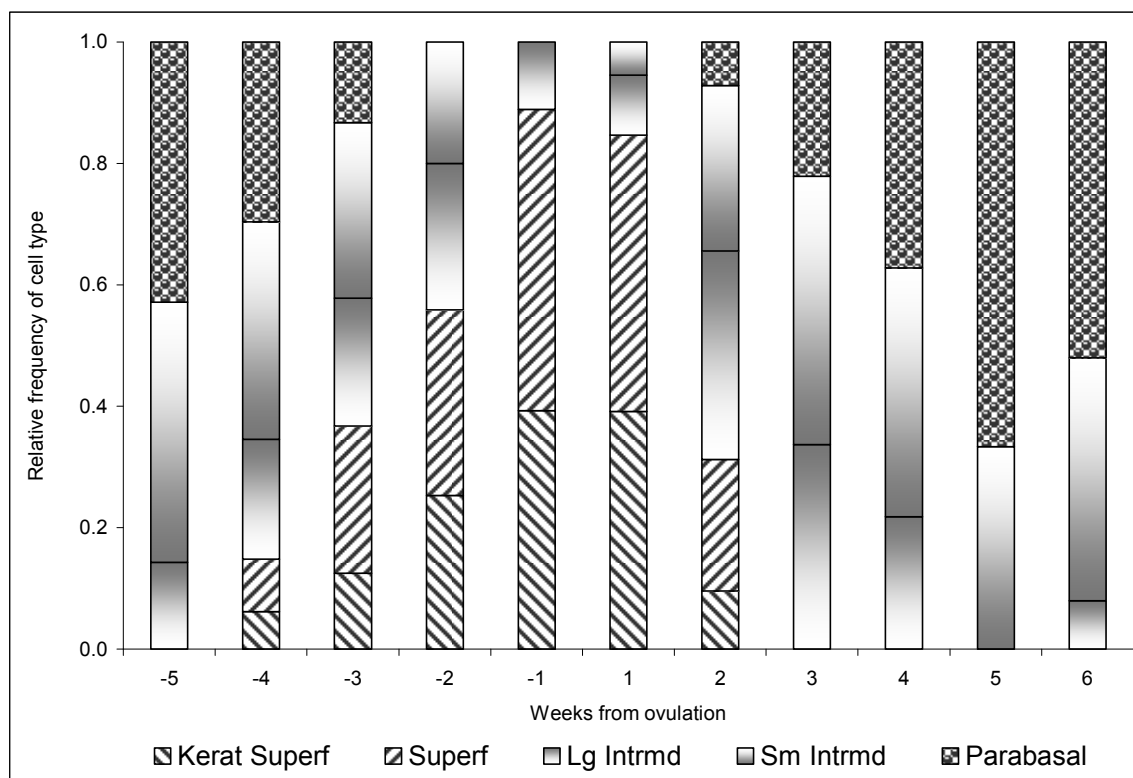
**FIG. 4.**—Weekly mean ( $\pm$  SE) serum progesterone (ng/ml) levels from 8 pregnant ( $n = 245$ ) and 8 non-pregnant ( $n = 482$ ) coyotes during 2000-2002 breeding seasons; aligned to the estimated day of ovulation (day 0). No inter-group statistical difference was detected. Number within column bar indicates the number of individuals included in a specific weekly data set. Missing columns are weeks for which there were insufficient data available.



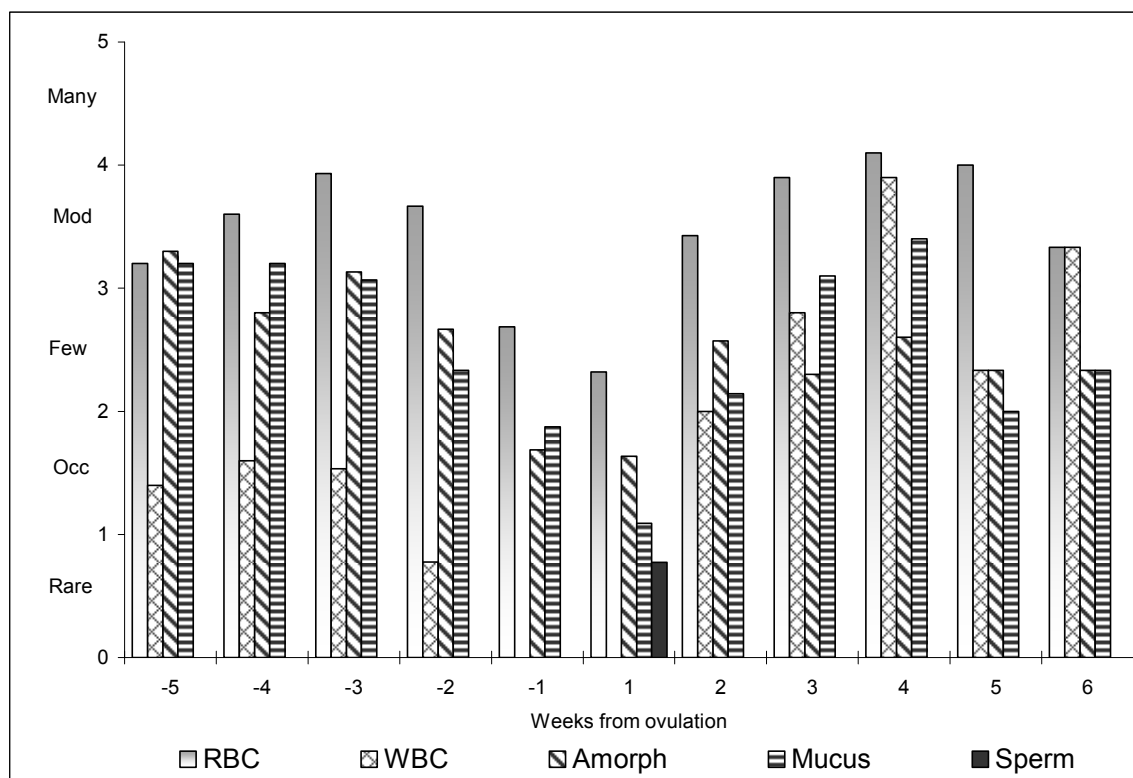
**FIG. 5.**—Weekly mean ( $\pm$  SE) serum levels of prolactin (ng/ml) from 5 pregnant ( $n = 85$ ) and 8 non-pregnant ( $n = 120$ ) female coyotes during 2000-2002 breeding seasons. Data aligned to the estimated day of ovulation (day 0). Asterisk (\*) indicates statistical difference detected between study groups. Also shown, plasma relaxin (OD = optical density) readings from 82 pregnant coyotes ( $n = 209$ ); seasons 2000-2003. Relaxin was  $<0.100$  OD for non-pregnant coyotes (data not shown).



**FIG. 6.**—Representative example of exfoliated epithelium and other inclusions commonly seen on a coyote vaginal smear in proestrus (x480). Dotted arrow indicates a parabasal cell, dashed arrow indicates an intermediate cell, and solid arrow indicates a red blood cell. Amorphous debris and mucus is conspicuous in the background.



**FIG. 7.**—Relative proportion of exfoliated vaginal epithelial cells viewed on vaginal smears ( $n = 133$ ) collected weekly from 18 coyotes during 2000-2002 breeding seasons. Data aligned to the estimated day of ovulation (day 0) including 5 weeks pre-ovulation to 6 weeks post-ovulation. Kerat Superf = keratinized (anuclear) superficial, Superf = nucleated superficial, Lg Intrmd = large intermediate, Sm Intrmd = small intermediate, Parabasal = parabasal cells.

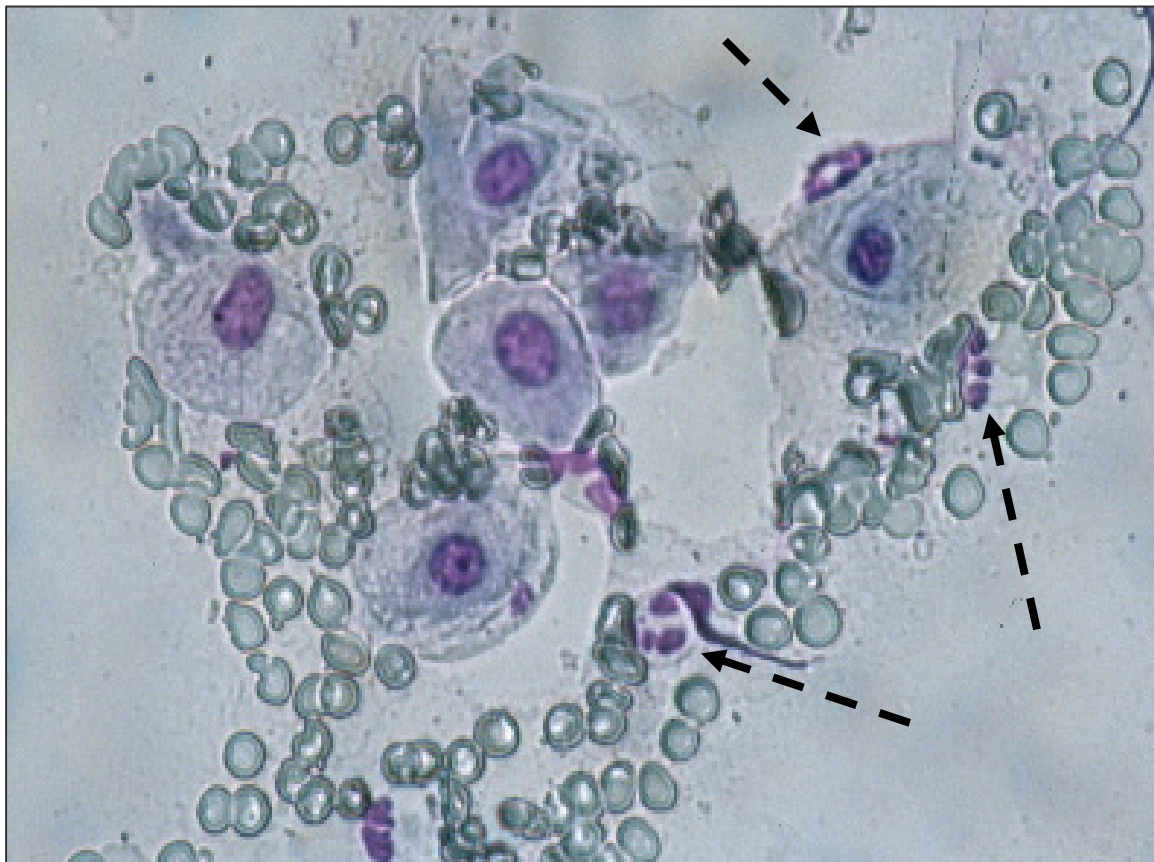


**FIG. 8.**—Inclusions other than epithelial cells viewed on weekly vaginal smears ( $n = 133$ ) from 18 coyotes during 2000-2002 breeding seasons. RBC = red blood cells, WBC = white blood cells, Amorph = amorphous debris, Mucus = mucus, and Sperm = spermatozoa. Data aligned to the estimated day of ovulation (day 0) including 5 weeks pre-ovulation to 6 weeks post-ovulation.





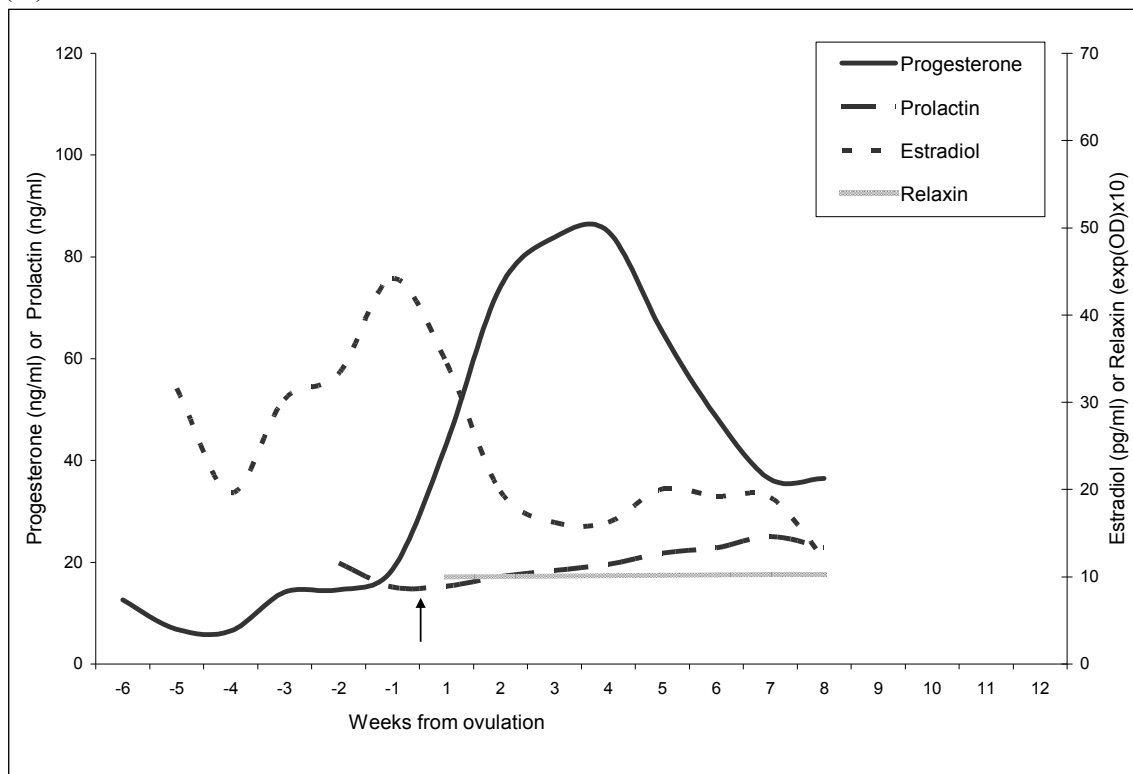
**FIG. 9.**—Representative example of a coyote vaginal smear collected in estrus (x480). Superficial cells with pyknotic nuclei and spermatozoa are shown; note the relatively clear background as compared to Figs. 6 and 10.



**FIG. 10.**—Representative example of exfoliated epithelium and other inclusions seen on a coyote vaginal smear collected in diestrus (x480). Dashed arrows indicate intact white blood cells (neutrophilic leukocytes). Parabasal and intermediate epithelial cells re-emerge. Red blood cells, mucus, and amorphous debris also reappear.

**FIG. 11.**—Relationship of weekly mean blood levels of estradiol (pg/ml), progesterone (ng/ml), prolactin (ng/ml), and relaxin (OD), aligned to the estimated day of ovulation (day 0). Solid arrow indicates ovulation. (A) pseudopregnant females; (B) pregnant coyotes. Columns indicate weekly number of copulatory ties observed during 2000-2003 breeding seasons; dashed arrow indicates day of parturition (day 64 post-ovulation). Solid bar indicates range of behavioral estrus (day -8 to day 10).

(A)



(B)

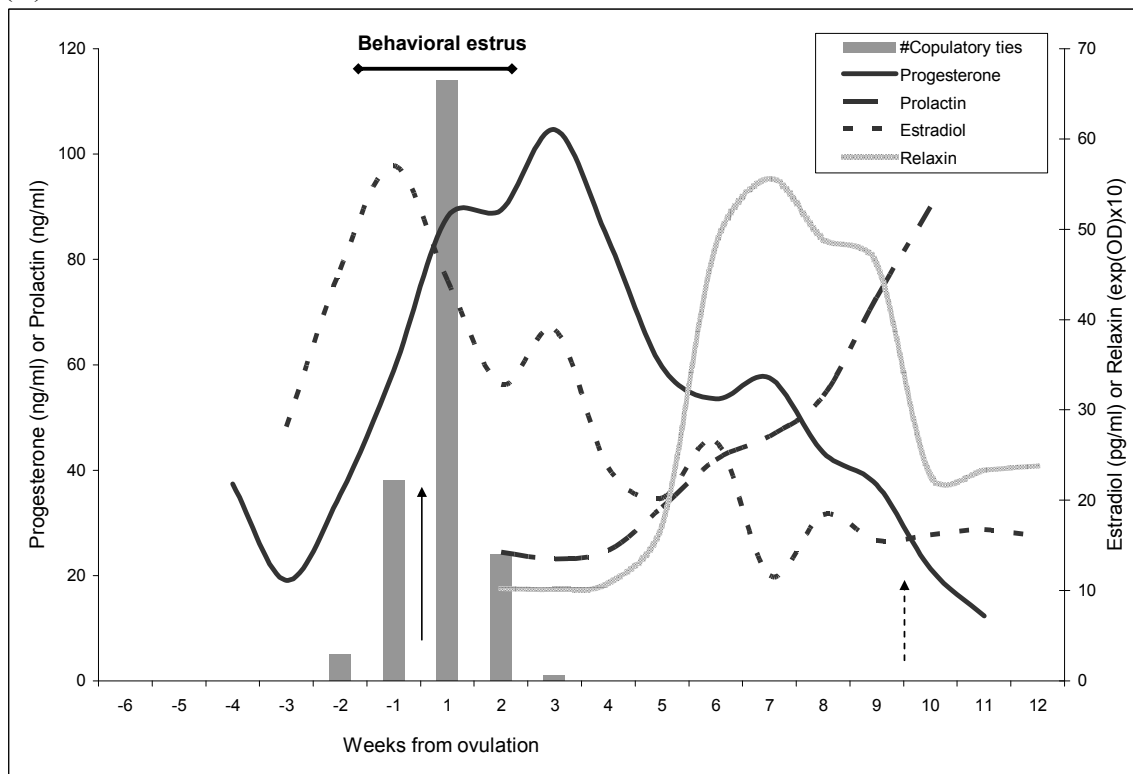
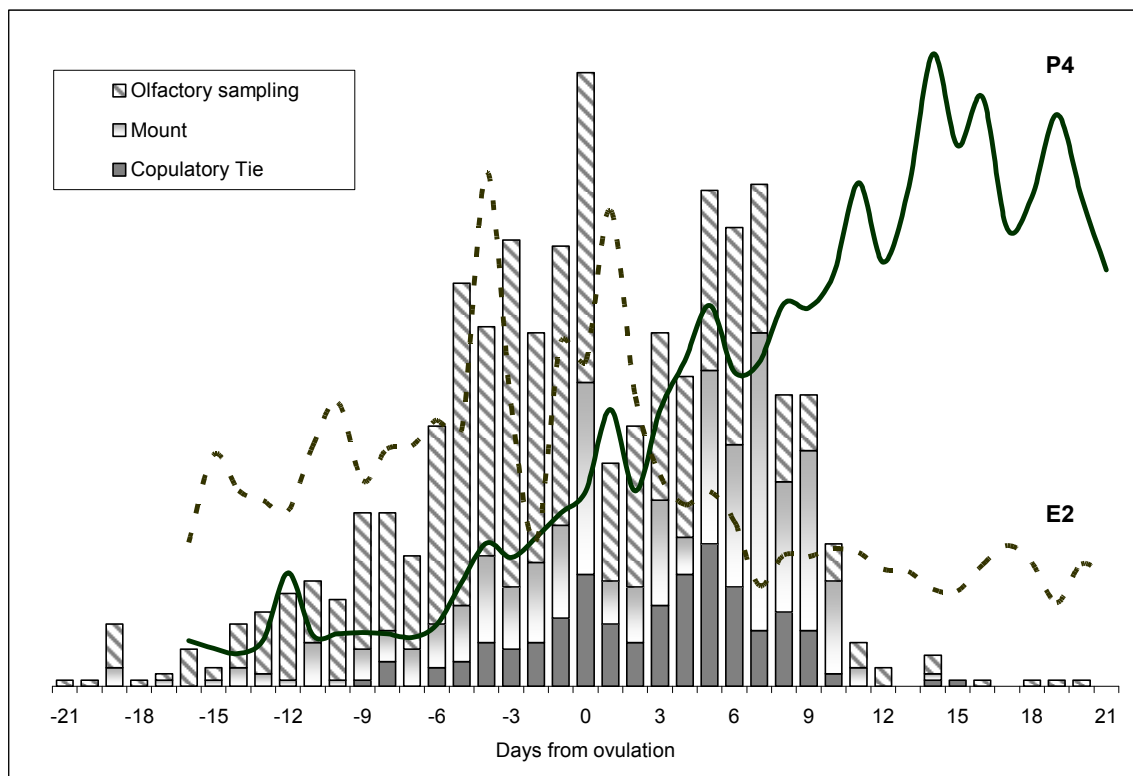
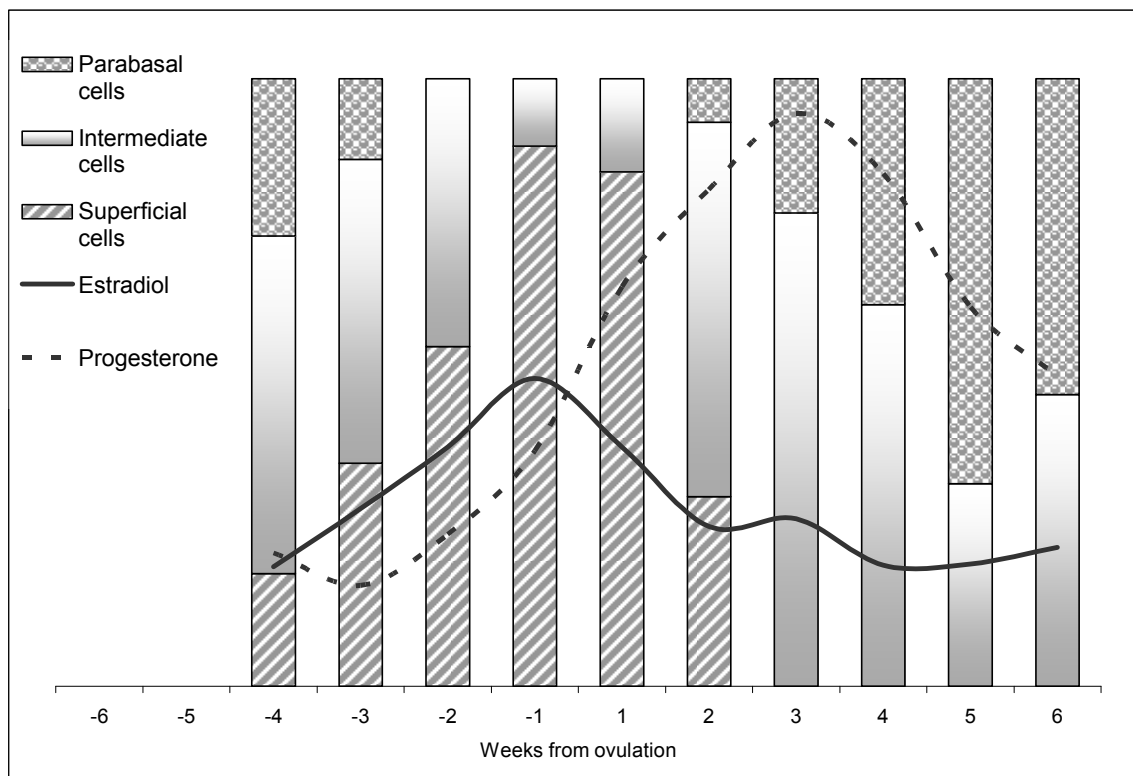


FIG. 11.

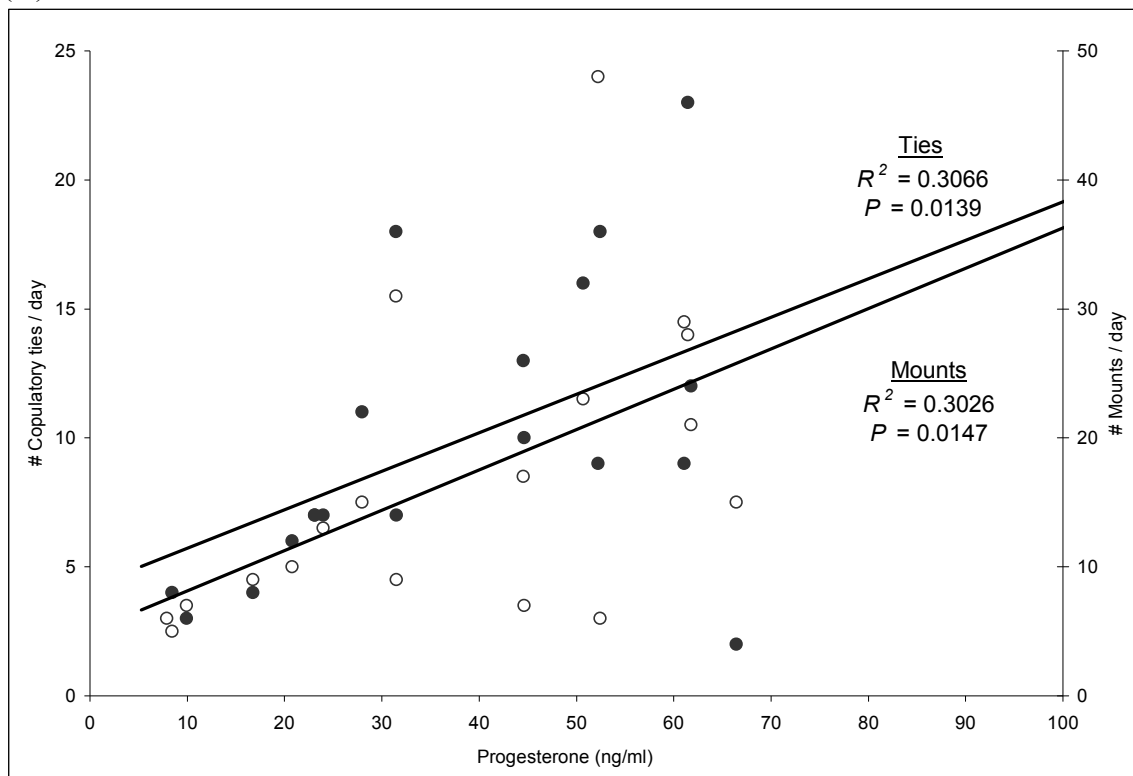


**FIG. 12.**—Daily mean blood levels of estradiol (pg/ml) and progesterone (ng/ml) overlaying sexually specific behaviors in coyote pairs (olfactory sampling, mounts, and copulatory ties) observed during 2000-2003 breeding seasons. Data aligned to the estimated day of ovulation (day 0).



**FIG. 13.**—Weekly mean blood levels of estradiol (pg/ml) and progesterone (ng/ml) and the relative proportion of epithelial cells observed on vaginal smears from coyotes during 2000-2002 breeding seasons, 4 weeks pre-ovulation to 6 weeks post-ovulation. Data aligned to estimated day of ovulation.

(A)



(B)

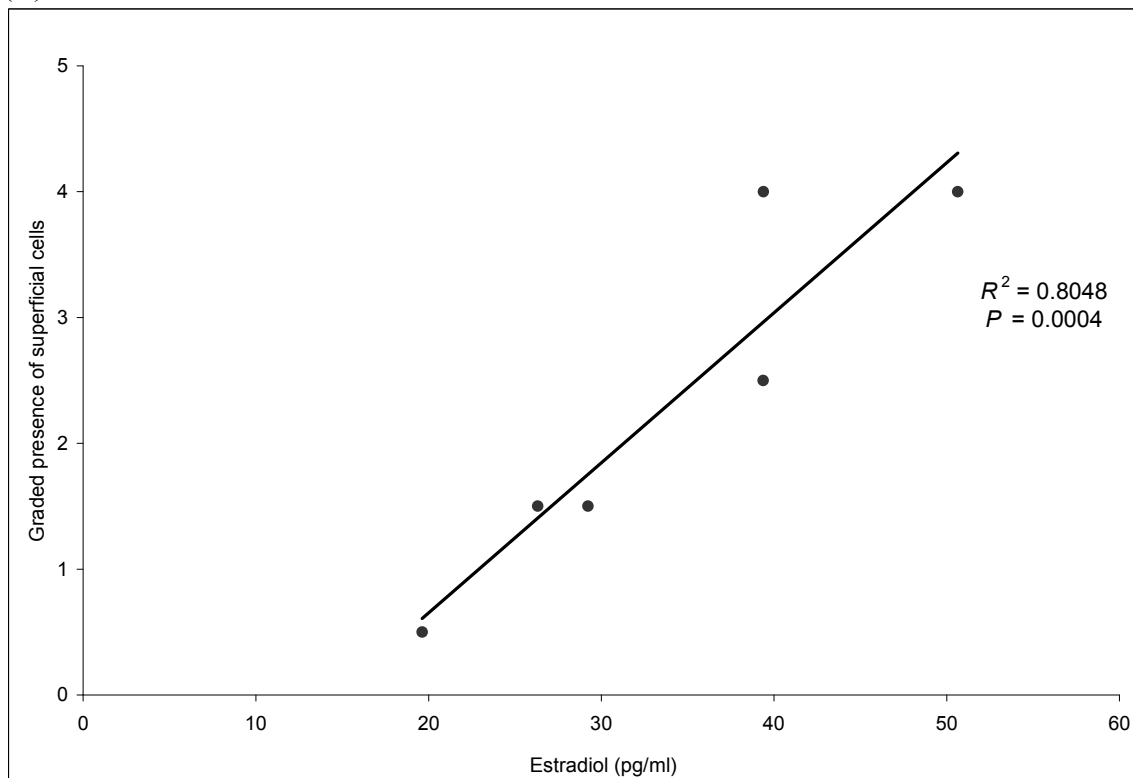
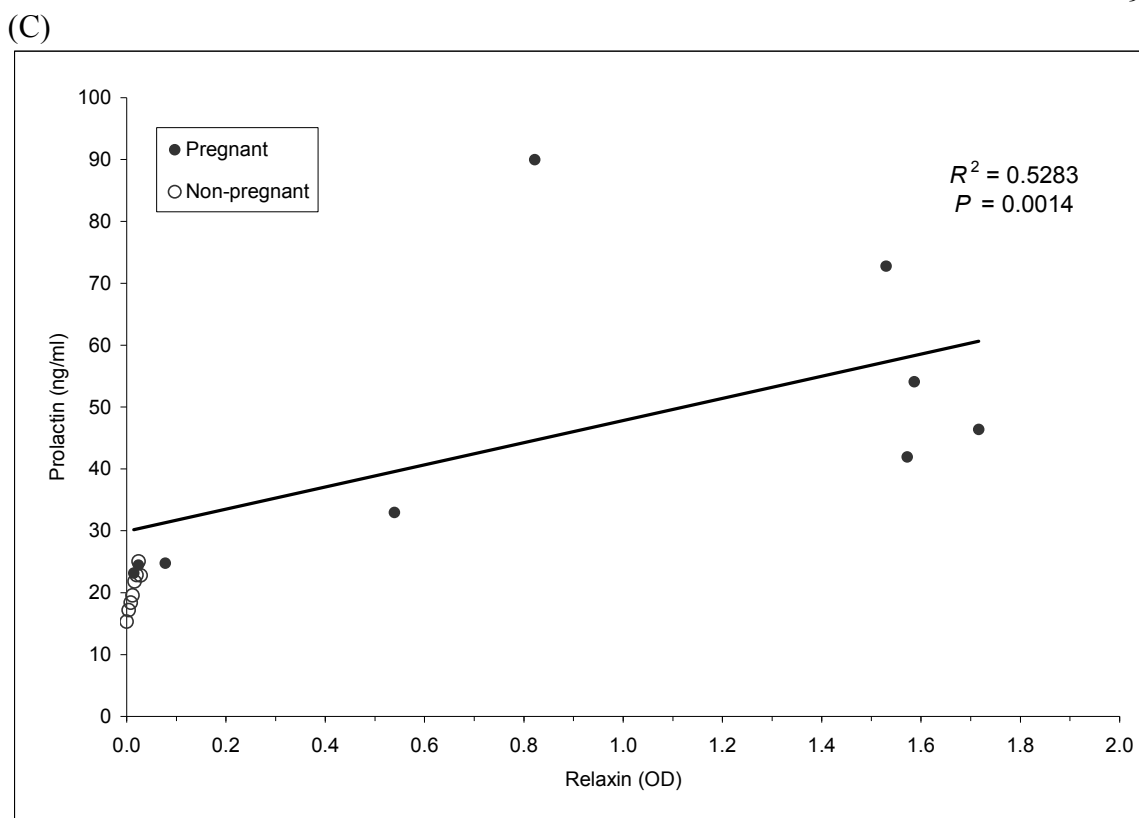


FIG. 14



**FIG. 14 Continued.**—Regression analyses showing statistically significant relationships between (A) daily number of observed copulatory ties (closed circles) or mounts (open circles) and mean progesterone blood level; (B) weekly mean estradiol level and gradation of superficial cells observed on vaginal smear; (C) weekly mean prolactin level and relaxin absorbance measurement (OD = optical density). Coefficient of determination ( $R^2$ ) and  $p$ -value are displayed for each model.



CHAPTER V  
INTEGRITY OF MATING BEHAVIORS FOLLOWING  
ESTRADIOL MANIPULATION<sup>3</sup>

**ABSTRACT**

Monogamy and biparental care of offspring are uncommon strategies among mammals, yet characteristic reproductive traits in wild *Canis* species. Coyotes (*Canis latrans*), for example, maintain perennial pair-bonds and both sexes share responsibility for territory defense and pup-rearing. Furthermore, coyotes are seasonally monestrous and breeding is dominated by each pack's alpha male and female. They are also opportunistic predators on domestic livestock. 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 involve possible induction of aberrant behavior, or uterine and hematopoietic pathologies. Herein we describe a study examining the influence of steroid contraception on intra-pair socio-sexual dynamics in a wild canid. We treated captive female coyotes in estrus with low-dose estradiol benzoate (EB), either before or immediately after ovulation. During treatment, mating behavior appeared temporarily suppressed resulting in a slight lengthening of estrus; but no deleterious physiological consequences were

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<sup>3</sup> Content and style of this chapter has been formatted for submission to the journal *Animal Behaviour* with the exception of language (American versus British english). Debra A. Carlson and Eric M. Gese, coauthors.

noted. Precise timing of treatment, however, proved crucial to successful contraception. After treatment, non-pregnant females (and their mates) resumed normal behavior including food begging and reflexive regurgitation characteristic of pregnancy (behavioral pseudopregnancy). Thus our evidence suggests that integrity of a coyote pair-bond might withstand temporary perturbation due to manipulation of reproductive hormones but also that affiliative pair-bond behavior remains intact and is not weakened by failure to reproduce.

## INTRODUCTION

Monogamy is a behavioral adaptation observed when individuals choosing a single mate for breeding derive greater reproductive benefit than would be gained from breeding with multiple mates (Kleiman 1977; Wittenberger & Tilson 1980; Clutton-Brock 1989). Although some species may utilize more than one mating strategy depending on environmental conditions, obligatory monogamy often appears as a more stable strategy within species that require more than one adult to help successfully rear offspring (Kleiman 1977; Clutton-Brock 1989).

Monogamy among mammals is uncommon, particularly when compared to other vertebrates such as birds (Wittenberger & Tilson 1980), but is the predominant mating system in canids (Kleiman & Eisenberg 1973; Kleiman 1977; Clutton-Brock 1989). For example, coyotes (*Canis latrans*) form long-term pair-bonds, sharing responsibility for territory defense (Camenzind 1978; Andelt 1985; Bekoff & Wells 1986; Gese 2001) as well as parental care of offspring (Gier 1968; Silver & Silver 1969; Mengel 1971; Camenzind 1978; Andelt 1985; Hatier 1995).

Confined to a single mating season each winter, coyote reproduction is restricted by the female's annual ovarian cycle, as well as seasonal fluctuation in male spermatogenesis (Hamlett 1938; Gier 1968; Kennelly 1978). Litters averaging 3-7 pups are typically born March-May in most North American latitudes after a gestation of 60-63 days (Hamlett 1938; Gier 1968; Knowlton 1972; Kennelly et al. 1977). Ovulation in coyotes is spontaneous and locally synchronous although rare cases of pregnant coyotes found in summer have been reported (Hamlett 1938). Observed variations might be explained by differences in habitat or altitude, but also by age. Juvenile coyotes (<12 months of age) may choose to disperse or be forced from their natal territory before their first winter; however some individuals remain with their parents as subordinate helpers defending younger siblings and territory resources (Andelt 1985; Bekoff & Wells 1986; Gese et al. 1989, 1996a; Gese 2001).

Juvenile females are usually anovulatory in their first winter; meanwhile yearlings (12-24 months), although reproductively competent, are nonetheless less fecund than adults (>2 years) (Hamlett 1938; Gier 1968; Kennelly & Johns 1976; Windberg 1995; Green et al. 2002; Sacks 2005). Since only dominant (alpha) adult coyotes within a territory seem to successfully rear offspring (Bekoff & Wells 1986; Gese et al. 1989), it is assumed alpha coyotes physically interfere with mating attempts among subordinates. But there is also evidence that a physiological stress hormone response associated with social status may suppress or delay ovulation in subordinate coyotes (Hodges 1990).

Alpha coyotes are also known to dominate and control access to food resources within their territory (particularly carcasses) (Gese et al. 1996b). While 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 the parents are exploiting a nutritionally rich and easily accessible resource for young dependent pups. As such, contraception represents a promising non-lethal solution to coyote-human conflicts. Accordingly, Bromley & Gese (2001a, 2001b) 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 bitches, but life-threatening adverse reactions may occur (Feldman & Nelson 2004). Estradiol prepares the reproductive tract for mating and pregnancy, primes progesterone receptors in reproductive tissues, and works synergistically with progesterone to affect zygote transport through the oviduct and promote early embryonic development (Harper 1994; Johnson & Everitt 2000). In the domestic bitch, smooth muscle contractions within the oviduct help retain the blastocysts until uterine conditions are favorable for implantation. As estradiol levels decline and ovarian progesterone synthesis increases, the oviducal musculature relaxes allowing the embryos to pass into the uterus, typically 8-10 days after ovulation (Holst & Phemister 1971; Tsutsui 1989). Exogenous estrogen given while an embryo is in passage through the oviduct has been shown to result in embryonic death (Kennelly 1969; Jöchle et al. 1975; Tsutsui et al. 2006), and may be attributed to alterations in the estradiol:progesterone ratio that affects oviducal embryonic development (Harper 1994; Feldman & Nelson 2004). 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

regimes, however, have been associated with severe complications, such as pyometra and bone marrow suppression (Bowen et al. 1985; Miura et al. 1985; Feldman & Nelson 2004).

In contrast to veterinary practice in the United States, low-dose (0.01mg/kg) EB is approved for use in domestic dogs in Europe (England 1998). Sutton et al. (1997) surveyed clinical veterinarians in the United Kingdom who treated 358 pet dogs (0.01mg/kg EB, sc or im) 3 and 5 days (sometimes 7 days) after a misalliance. Among the domestic bitches treated, 95.5% failed to whelp, and there were no reported cases of anemia, leukopenia or thrombocytopenia. Meanwhile, participating veterinarians reported pyometra in 7.3%, but since pyometra is not a rare disease among intact domestic bitches, it is uncertain if this rate of incidence exceeded expectations for a subset of the domestic population. Thus, since drug induced pyometra and bone marrow aplasia appears to be associated with long-acting estrogen compounds, high dosages, or when products were given during the luteal phase (diestrus), it is possible that low-doses of a short-acting product (such as EB) might not provoke the same deleterious responses.

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 & 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 higher sexual behavior scores. Also, a positive effect

was more pronounced if estradiol was withdrawn at the time progesterone was given.

Among the different study treatment regimes, bitches with estradiol continuing along with progesterone treatment displayed protracted estrous behavior, while those receiving only estrogen products showed brief transient or no response.

In his summation, Hamlett (1938) was unwilling to definitively conclude that the coyote was monestrous, or could not breed again if she failed to become pregnant in her first estrus. Later work investigating reproduction in coyote and dog *C. familiaris* hybrids described F<sub>1</sub> offspring as fertile, but with breeding seasons out of phase from their wild progenitors (Gier 1968; Silver & Silver 1969; Mengel 1971). Furthermore, one hybrid female reportedly experienced a foreshortened inter-estrus period (only 6 months). The “estrus” commonly referred to in these studies was behavioral and it is therefore unknown when ovulation actually occurred. But a similar case of a possible “multiple estrus” was reported by Harrington et al. (1987), thus enforcing a possible hypothesis that the coyote could, under certain circumstances, be polyestrous. This study, therefore, was designed to address two questions in the reproductive biology of the coyote. First, do coyotes have a truly monestrous ovarian cycle, implying that if a female fails to become pregnant in her first estrus she will not experience a foreshortened inter-estrus interval and ovulate again in the same year? Second, what influence would steroid hormone contraceptive therapy have on the intra-pair relationship of a wild canid species with rigorous social and mating behaviors?

## METHODS

### Animals

Coyotes were captive born or wild caught as pups, and reared at the National Wildlife Research Center (NWRC) facility in Millville, Utah, U.S.A. (41°68' N, 111°82' W). All animals were housed in outdoor enclosures with natural lighting. Male-female pairs resided in 0.1 hectare pens with access to sheltered den boxes. Three pens formed a clover-shaped cluster separated by double fencing and concrete barriers; 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, Utah, U.S.A.) once daily, and fasted one 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 and the NWRC.

Within this colony, coyotes appeared to be synchronized and typically entered estrus mid-January to mid-February (Chapter IV this text). Pairs recruited into this study were either established (resided with each other during a previous breeding season) or were introduced in October to allow formation of a pair-bond before the breeding season began. All females were considered fertile, having produced live pups in previous years. During 2002-2003, the female coyotes ranged from 3-11 years of age and weighed  $10.9 \pm 0.4$  kg at the time of treatment.

## **Treatment protocols**

### *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 0.01mg/kg EB (Mesalin®, oestradiol benzoate, 0.2mg/ml, Intervet UK Ltd., Milton Keynes, Buckinghamshire, U.K.). Each control female received 0.5ml of 0.9% sterile normal saline by interscapular subcutaneous injection.

### *Protocol 2*

In 2003, 1 treatment female was replaced and another added to the cohort. Otherwise, 4 females from 2002 remained in the treatment group for this phase of the experiment. 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 level suggested the female had ovulated (Chapter IV this text). Each treatment female (n = 6) then received an interscapular subcutaneous injection of 0.01mg/kg EB (Oestradiol Benzooat, oestradiol benzoate, 0.2mg/ml, Intervet International B.V., Boxmeer, N.A.) with all females beginning treatment within 2 days of the estimated day of ovulation (day 0). Following the initial dose, 2 additional post-ovulation treatments were given 2 days and 4 days after the first treatment. By the same schedule, control females (n = 3) received a 0.5ml interscapular subcutaneous injection of 0.9% sterile normal saline.



### *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 - July for signs of latent adverse effects. Results collected from the study animals (treatment and control groups) in this experiment were compared to parameters previously collected from cohorts in an associated longitudinal study (Carlson unpublished data). Within this study, and under the conditions described above, no adverse effects were noted following the administration of either Mesalin® or Oestradiol Benzoat to coyotes.

### **Mating behaviors**

Continuous scanning observations of the coyote pairs were conducted daily throughout available daylight hours, January through March, 2002 and 2003. 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 seconds per pen, all pens were viewed at least once every 5-10 minutes. 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 minutes); 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 & Mendelsohn 1971; Bekoff & Diamond 1976) 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).

### **Specimen collection and handling**

Peripheral blood samples were collected from the cephalic or saphenous veins by venipuncture. Samples were collected during 0800-0930 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 minutes. The serum was separated from the blood cells and divided into aliquots for storage. Sample aliquots to be tested within 24 hours were stored at 2-7°C, while others were frozen at  $\leq -20^{\circ}\text{C}$  for later use.

In 2002, sampling for progesterone levels 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, blood sampling began the day after the first observed copulatory tie and was repeated every other day until the female's rising progesterone levels suggested ovulation had occurred. After the first dose of EB (or normal saline) 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 weeks and 4 weeks after treatment was completed. A pattern of sustained elevation in serum progesterone was similar to the endocrine profile described for other coyotes within this colony (Chapter IV this text) and thus suggested that ovulation had occurred in the current study subjects.

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 (Chapter III this text), 90-100% of pregnant coyotes tested positive for relaxin on days 25-28 of gestation; while 100% of the coyotes, later seen with pups, remained relaxin-positive between day 28 and parturition. 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  $\leq -20^{\circ}\text{C}$  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 leukocyte differential were performed within 8 hours 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.

### **Laboratory assays**

Quantitative progesterone blood levels were assayed by competitive binding enzyme immunoassay (EIA) (Progesterone EIA, DSL-10-3900, Diagnostic Systems Laboratories, Inc., Webster, Texas, U.S.A.) using the procedure described elsewhere (Appendix A) and validated for coyotes (Appendices F). 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 peri-ovulatory progesterone blood levels. All samples were tested in duplicate with an intra-assay coefficient of variation (CV) threshold  $\leq 10\%$ . 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, California, U.S.A.). Using the procedure validated (Appendix C) and described for the coyote (Chapter III this text), the presence of relaxin was characterized by the formation of a blue color

within a micro-titer well, with color development increasing in intensity as a female progressed through her pregnancy. In contrast, 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 parameters were determined by manual laboratory methods (Davidsohn & 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, New Jersey, U.S.A.) 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 5,000rcf. 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 (x1000, 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 (Appendix D).

### **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 19 mated pairs during 2000-2003 breeding seasons; including data from animals subsequently recruited into the treatment groups. Since the behavioral patterns

of the experimental control animals were found to be consistent 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 cohort referred to as colony.

To estimate the day of ovulation, we similarly employed historical endocrine data for interpretation of serum progesterone levels. The 7 individual female coyotes treated with EB during the experimental trials (Protocol 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 (Chapter IV). The previously collected data, therefore, provided a reference against which current individual progesterone levels could be compared, helping predict if (and when) ovulation had occurred in each coyote. For control females, if historical data was unavailable, predictions were based on the apparent rate of change in successive progesterone levels. Prior experience with the quantitative progesterone EIA showed serum progesterone levels would nearly double around the time of ovulation (increase from day -1 to day 1; mean  $CV \pm SE = 0.354 \pm 0.055$ ) (Chapter IV this text).

## RESULTS

### **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 and 4 days later. Consequently, 5/5 control females treated with normal saline (NS) produced live pups, and 4/5 females treated with EB also became pregnant and had healthy litters. Furthermore, mean ( $\pm SE$ )

litter size in the control group ( $5.1 \pm 0.4$  pups) was consistent with other colony litters ( $5.3 \pm 0.3$  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 ( $6.0 \pm 0.9$  pups).

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

After treatment and during pregnancy, mean ( $\pm$  SE) peripheral WBC and HCT for control coyotes was  $11.9 \pm 0.9 \times 10^3/\text{mm}^3$  and  $50 \pm 1\%$ , respectively; mean rectal temperature was  $101.8 \pm 0.2^\circ\text{F}$ . Similarly, mean physiological parameters measured for coyotes treated with EB in Protocol 1 were: WBC,  $11.5 \pm 0.5 \times 10^3/\text{mm}^3$ ; HCT,  $49 \pm 1\%$ ; and temperature,  $102.0 \pm 0.2^\circ\text{F}$ . At the individual level, average variation (represented as %CV from baseline pre-ovulation to prepartum) in WBC throughout pregnancy was 21.5% for control coyotes and 21.7% for EB treated females.

Among Protocol 2 female coyotes, diestrous hematology parameters were: WBC,  $8.8 \pm 0.6 \times 10^3/\text{mm}^3$  and HCT,  $50 \pm 1\%$ ; while mean temperature was  $101.5 \pm 0.2^\circ\text{F}$ . It should be noted that although the WBC for control and Protocol 1 coyotes appears slightly higher than Protocol 2, this difference was an expected normal physiological response to pregnancy. Accordingly, mean WBC among control and Protocol 1 coyotes fell in summer (July 2002) to  $9.5 \pm 0.4 \times 10^3/\text{mm}^3$  and  $8.4 \pm 0.5 \times 10^3/\text{mm}^3$  respectively.

## 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 ( $\pm 1.4$  SE) days (Chapter IV this text). Comparison of EB treated coyotes with colony or control 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 control 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 2:  $P|t|_{0.05(2),12} \geq 1.58 = 0.139$ ,  $F_{5,7} = 1.09$ ). Nevertheless, 1 coyote pair in Protocol 1 was observed in a copulatory tie on day 11 post-ovulation, while 3 pairs in Protocol 2 copulated on day 11 and 2 pairs on day 12 (Fig. 15). By comparison, only 2/182 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 a pulse of increased copulations near the day of ovulation (Fig. 15). 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. 16A) 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 to themselves during the 2 experimental seasons (Fig. 15) also illustrates how active these particular pairs



typically were prior to ovulation, and the suppressive influence of treatment (Figs. 16A and 16B).

During treatment in Protocol 2, the coyotes again exhibited a suppression of sexual behavior (Fig. 15). Initially, this cohort's activity pattern fell in accordance with a lull historically seen during day 1-2 post-ovulation (Fig. 16B), but the coyotes in Protocol 2 subsequently displayed a significant decline 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 fell 57% from 1.17 to 0.50 copulations per pair.

Nevertheless, 4/5 pairs in Protocol 1 and 4/6 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 (Figs. 16A and 16B). 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 4/5 females in this cohort became pregnant.

In contrast, 5/6 pairs in Protocol 2 resumed copulating after treatments were terminated (day 5-6), but only 1/6 females became pregnant. This cohort was also distinctive because of atypical activity after day 10 post-ovulation. As previously mentioned, 3 pairs in Protocol 2 copulated on day 11; however, 1 pair did not tie between the day following the first treatment (day 2) and day 11 (which lasted <1 minute). 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 12.

Other mating behaviors, meanwhile, appeared to be less affected by treatment with EB (Fig. 17). 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 copulatory ties).

## DISCUSSION

In this experiment, pregnancy was averted through administration of low-dose estradiol benzoate; meanwhile, perturbation of sexual behavior appeared transient, and there was no adverse affect on hematopoiesis or subsequent fertility. Timing, however, was found to be critical. When the initiation of treatment was based on the first copulatory tie (the start of estrus) reproduction was unimpaired. In fact, 4/5 females in Protocol 1 ovulated during the treatment period; and since treatments in these cases ended by day 2 post-ovulation, the coyote pairs resumed normal sexual activity with adequate time for fertilization to occur.

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 levels 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.

Observations from Protocol 1 suggested several key points: (1) that the treatment would not be effective if given before ovulation and (2) that the dose of estradiol given was not sufficient to fully suppress ovulation or sexual behavior. Also, the evidence suggested the low-dose steroid preparation was short-acting and might be safely administered in mid-estrus. Thus in Protocol 2 administration of EB was postponed until after ovulation.

We assumed the coyotes had ovulated when a significant and sustained increase in individual serum progesterone was detected and hormone levels appeared consistent with data previously recorded (Fig. 18). Also, we presumed there was an opportunity for fertilization since all females copulated after ovulation, although the pattern of sexual activity might vary. 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 4 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 this study to address the specific mechanism by which EB prevented pregnancy, our observations suggest that fertilization could have occurred, and that 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 females 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) was weaker 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 exposure. But with embryonic demise incomplete, a placenta (the site of relaxin synthesis in canines) could, nonetheless, have been established. 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 high number of copulations recorded in late estrus, 7 times during day 10-12 post-ovulation; and these ties were unusually short, only 1-3 minutes each. Interruption of sexual activity (split estrus) may be attributed to the iatrogenic estradiol surge (since most of the females displayed a suppression of mounts and ties). But the high incidence of extended estrus (which occurred in 3/6 non-pregnant females as well as the failed pregnancy case described above) can not be adequately explained in this 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. Such a rebound effect, however, would only appear unusual when it fell outside the established parameters for a normal estrus (i.e., >10 days post-ovulation). Otherwise, if a rebound occurred during the expected estrus time-frame, it 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. Meanwhile, a begging behavior unique to diestrus emerged, performed by both pregnant and non-pregnant females. Characteristically, a female addressed her mate in a submissive juvenile-like posture reminiscent of pups begging food from an adult. The female would hold her tail, neck and head low, below the top-line of her back; her tail would wag rapidly and she would bite and lick at the male's lower jaw and mouth. On several occasions a reflexive regurgitation was recorded by the observers; in other cases, because of the animal's orientation, it was not possible to see food expelled but the female would stop begging and appear to be consuming something (presumably regurgitate) off the ground where the male had been. Sometimes the males would move 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 (both pregnant and non-pregnant) sporadically throughout the following weeks (Fig. 19) (termination of the regular data collection schedule unfortunately precluded discovery of when begging ceased in either cohort).

Begging by non-pregnant coyotes is particularly interesting because it represents a behavioral component to covert (physiological) pseudopregnancy previously described

for coyotes (Chapter IV this text). For example, Figure 20 graphically shows the serum progesterone levels measured in a single female during 4 consecutive breeding seasons (2000-2003). As indicated, this coyote was pregnant in 2 of the years shown, but progesterone levels measured during the luteal phase of her non-pregnant ovarian cycles were comparable in concentration and duration to the gravid cycles. In Protocol 2, this female did not become pregnant after treatment with EB, yet she begged food and received regurgitated meat from her mate.

All non-pregnant coyotes remained with their mates and were periodically observed for recrudescence 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 remained consistent with other colony pairs throughout the fall. Furthermore, hematology samples collected in the summer remained within normal species and seasonal parameters; and the females were reproductively active and fertile in subsequent breeding seasons.

To our knowledge this is the first successful use of low-dose estradiol benzoate as a contraceptive 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 at low doses) on endogenous progesterone may have deleterious physiological consequences; therefore treatment with EB should only be attempted when the female's position within the ovarian cycle 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). Such behavioral consistency and longevity should serve as reinforcement for the pair's long-term social bond, thereby promoting social monogamy despite reproductive failure. Although the females were shown to be strictly monestrous and would not breed again during the summer, perpetuation of the pair-bond would still benefit the reproductive fitness of free-roaming coyotes. Territory maintenance and defense requires both the male and female's vigilance year-round, and since 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.

Thus this study provides supporting evidence that the coyote is a seasonally obligated monestrous species; and that non-pregnant females become physiologically and behaviorally pseudopregnant after ovulation. Contraceptive methods applied in winter might therefore be expected to extinguish reproduction for the current season without long-term effects on a pair's future reproductive potential. However whether permanent fertility control or sterilization would destabilize the monogamous pair-bond over time requires further investigation.

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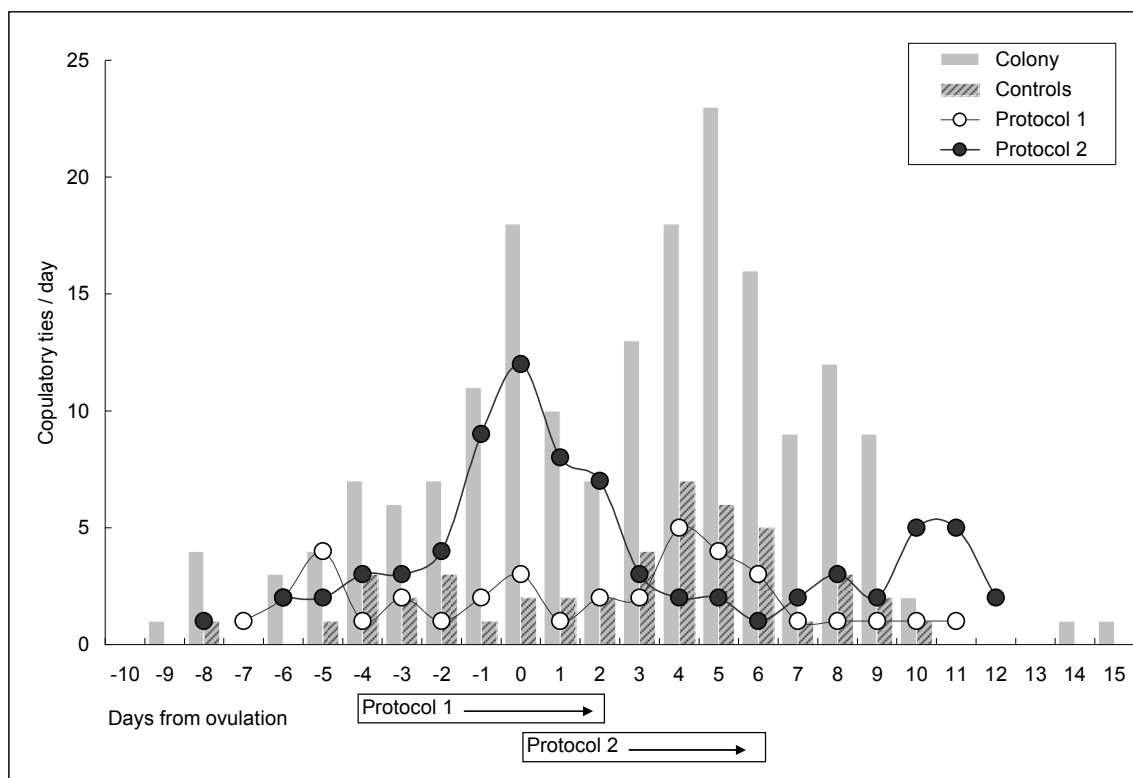
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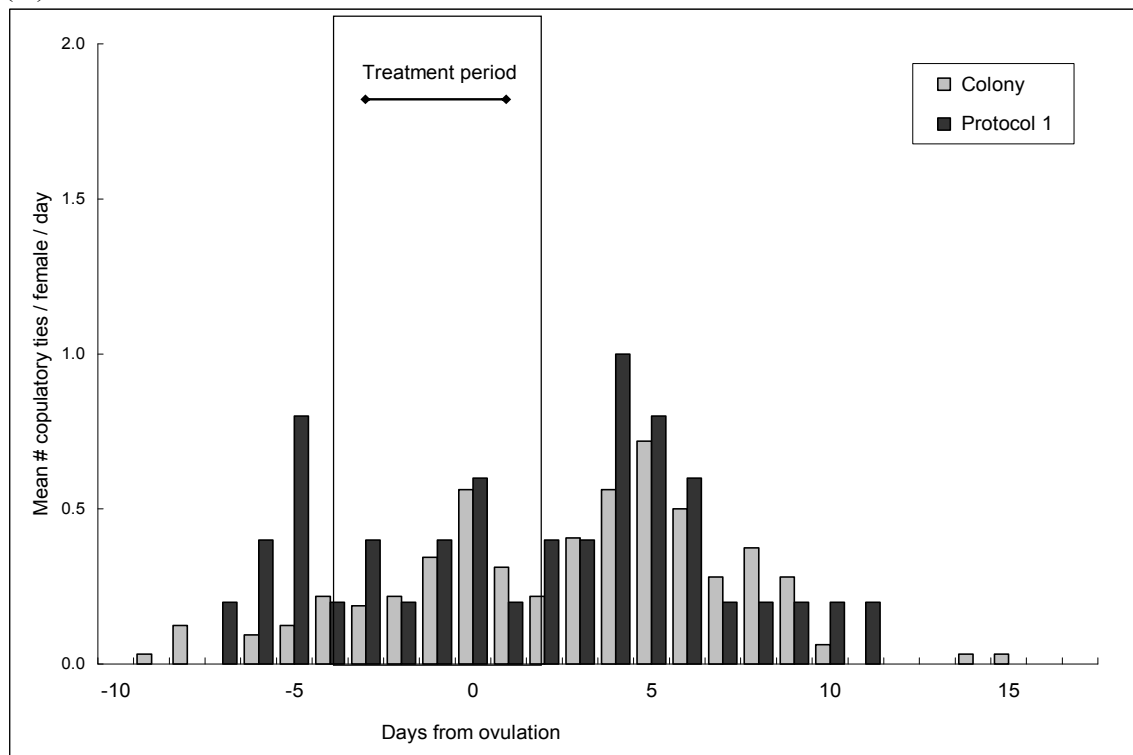
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**Figure 15.** Number of copulatory ties (aligned to the estimated day of ovulation) observed each day between coyote mated pairs. Colony, 2000-2003 breeding seasons, represents generalized pattern of behavior. Controls, 2002-2003, given 0.5ml normal saline showed no placebo effect and are grouped together. Duration of treatment (0.01mg/kg estradiol benzoate) indicated below x-axis; Protocol 1 (2002) started after first observed tie; Protocol 2 (2003) started after ovulation (day 0).

**Figure 16.** Mean number of copulatory ties observed per female per day and relative to the estimated day of ovulation (day 0), (A) Protocol 1, 2002 breeding season, (B) Protocol 2, 2003 breeding season. Colony (including control animals) represents mean number of ties per female per day observed during 4 consecutive breeding seasons, 2000-2003. Shaded area represents span of treatment (0.01mg/kg estradiol benzoate) period.

(A)



(B)

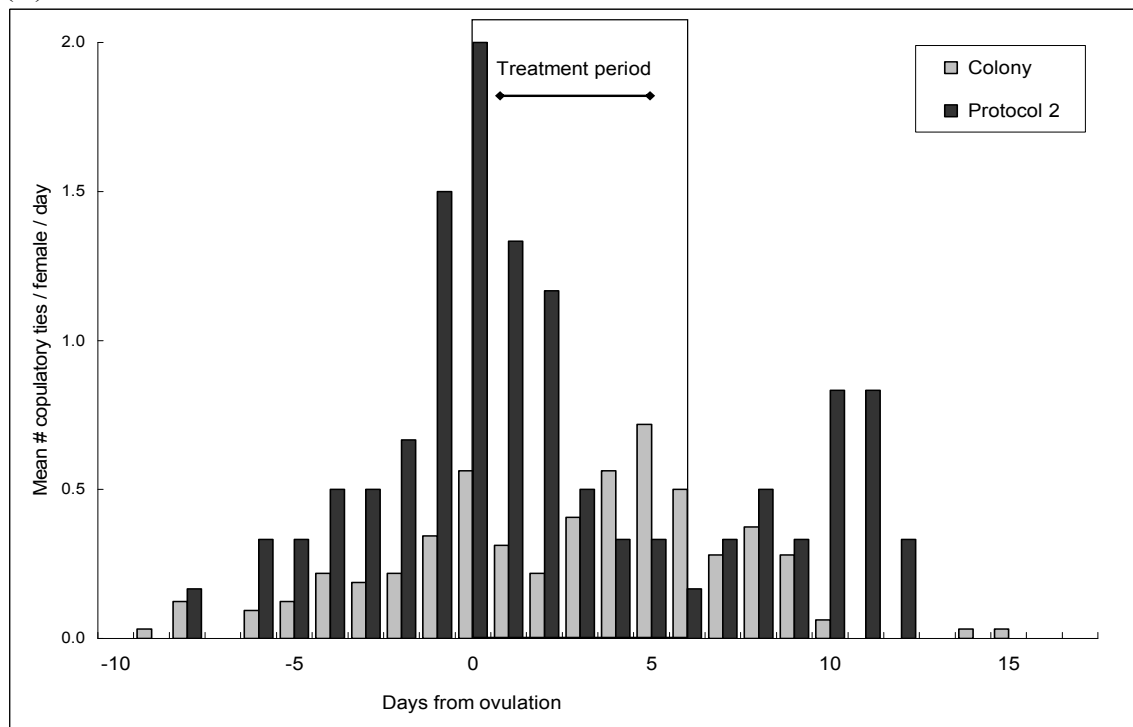


Figure 16.

**Figure 17.** Number of affiliative (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 (including experimental control animals) 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 0-6.

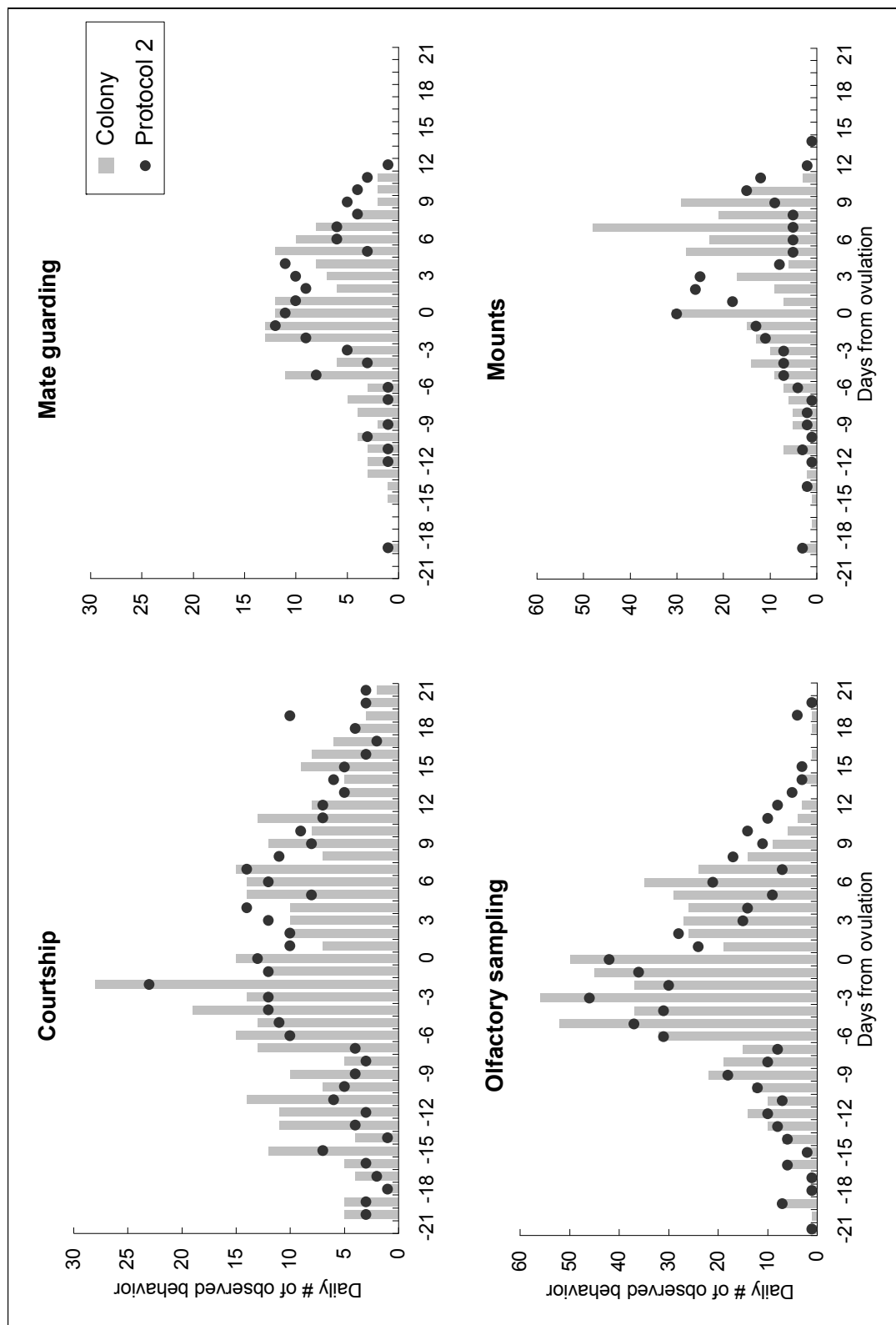
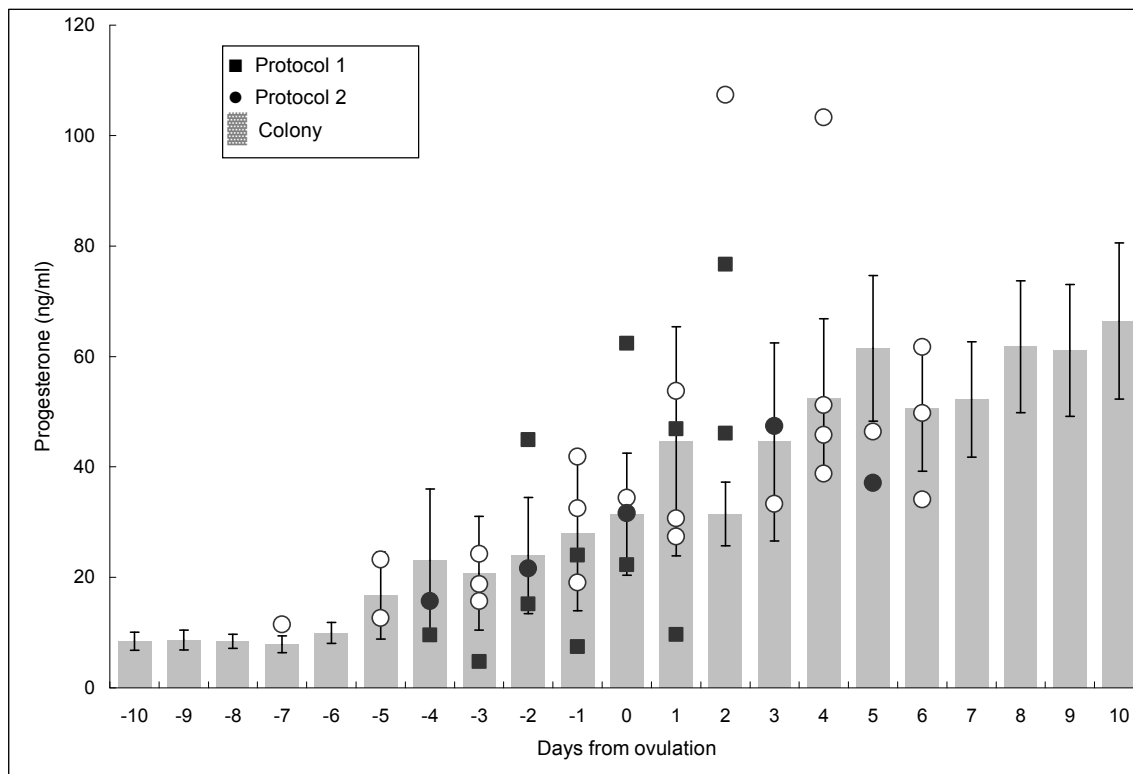


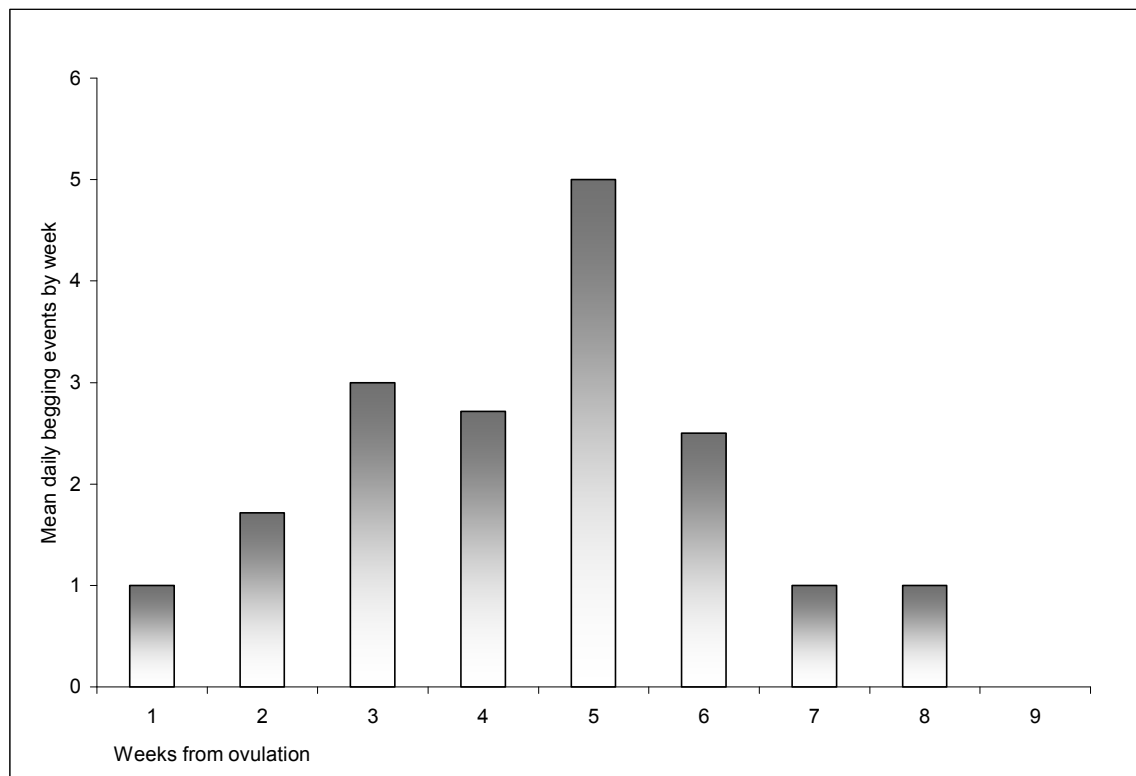
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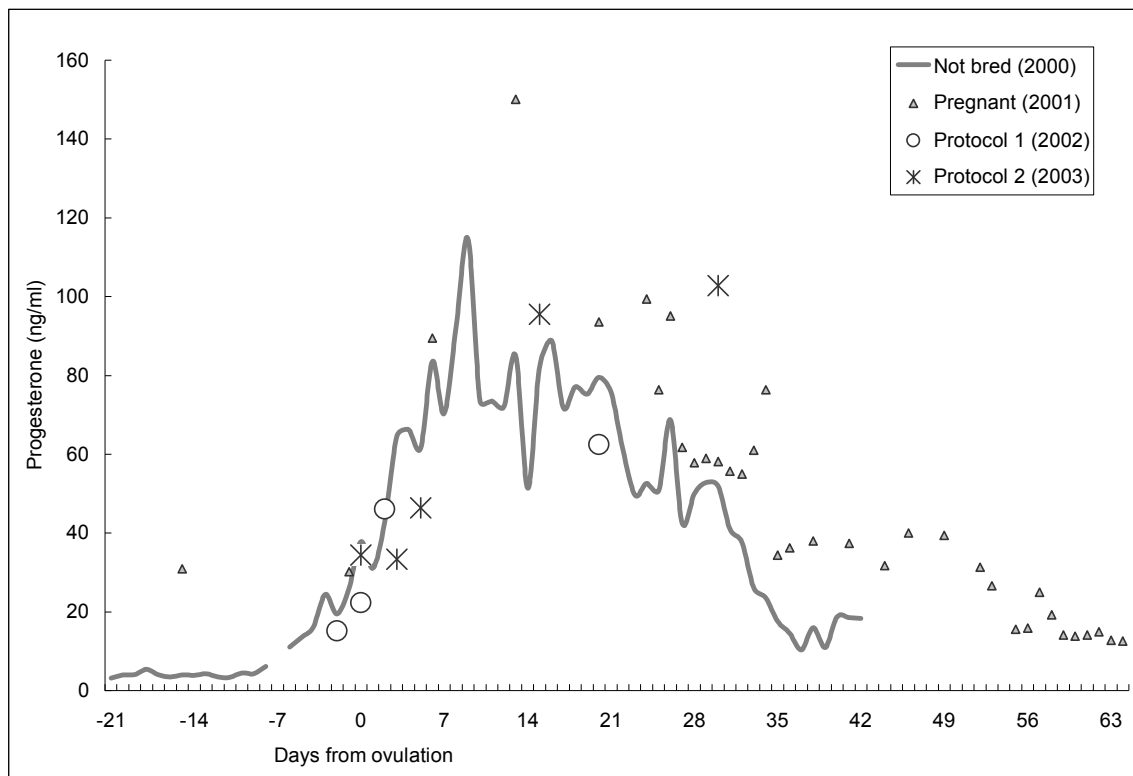


**Figure 18.** Mean  $\pm$  SE serum progesterone (ng/ml) levels measured from study animals before experimental treatment (Colony) during 2000-2001 breeding seasons.

Progesterone levels from the same individual coyotes during treatment with 0.01 mg/kg estradiol benzoate; open circles/squares represent levels from coyotes that ovulated but did not become pregnant during 2002 (Protocol 1) and 2003 (Protocol 2). Closed circles/squares represent hormone levels from treated coyotes that did become pregnant during 2002 and 2003. Data aligned to estimated day of ovulation (day 0).



**Figure 19.** Weekly mean number of events (post-ovulation) involving EB treated non-pregnant female coyotes begging food from their mates; Protocol 2 cohort.



**Figure 20.** Quantitative serum progesterone (ng/ml) measurements from a single female coyote during 4 consecutive breeding seasons, 2000-2003. Reproductive history: 2000, not bred; 2001, pregnant; 2002, pregnant after treatment with 0.01mg/kg estradiol benzoate per Protocol 1; 2003, not pregnant after treatment with EB per Protocol 2.

## CHAPTER VI

INFLUENCE OF EXOGENOUS GnRH ON REPRODUCTIVE SEASONALITY  
AND MATING BEHAVIORS<sup>4</sup>

## ABSTRACT

Seasonal reproduction in animals is recognized as a life history trait conferring increased fitness to those individuals that produce offspring when environmental conditions and acquisition of resources are optimal. While many species possess this adaptation, most are capable of initiating a second ovarian cycle if the first breeding effort fails. Wild canids such as the coyote (*Canis latrans*) or wolf (*Canis lupus*), however, are unusual among mammals; they are socially monogamous and both sexes are reproductively active only in winter. Thus wild canid pair-mates rely upon social and physiological synchrony to successfully breed during the female's single estrus. Using captive coyotes as a model wild canid, we challenged the seasonality of the coyote by evoking an out-of-season estrus in the fall using a gonadotrophin-releasing hormone (GnRH) analogue. In addition to stimulating an ovarian endocrine response, socio-sexual behaviors reminiscent of winter mating behaviors were solicited. Herein we describe the un-seasonal behaviors that were induced, but also report additional consequences experienced during the subsequent native winter breeding season. Specifically, exogenous GnRH given in October suppressed and delayed the emergence of expected sexual behaviors the following winter. Furthermore, while 8/12 females produced

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<sup>4</sup> Content and style of this chapter has been formatted for submission to the journal *Animal Behaviour* with the exception of language (American versus British English). Debra A. Carlson and Eric M. Gese, coauthors.

healthy litters in the spring, 4/12 young naïve coyotes failed to copulate or become pregnant. Our evidence therefore suggests that perturbation of reproductive hormones prior to ovulation may have profound implications on intra-pair socio-sexual relationships; and even if fertility is not disrupted physiologically, endocrine manipulations may induce behavioral disruptions with potentially deleterious consequences for breeding members of a monogamous species.

## INTRODUCTION

Seasonal fluctuations in various environmental features often act as cues, allowing an animal to coordinate arrival of its offspring with the availability of resources necessary for optimal survivability (Bronson & Heideman 1994). Environmental cues may include triggers such as temperature, rainfall, or day-length; yet mechanisms controlling reproduction ultimately depend upon a complex of physical, dietary, and social factors. Some factors (such as food, temperature, or light) may act directly and provocatively on an animal's reproductive system; or they might alert the animal to an imminent change in environmental conditions. Regardless, an individual must be appropriately prepared, physically and socially, before reproduction can proceed.

Wild canids are described as spontaneous ovulators, seasonally monestrous, and socially monogamous (Kleiman & Eisenberg 1973; Asa & Valdespino 1998); characteristics that collectively form a unique reproductive strategy among mammals. Some plasticity exists, however, and has been ascribed to changes in social or environmental conditions (Porton et al. 1987; Sillero-Zubiri et al. 1998; Kitchen et al. 2006; Asa et al. 2007). Canids therefore appear to have the adaptive ability to modify

tactics when necessary to ensure continued reproductive fitness; or when the inherent strategy is no longer under selective pressure. For example, with domestication dogs (*Canis familiaris*) have become generally aseasonal and polygamous. Male dogs are fertile all year, and the inter-estrous intervals of bitches can range from 5-12 months depending on the breed (Concannon 1993).

Among the wild North American congeners of the dog, gray wolves (*Canis lupus*) and coyotes (*Canis latrans*) are restricted to a single breeding season, extending from late January through March-April depending on latitude (Hamlett 1938; Gier 1968; Mech 1970; Kennelly & Johns 1976; Seal et al. 1979). However, possible plasticity in the coyote's reproductive seasonality is implied from studies of coyote-dog hybrids (Gier 1968; Kennelly & Roberts 1969; Silver & Silver 1969; Mengel 1971; Gipson et al. 1975). Specifically, these authors described cases of hybrid (F1) females entering estrus and mating in the fall (October-December), with one female breeding again in May (Gier 1968).

The mechanism controlling reproductive seasonality in wild *Canis* has not been elucidated although presumably it is similar to that described in other species. Photoperiod activates neuroendocrine messengers which in turn stimulate a cascade of physiological and behavioral events; however hormone receptors have varying sensitivity for activation depending on photoperiod and sequence of exposure to endocrine factors (Turek & Van Cauter 1994; Parvizi 2000). Timing of reproduction also involves synchronization of often different intrinsic biological rhythms. In other words, exogenous environmental cues (physical or social) synchronize endogenous circadian and circannual rhythms; yet conversely, the same factors may be ineffectual at suppressing or

provoking a biological process from its entrained pattern (Turek & Van Cauter 1994; Parvizi 2000).

For example, Kreeger et al. (1991) reported contrasting responses (depending on the phase of the reproductive cycle) to melatonin treatment in gray wolves. Melatonin suppressed prolactin secretion during peak levels in May-June, but had no effect when given October-December. Meanwhile, pinealectomy in wolves neither abolished seasonal breeding (Asa et al. 1987) nor the prolactin circannual pattern (Kreeger et al. 1991) (both papers suggested an alternate site of melatonin synthesis may have been a possible factor). Yet, an adult female wolf held in a 12L:12D environment for 9 months, failed to ovulate or display estrous behavior upon return to natural light in February (Seal et al. 1979).

Seasonal changes in photoperiod are communicated to the hypothalamus by shifting levels of melatonin (Turek & Van Cauter 1994). Gonadotrophin-releasing hormone (GnRH), synthesized and secreted by neurons within the hypothalamus, is transported to the pituitary through the hypothalamic-hypophyseal portal system. Alternatively known as luteinizing hormone-releasing hormone (LH-RH), pulses of GnRH stimulate synthesis of the gonadotrophins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), which in turn stimulates gonadal steroid synthesis. However the system involves negative endocrine feedback as well as positive; for example, release of gonadotrophins and ovarian hormones suppress further GnRH secretion from the hypothalamus (Turek & Van Cauter 1994). Consequently, experimental manipulations (using photoperiod or pharmaceuticals) may precipitate disparate effects when applied at different moments in an individual's reproductive cycle (Parvizi 2000).

Administration of exogenous GnRH has been used to advance estrus and ovulation, but paradoxically, also as a contraceptive. In the domestic bitch, serial injections, pulse infusion, or continuous infusion of GnRH (or GnRH agonist) given in anestrus provoked increases in serum LH and estrus (Cain et al. 1990; Concannon et al. 1997). But sustained elevation of GnRH (analogue or agonist) eventually suppressed further secretion of LH (Concannon 1989) postponing estrus and ovulation (Vickery et al. 1989; Trigg et al. 2001). In studies investigating the anti-fertility effect of GnRH, researchers noted signs of proestrus and estrus were often observed before the desired suppressive effect was achieved in dogs (Vickery et al. 1989; Trigg et al. 2001; Wright et al. 2001) and wolves (Bertschinger et al. 2001); and 2 of these studies (Bertschinger et al. 2001; Wright et al. 2001) reported pregnancies following treatment.

The goal of this study was to disrupt the estrous cycle of the coyote and explore the physiological and behavioral consequences that might arise by desynchronizing pair-mates. We hypothesized that in the fall coyotes were physiologically prepared to initiate a new ovarian cycle; if true, administration of GnRH during late anestrus would evoke a premature estrus. We could not, however, predict to what degree a pure-bred coyote might be sensitive to treatment protocols used in domestic dogs, or to what extent entrained mechanisms controlling seasonality in this species might modulate the effects of exogenous GnRH. Therefore 2 treatment products were employed: an implantable GnRH analogue, deslorelin, and an injectable porcine derived GnRH, gonadorelin. After treatment ovarian response was monitored by weekly measurement of serum estradiol and progesterone, and hormone levels in GnRH treated females were compared to control females given normal saline. In addition, behavioral observations conducted throughout



the fall recorded the influence of exogenous GnRH (or induced steroid synthesis) on socio-sexual behaviors. Finally, observations of GnRH treated coyotes during the subsequent native breeding season were conducted to describe the residual affect (if any) hormone manipulation might exert on intra-pair interactions and reproduction. Herein we describe our results and discuss how reproductive hormone manipulation might impact normal pair-mate interactions, emphasizing the importance of healthy mating behavior as well as physiology for successful reproduction in wild canids.

## METHODS

### Animals

Coyotes were captive born or wild caught as pups, and reared at the National Wildlife Research Center (NWRC) facility in Millville, Utah, U.S.A. (41°68' N, 111°82' W). All animals were housed in outdoor enclosures with natural lighting. Male-female pairs resided in 0.1 hectare pens with access to sheltered den boxes. Three pens formed a clover-shaped cluster separated by double fencing and concrete barriers; 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, Utah, U.S.A.) once daily, and fasted one 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 and the NWRC.

Eighteen mated coyote pairs recruited into this study were either established (10/18 pairs resided with each other during a previous breeding season) or recently introduced the month prior to initiation of treatment and observations (8/18). Sexually experienced females (12/18) ranged in ages 3 – 6 years old, while maiden coyotes (6/18) were 18 months – 3 years of age at the time of treatment (October 10-12, 2002). Average weight of female coyotes was 11.1 kg (range: 7.6 – 13.8 kg).

Previous studies (Chapter IV this text) have noted the ovarian cycles within this colony to be synchronous. During the 2000-2003 breeding seasons, the coyotes commonly entered estrus mid-January to mid-February. Behavioral estrus (the period of sexual receptivity when a female permits her mate to copulate) ranged from 8 days before ovulation to 10 days after ovulation; but at the individual level, females remained receptive an average  $7.6 \pm 1.4$  days.

### **Treatment groups**

#### *Deslorelin*

Deslorelin (6-D-Tryptophan-9-(N-ethyl-L-prolinamide)-10-deglycinamide), a synthetic analogue of GnRH (LH-RH), has been incorporated into a biocompatible inert matrix and formed into an implantable pellet for sustained-release (developed by Peptech Animal Health, North Ryde, NSW, Australia). In this study, the commercially available product, Ovuplant™ (distributed by Fort Dodge Animal Health, Fort Dodge, Iowa, U.S.A.), provided 2.1mg deslorelin acetate in a short-acting subcutaneous (2.3mm x 3.6mm) pellet.

In the first treatment group, 6 female coyotes each received a single interscapular Ovuplant™ pellet (mean dose: 0.2 mg/kg deslorelin per animal) in early October 2002. To prepare the insertion site, a small patch of fur was clipped, and the skin cleaned with alcohol and providone-iodine then allowed to dry. A small incision ( $\leq 0.5$ cm) made with a sterile surgical blade eased initial penetration of the implanter syringe needle through the epidermis; the pellet was then placed in the subcutaneous space according to the manufacturer's instructions. The implant site was subsequently inspected each time the coyote was handled but no gross adverse reactions were noted.

Within this cohort, 3/6 females were sexually experienced and residing with their established mates; also each had whelped a healthy litter the previous spring (March-April, 2002). Among the maiden females (3/6), 1 was 2 years of age, and 2 were 18 months old; the males selected to be their mates were also sexually naïve.

#### *Gonadorelin*

In the second treatment group, 6 female coyotes were given daily intramuscular injections, 2.0 $\mu$ g/kg gonadorelin diacetate tetrahydrate (Cystorelin® distributed by Merial Ltd., Iselin, New Jersey, U.S.A.) for 3 consecutive days, October 10-12, 2002. Gonadorelin is a GnRH (LH-RH) porcine hypothalamic extract with a short in vivo half-life. Thus daily administration of gonadorelin was intended to mimic the endogenous GnRH pulses that naturally evoke reproductive recrudescence.

Within this cohort, 3/6 females were sexually experienced and in an established pair-bond; furthermore all 3 had been pregnant the previous spring (March-April, 2002).

The maiden females (3/6) were 3 years or 18 months old (1 and 2 females respectively), and were introduced to sexually mature but equally naïve males in September 2002.

#### *Normal saline*

Six female coyotes received single interscapular subcutaneous injections of 0.5 ml sterile 0.9% physiological normal saline in early October. Within this control cohort, 4/6 pairs were established, and the females had been pregnant the previous spring. Meanwhile the other 2 females, although experienced and pregnant in 2001, were paired with new mates in September 2002 (1 male was sexually experienced, the other naïve).

#### **Mating behaviors**

The coyote pairs were habituated to low level human activity prior to the beginning of the study, and behavioral observations began a week prior to treating the females in early October. All enclosures could be viewed through binoculars or spotting scope from sites 100–500 m away and were continuously scanned: from 0800-1000 and from 1500 until visibility was lost in the evening, October – December, 2002; and throughout available daylight, January – March, 2003.

Observers would continuously scan the enclosures, viewing one pen, documenting any interactive behavior occurring between the mated coyotes then scan the next pen. Because this process rarely took more than 30 seconds per pen, all pens were viewed at least once every 5-10 minutes. Also, 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 minutes). 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 & Mendelsohn 1971; Bekoff & Diamond 1976) 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. Observed affiliative social behaviors included: (a) courtship (non-antagonistic play-wrestling and play-chases, allo-grooming such as licking the face, ears or back, also body-rubs, 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).

### **Specimen collection and handling**

To evaluate ovarian response to the GnRH treatments, blood samples for quantitative estradiol and progesterone assays were routinely collected; immediately prior to treatment (initial baseline) and weekly thereafter for 9 weeks (October 10 - December 19). Further sampling however was temporarily suspended until the coyotes' native breeding season and estrus began (January-February 2003). In winter, a serum sample was collected 1-3 days after a mated pair's first observed copulatory tie; and another 2 weeks later. In the event a pair(s) was not observed in a copulatory tie, a sample was

collected on a random day in mid-February (approximately 64% of pairs were observed in a tie, January 24 - February 13) followed by a second sample 2 weeks later.

Peripheral blood samples were collected from the cephalic or saphenous veins by venipuncture. Samples were collected during 0800-0930 before the animals were fed and without sedation or anesthesia. For quantitative estradiol and progesterone analysis, whole blood was collected in an evacuated tube and allowed to clot at room temperature (20-24°C) for 30-120 minutes. Serum was separated from the blood cells by centrifugation, divided into aliquots then stored at  $\leq -20^{\circ}\text{C}$  until testing. .

Pregnancy was determined by the presence or absence of relaxin in plasma, therefore anti-coagulated (sodium heparin or lithium heparin) whole blood samples were also collected. In a previous study, relaxin was detectable after day 28 of gestation in the plasma of all coyotes later seen with pups; while non-pregnant coyotes were consistently negative (Chapter III this text). Thus in the present study, heparinized samples were collected 4–5 weeks after the first observed copulatory tie, and females initially testing negative were resampled 2 weeks later. Samples were promptly centrifuged and the separated plasma was stored at  $\leq -20^{\circ}\text{C}$  until testing.

### **Laboratory assays**

Quantitative progesterone blood levels were assayed by competitive binding enzyme immunoassay (EIA) (Progesterone EIA, DSL-10-3900, Diagnostic Systems Laboratories, Inc., Webster, Texas, U.S.A.) using the procedure described elsewhere (Appendix A) and validated for coyotes (Appendix F). All specimens from an individual coyote, collected in the fall and winter, were tested together in a single run. Samples

were tested in duplicate with an intra-assay coefficient of variation (CV) threshold  $\leq 10\%$ . Kits from a single reagent lot were used and the inter-assay mean CV was 7.8%.

Serum estradiol was quantitatively measured by radioimmunoassay (RIA) at the Colorado State University Endocrine Laboratory (ARBL/Foothills Campus, Fort Collins, Colorado, U.S.A.). In this double-antibody assay, ether-extracted estradiol from coyote sera competed with  $^{125}\text{I}$  labeled estradiol-17 $\beta$  for a fixed amount of rabbit anti-estradiol antibodies. Anti-rabbit IgG was added and the level of radioactivity in the captured antigen-antibody precipitate was measured. Samples were compared to a standard curve; the level of radioactivity being inversely proportional to the quantity of estradiol present in the unknown coyote sera. The stated “lowest detectable limit of estradiol” by this assay was 2.62 pg/ml.

Canine relaxin was qualitatively assayed by solid-phase enzyme-linked immunoassay (ELISA) (ReproCHEK™, Synbiotics Corporation, San Diego, California, U.S.A.) using the procedure validated (Appendix C) and described elsewhere for the coyote (Chapter III this text). Relaxin present in the plasma of pregnant coyotes produced a blue color within micro-titer wells; meanwhile plasma from non-pregnant coyotes produced distinctively weaker (or no) color development by comparison. All initial-negative or indeterminate results were confirmed by retesting with a new sample.

### **Data analysis**

Coyote mating behaviors were categorized, aligned by the day of treatment (in fall) or the estimated day of ovulation (in winter) for each individual female then compiled by study cohort. In addition to inter-group comparisons, patterns of behavior

recorded in this study were also compared to data similarly collected and documented for the captive colony at large during 2000-2003 breeding seasons (Chapter IV this text). Since the social and sexual behavior of the experimental control animals in winter did not appear affected by participation in the fall portion of the study, their data were included in data representing expected coyote breeding behavior, hereafter (unless otherwise noted) referred to as colony. Accordingly, the patterns of mating behavior in winter among deslorelin and gonadorelin treated animals showed similar deviations from the expected estrous profile, therefore the 2 treatment groups were combined for contrast to other coyotes within this colony (exception: 2 deslorelin and 2 gonadorelin treated animals were excluded from winter behavioral analysis because, unlike the rest of the cohort, these females did not copulate or become pregnant).

The approximate day of ovulation for an individual may be estimated by back-calculating from the day of parturition or by monitoring changes in serum progesterone levels (Chapter IV this text). In the current study, all colony and control coyote pairs produced healthy full term litters, as did 4/6 deslorelin and 4/6 gonadorelin treated females. Therefore the estimated day of ovulation for these individuals was based on an assumed gestation of 62 days. However to evaluate the possibility of a residual treatment effect on the 4 females that did not copulate or produce pups, we compared the progesterone levels of these individuals to progesterone profiles previously described in an affiliated study of mated female coyotes in this colony (Chapter IV this text).

Multivariate analysis of variance (MANOVA) and repeated-measures statistical procedures were used to analyze steroid hormone profiles and detect differences between study groups, and between successive weeks (Statistical Analysis System, SAS®, version



8.2, SAS Institute Inc., Cary North Carolina, U.S.A.). Unless otherwise noted, we assumed a level of statistical significance  $< 0.05$ .

## RESULTS

### Fall

Short-acting exogenous GnRH was given to 12 female coyotes (deslorelin,  $n = 6$ ; gonadorelin,  $n = 6$ ) in anestrus, approximately 34-38 weeks after their last ovulation and 15-18 weeks before their next estrus. Within a week after treatment (week 0 to week 1) an ovarian hormone response was detected but only in the deslorelin cohort ( $F_{18,14} = 2.31$ ,  $P = 0.059$ ). Deslorelin mean serum estradiol levels increased significantly ( $F_{2,15} = 11.76$ ,  $P = 0.001$ ) from baseline pre-implant concentrations ( $< 2.6$  pg/ml) to  $22.4 \pm 6.3$  pg/ml (mean  $\pm$  SE). In contrast, females in the control group remained relatively unchanged (intra-group mean estradiol,  $5.7 \pm 2.9$  pg/ml to  $3.4 \pm 2.1$  pg/ml) during this period, and estradiol levels within the gonadorelin treatment group were consistently  $< 2.6$  pg/ml (Fig. 21).

A change in mean serum progesterone levels also suggested an ovarian response, but as with estradiol, only in the deslorelin group ( $F_{18,14} = 2.62$ ,  $P = 0.037$ ). Two weeks after deslorelin implant progesterone surged from  $22.3 \pm 6.5$  ng/ml to  $46.5 \pm 17.5$  ng/ml (mean  $\pm$  SE). While this episodic pulse (from week 1 to week 2) was statistically borderline ( $F_{2,15} = 3.23$ ,  $P = 0.068$ ) it was notably absent in the normal saline or gonadorelin profiles (Fig. 22).

Concurrent with endocrine expression, coyotes treated with deslorelin solicited and displayed (albeit sporadically) socio-sexual behaviors commonly restricted to the

breeding season (Fig. 23). Specifically, physical interactions reminiscent of courtship (such as body-rubs, hip-pushes and face-licking) were seen during the first week after implant. Later in the second and third weeks females tolerated their mates' olfactory investigations; subsequently soliciting attention from their mates with diverted tails, and permitting the males' anogenital sniff/lick and pre-coital mount attempts. Also during this period 2 males became defensive, shadowing their mates or standing over them. In one particular case the male became aggressive, threatening the neighboring male by charging the fence with hackles, ears and tail raised. Periodically his threat displays also included reprimanding his mate when she went near the fence, snapping at her and driving her back.

In contrast, neither the control nor the gonadorelin group engaged in any unseasonal behavior; the majority of their activities being independent from their mate. Routine activity usually consisted of patrol and investigation of their enclosures or surveillance of regular maintenance activities by humans. Occasionally agonistic interactions over food or play objects were seen, but time spent in close proximity to a mate was short and inevitably focused on a task without specific affiliative or sexual intent.

The deslorelin cohort was most remarkable because of a brief period of out-of-season mounting and copulation, atypical among coyotes in the fall (Fig. 23). During week 3 post-implant, 4 deslorelin pairs were observed in pre-coital mounts, and 2 pairs ultimately tied on days 20-21 (1 copulatory tie each pair); remarkably 1 of these latter pairs was previously inexperienced. In addition, 1 coyote pair engaged in several bouts of pre-coital mounting (most intensively on days 21-22) including sustained pelvic

thrusting and remounts. But in this case, the male became increasingly exhausted before ejaculation. A copulatory lock was never confirmed for this pair due to loss of visibility at nightfall, and the following day they did not engage in any further sexual activity.

Ironically, 2 cases within the deslorelin group failed to be stimulated; their lack of activity following treatment resembled control and gonadorelin pairs more than those of their cohort. The 2 naïve 18-month old coyotes in this group experienced 2-3 fold increases in progesterone levels similar to other females treated with deslorelin. However, compared to the increasingly intimate behavior demonstrated by the other females, the younger coyotes rarely engaged their mates. These females were observed in typical agonistic displays of passive and active submission (rolling-over or rapid chin-licking with head and tail held low below the top line) but we never witnessed elements of courtship such as allo-grooming, non-agonistic body contact, or role reversal in play.

To determine if any observed (or unseen) copulations could have lead to fertile matings, all females were tested and found negative for relaxin in December.

### **Winter**

Deslorelin induced behavioral and physiological effects in the fall were transient and short-term, returning to normal (i.e. consistent with those of control and gonadorelin pairs) before December. Yet as the coyotes entered their native breeding season (January-February) evidence suggested another affect of GnRH treatment. Emergence of affinitive and appetitive behaviors within the deslorelin and gonadorelin groups appeared suppressed relative to the colony at large, particularly during the week prior to ovulation.

While activity within other colony pairs intensified, pre-ovulatory courtship ( $F_{19,17} = 5.56$ ,  $P = 0.001$ ), olfactory sampling ( $F_{20,12} = 41.48$ ,  $P < 0.001$ ), mate-guarding ( $F_{15,14} = 19.01$ ,  $P < 0.001$ ) and mounting attempts ( $F_{16,8} = 19.83$ ,  $P = 0.001$ ) among GnRH pairs appeared relatively steady (Fig. 24).

In addition, the near absence of pre-ovulatory copulations among GnRH treated coyotes was unexpected and varied significantly from the colony ( $F_{20,10} = 3.49$ ,  $P = 0.047$ ), resulting in an atypical pattern of sexual activity (Fig. 25). Among other colony pairs, 23.6% (43/182) of all observed copulatory ties occurred before ovulation; however within the GnRH groups only 1/58 (1.7%) pre-ovulatory tie was witnessed. Nevertheless, the length of behavioral estrus at the individual level was not statistically different ( $P|t_{0.05(2),23} \geq 1.34 = 0.194$ ,  $F_{17,7} = 8.97$ ) between GnRH treated coyotes (mean  $\pm$  SE,  $5.5 \pm 0.7$  days) and other colony females ( $7.6 \pm 1.4$  days), and ultimately fecundity was not impaired.

Among GnRH pairs observed in copulatory ties (8/12) in winter, all produced healthy full term litters in spring; and litter size for GnRH females (mean  $\pm$  SE,  $5.5 \pm 0.7$  pups) was not significantly different ( $P|t_{0.05(2),42} \geq 0.46 = 0.644$ ,  $F_{7,35} = 2.14$ ) from the colony at large ( $5.4 \pm 0.3$  pups). There were, however, 4 notable exceptions suggesting that GnRH may have had a more profound influence on sexually naïve females. Two deslorelin treated and 2 gonadorelin treated females were never observed in a copulatory tie, nor did they become pregnant. In addition to a lack of experience, these 4 females were coincidentally 22 month old litter-mates.

## DISCUSSION

Anestrus is a relatively quiescent phase in the canine ovarian cycle. The female's reproductive system recovers and repairs in preparation for the next cycle; and reproductive behavior is typically absent. It also is the phase within the ovarian cycle which determines the overall inter-estrous length for an individual (Concannon 1993); and is therefore the likely phase during which a wild canid regulates its reproductive seasonality. While specific physiological mechanisms controlling reproductive behavior and seasonality in wild canids are not well understood, evidence from this experiment suggests: (1) that the female coyote is physiologically and behaviorally prepared for mating in the fall, 4 months prior to the native breeding season; (2) that GnRH appears to be an important influence in coyote sexual behavior (either directly or through stimulation of pituitary-ovary hormone synthesis); and (3) that sexually experienced males can recognize the physiological and behavioral changes in their mates and react accordingly.

In this experiment, coyotes treated with a subcutaneous implant of deslorelin responded with increased secretion of ovarian steroid hormones. Elevated serum estradiol levels were detected approximately 9 - 12 days after treatment, followed by a rise in progesterone 18 – 26 days post-implant. This consecutive pattern of hormone synthesis (estradiol followed by progesterone) was reminiscent of the pre-ovulation endocrine profile previously reported for the coyote (Chapter IV this text); and suggested follicular stimulation within the ovary. During a normal ovarian cycle, however, progesterone remains elevated for approximately 9 weeks; rising before ovulation and

reaching peak levels 3 – 4 weeks post-ovulation. By contrast, deslorelin induced progesterone surges appeared dampened and transient; presumably because either ovulation did not occur or the corpora lutea could not be sustained because of the persistent presence of GnRH.

Although only single pulses of estradiol and progesterone were detected, it appears that the steroids nonetheless exerted a positive stimulating effect on the coyotes' socio-sexual behaviors. The subsequent expression of mating behaviors was also interesting because of the males' involvement. Affinitive behaviors (similar to courtship activity seen in winter) were first to emerge; specifically body-bumps, hip-pushes, allo-grooming, and play-solicitation. Such physical contact appears as ritualized social interactions but without obvious agonistic intent. In other words, contact did not include aggressive or passive gestures, nor end with one or the other individuals being dominant or submissive. Instead, the coyotes made contact and then separated equitably; or sometimes roles were reversed in obviously exaggerated but non-aggressive play.

Next, the appearance of appetitive and overtly sexual behaviors (such as male sniff-lick investigation of a female's anogenital region, mounting attempts, and copulations) was also interesting because: (1) emergence of proceptive and receptive behaviors in the females implied that the episodic pulses of estradiol and progesterone had sufficiently stimulated neural receptors in the female's brain; (2) that the neural receptors responsible for such behavior were sensitive and available to steroid influence in the fall; and (3) that physiological and/or behavioral changes in the female were detectable by the male and evoked appropriate responses from him (suggesting either direct physical or chemical stimulation by the female, or action based on memory).

Sexual interactions require interest, participation, and cooperation of both partners; and the behaviors we witnessed were not solely female initiated. To the contrary, males appeared stimulated by, and responsive to their mates.

It is difficult to predict the influential range of exogenous GnRH on either the hypophyseal-gonadal complex or the neural networks evoking mating behavior, because hypothalamic-pituitary-ovarian hormones initiate a cascade of cellular activity that is sometimes one-way and non-reversible (Pfaff et al. 1994). In the case of the GnRH treated coyotes, immediate physiological and behavioral responses were undetectable within the gonadorelin cohort, and ephemeral in the deslorelin group; but a long-term consequence was realized in the behavioral suppression observed at the beginning of the winter breeding season. All pre-ovulatory affiliative, appetitive and sexual behaviors were depressed in comparison to the pattern predicted by our previous observations of other colony pairs. Females treated with GnRH in the fall rarely solicited their mates' attention and often rebuffed their mates' sexual overtures in the winter. Interestingly, male initiated anogenital olfactory investigations, mate-shadowing and pre-copulatory mounting attempts were also reserved.

Spontaneous ovulation in winter occurred nevertheless; and copulations were well-timed because 8/12 GnRH treated coyotes became pregnant and delivered healthy pups. A review of historical records (for non-maiden coyotes) revealed no obvious discrepancy between the estimated day of ovulation in 2003 and previous seasons. Thus treatment in the fall with deslorelin or gonadorelin (at dosages described above) did not appear to ultimately delay or suppress ovulation in the subsequent breeding season. However, while most GnRH treated animals experienced a normalized (albeit altered)

estrus, 4 coyote pairs appeared to be more severely affected. These animals were all naïve 2 year olds (as were their mates), and we never saw them copulate nor did any of the females become pregnant.

In these particular cases, the males and females were very interactive (non-sexually): engaging in physical non-agonistic contact, playing and chasing. But the females rarely solicited their mates (with diverted tail), and male olfactory investigation (vulval sniff/lick) was also uncommon. Furthermore, when a male attempted a pre-coital mount, the females immediately employed a variety of evasive tactics, such as: passively sitting, lying down or running off; aggressively growling and snapping; or spinning and diverting his attention with play (play-bow, feigned charges or mock wrestling).

Unfortunately the reason for reproductive failure in the younger coyotes cannot be adequately explained within the context of this study. Inexperience is a likely cause. Yet within the deslorelin cohort there was a 3 year old naïve pair that successfully bred; and another inexperienced pair (4 year old female with a 2 year old male) in the gonadorelin group reproduced. Alternatively, it is possible that none of these females actually ovulated, although random serum samples (collected from 11 February to 7 March 2003) were within or exceeded the expected seasonal range for progesterone. Interestingly the females were sisters, and at least 2 of them have successfully bred since this study. We therefore speculate that decreased sensitivity to rising steroid levels (as observed in the other GnRH treated coyotes) contributed to the inhibited sexual receptivity of the females; and without prior experience, the females may have been confused by their mates' attempts to copulate, misunderstanding them to be non-sexual agonistic gestures or play solicitations.



The positive and negative responses of coyotes treated with exogenous GnRH in this study advances our understanding of reproductive behavior in this species (and possibly other wild canids), and also raises questions for future consideration. If a GnRH product is proposed for use as a contraceptive in coyotes, managers need to consider the potential consequences on seasonal reproduction. The evidence presented herein suggests that deslorelin can activate ovarian hormone synthesis (presumably through the normal hypophyseal-gonadal pathway), and precipitate socio-sexual features characteristic of normal coyote mating behavior. But it is currently unknown if there can be a fertile mating with males in the fall, and if so, what effect a seasonal shift would impose on survivability of pups born in winter.

Alternatively, if another regulator (such as prolactin, oxytocin, or melatonin) is involved and the females do not ovulate after GnRH treatment, our evidence also suggests that the neural networks (responsible for affiliative and appetitive behavior) may not perform normally, or be appropriately sensitive to further stimulation, during normal estrous recrudescence. Future studies are required to gain insight into the impact such socio-sexual disturbances could have on the coyote pair-bond. To date we do not understand the importance of the elaborate socio-sexual interactions observed in pro-estrus and pre-ovulatory estrus (Kleiman & Eisenberg 1973), or the mechanisms of coyote monogamous pair-formation and reinforcement. Dissolution of perennial pair-bonds could be a detrimental consequence of treatment. Monogamy is an important reproductive strategy closely linked to paternal care of young pups (Kleiman 1977), and obviously remains important to free-roaming coyotes since it is the predominant tactic in this species. Coyotes continue to be persecuted in rural ranching communities while

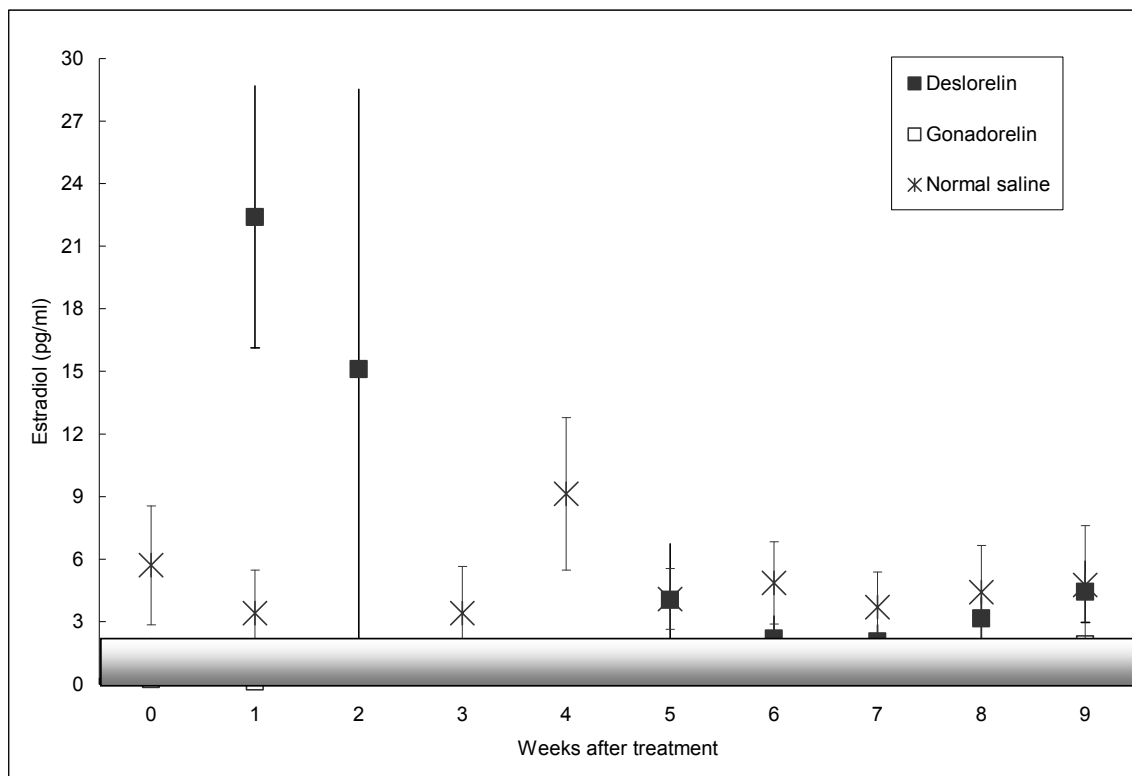
increasingly finding refugia in urbanized areas. Whether selective pressure on monogamy and paternal care in coyotes might shift under different environmental conditions represents a speculative but intriguing philosophical discussion.

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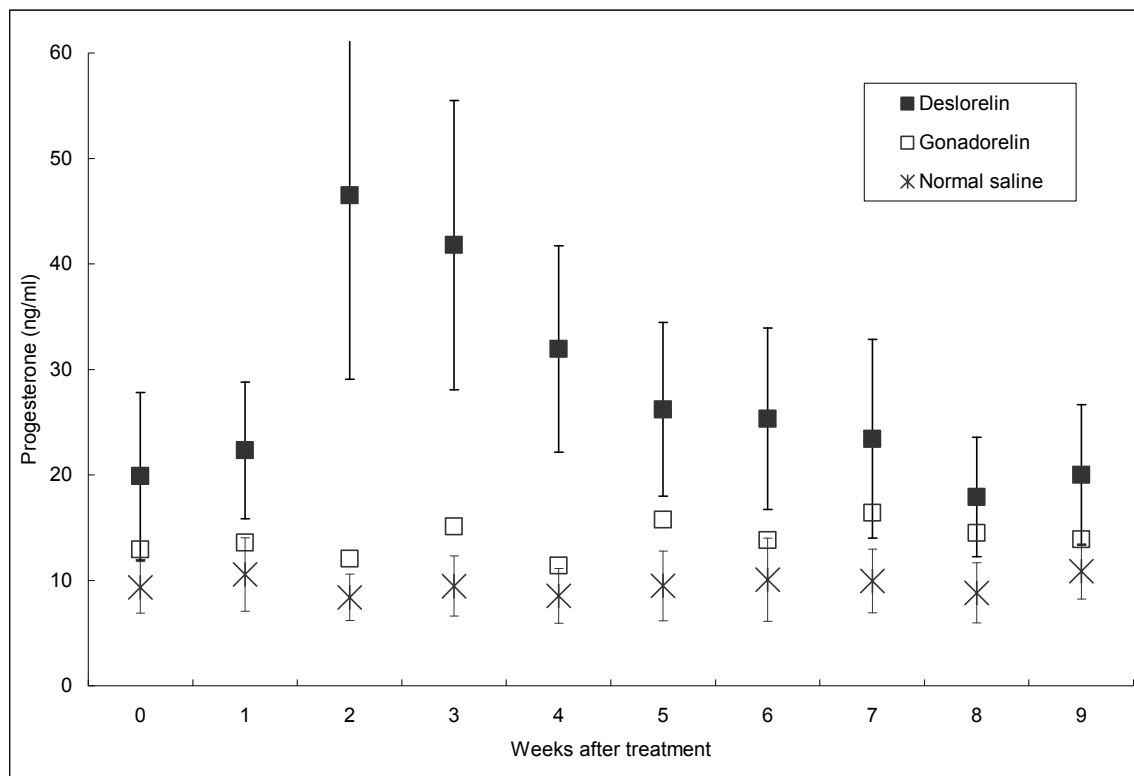
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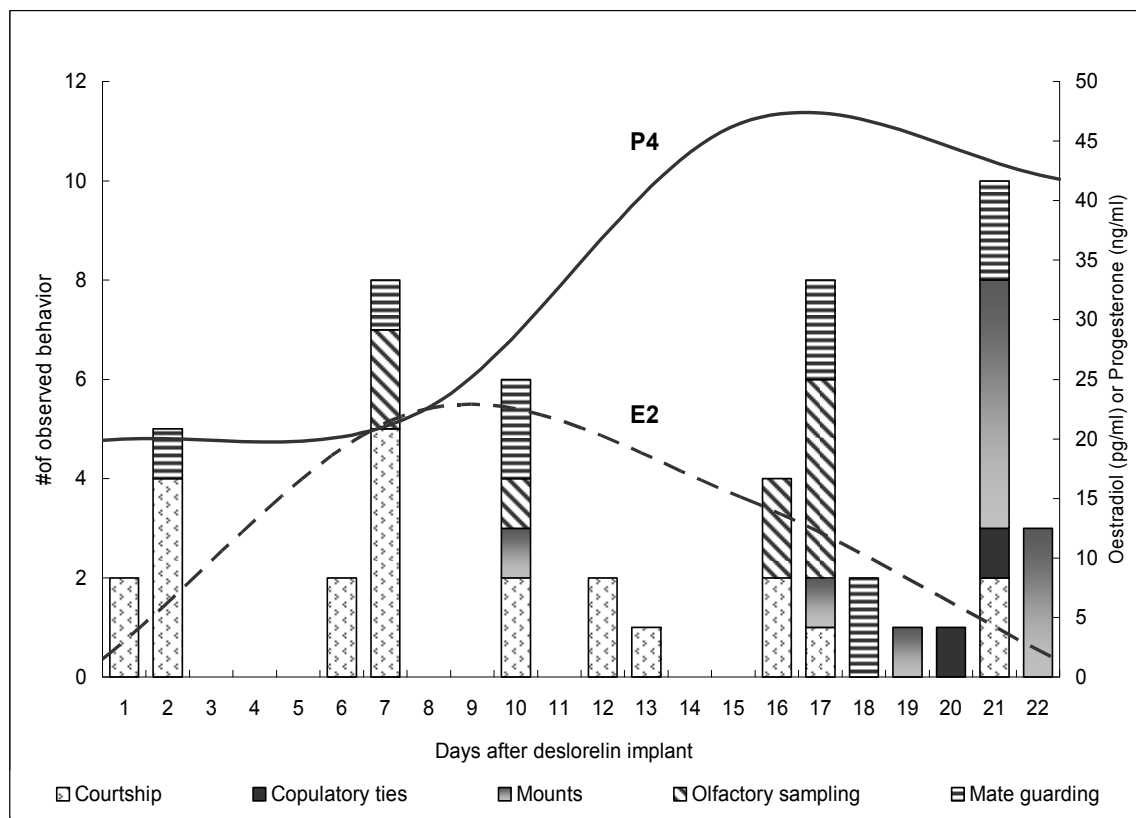
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**Figure 21.** Weekly (mean  $\pm$  SE) serum estradiol levels (pg/ml) in female coyotes sampled after treatment with deslorelin, gonadorelin, or normal saline; October-December, 2002. Week 0 represents pre-treatment baseline levels. Semi-transparent bar represents area below the detectable limit of the RIA (<2.62 pg/ml). All gonadorelin intra-group mean estradiol levels were <2.6 pg/ml.



**Figure 22.** Weekly (mean  $\pm$  SE) serum progesterone levels (ng/ml) in female coyotes sampled after treatment with deslorelin, gonadorelin, or normal saline; October-December, 2002. Standard error bars are not displayed for the gonadorelin group. Week 0 represents pre-treatment baseline level.



**Figure 23.** Social and sexual behaviors observed among 6 mated coyote pairs following treatment of the females with deslorelin in October 2002. Mean steroid hormone levels are overlaid, demonstrating temporal relationship between behavior and hormones.

**Figure 24.** Mating behaviors observed during the coyotes' native breeding season, January-March; aligned to the estimated day of ovulation. Colony data represents 32 (untreated) coyote pairs observed during 4 seasons, 2000-2003. Post-GnRH treatment data combines observations of 4 deslorelin post-implant pairs and 4 gonadorelin post-treatment pairs, January-February 2003. All colony and post-treatment females represented herein became pregnant.



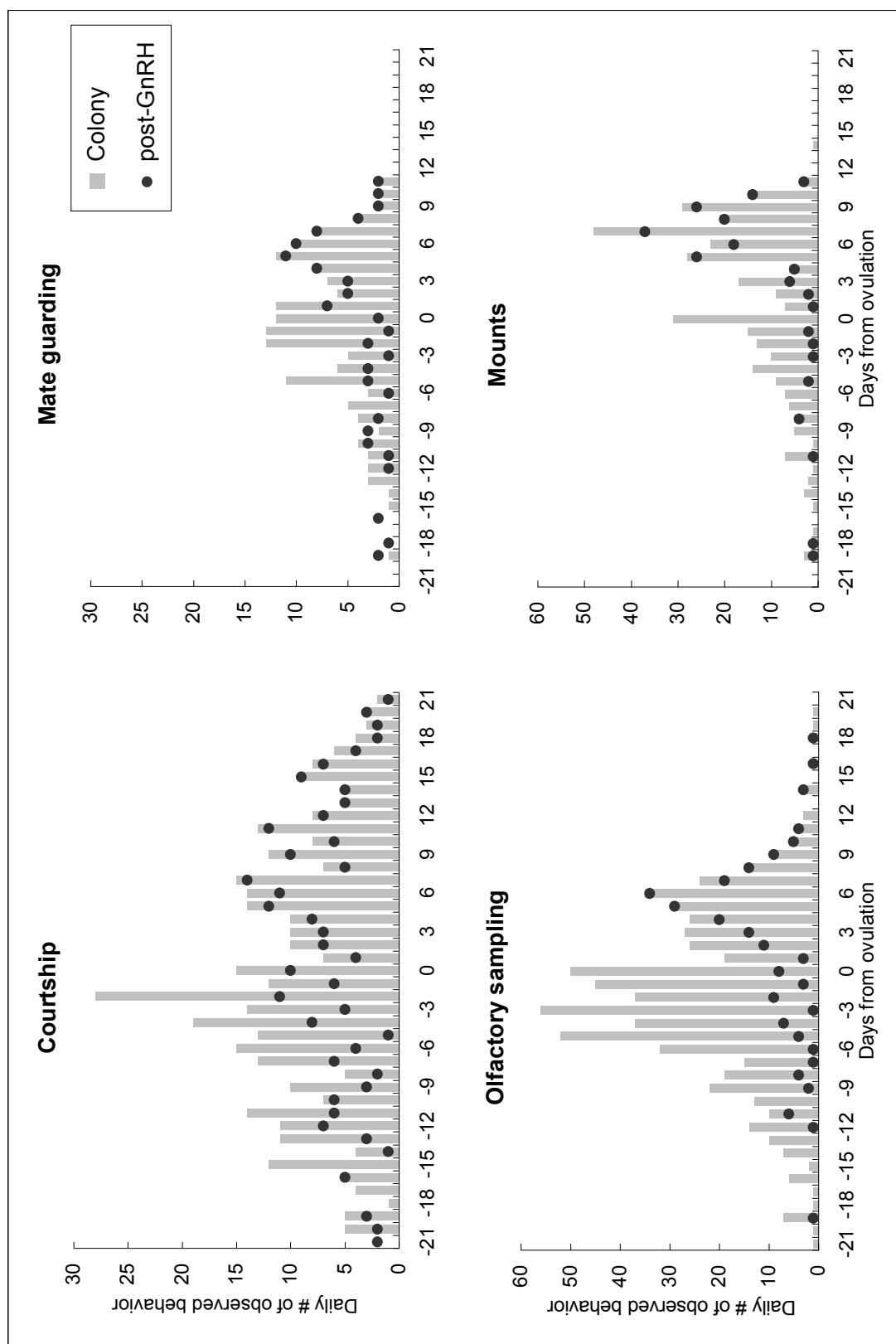
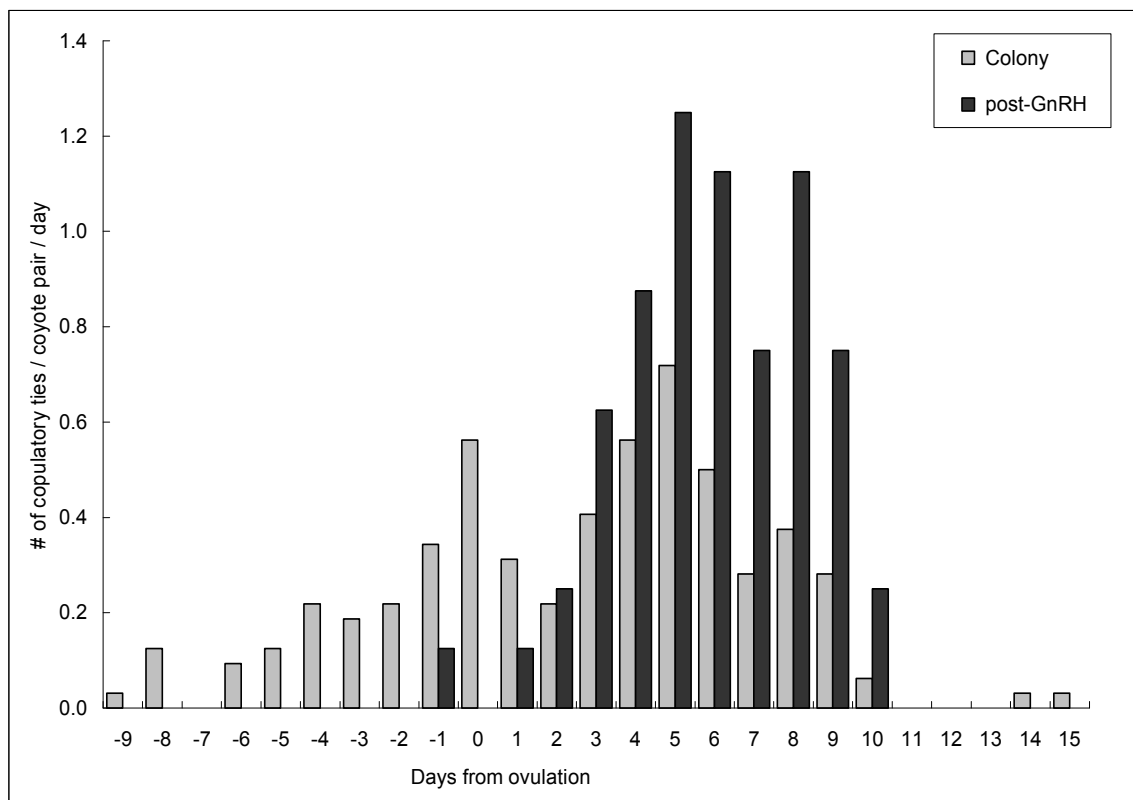


Figure 24.



**Figure 25.** Frequency of copulatory ties observed during the coyotes' native winter estrus; aligned to the estimated day of ovulation. Colony data represents 32 (untreated) coyote pairs observed during 4 breeding seasons, 2000-2003. Post-GnRH treatment data combines frequency of ties observed in 4 deslorelin post-implant pairs and 4 gonadorelin post-treatment pairs (2 pairs in each cohort failed to tie), January-February 2003.

## CHAPTER VII

## SUMMARY

Collectively, physiological results from this project support previous hypotheses that the coyote is seasonally monestrous, ovulates spontaneously, and experiences a prolonged luteal phase (obligatory covert pseudopregnancy). Females sequestered from their mates prior to estrus ovulated during the normal breeding season, and elevated blood levels of progesterone after ovulation were sustained for a period comparable to gestation. Yet while progesterone levels were indistinguishable between pregnant and pseudopregnant coyotes, pregnant coyotes maintained distinctively higher prolactin levels after mid-gestation. Concurrently, relaxin was detectable in plasma from pregnant but not pseudopregnant coyotes.

Although pregnancy was averted with low-dose estradiol benzoate, affiliative behavior between pseudopregnant females and their mates appeared similar to social interactions observed in the pregnant cohort. Unexpectedly this also included the (non-pregnant) females' successful solicitation of food. Thus future questions for investigation might be: What role, if any, do reproductive hormones play in observed begging and regurgitation behaviors? Is begging simply a compulsive remnant of juvenile behavior on the part of the female – while reflexive regurgitation an overt manifestation of paternal tendencies in male coyotes? Is food begging and regurgitation influential in maintaining a pair-bond?

Since social monogamy and active paternal care are conserved features of coyote reproductive behavior, these traits must ultimately promote the individual fitness of both

sexes. Biparental care might also be an important factor in the coyote's resiliency to predation by humans; e.g. a surviving parent can compensate (at least to some degree) after the loss of his/her mate and successfully defend, feed and train young pups. If this is true, then presumably coyotes employ some mechanism for assessing the "parental aptitude" of potential mates or continued commitment of existing ones. Logically, one would expect that potential mates should be appraised during proestrus (if not earlier). But coyotes are long-lived and can rear a litter of pups to independence within a year. Therefore, individuals may be able to afford an extended "evaluation period" for new mates, or reassessment of existing partners. Thus the myriad of behaviors observed in mated pairs (whether pregnant or not) may represent some selective components in the coyote's mating strategy.

Early interruption of pregnancy with exogenous estradiol released treated coyotes from the physiological constraint of gestation and lactation; however, the inter-estrus interval of non-pregnant coyotes remained unaltered (the females did not breed again until winter). While this implies that the ovarian cycle is immutable, the second experiment with GnRH suggested that reproductive seasonality of the coyote could actually be facultative rather than obligatory. Admittedly, while ovarian steroid synthesis was stimulated following treatment with deslorelin, further investigation is necessary before concluding that a pure-blood coyote could ovulate in the fall. But the behavior of coyotes after treatment with deslorelin was interesting for various reasons and present further questions. For example: If a female could be induced to ovulate before winter, would her mate be sufficiently stimulated to fertilize her (recalling that spermatogenesis

is also seasonal)? If the answer is “yes” then another research challenge might be to decipher the environmental cues regulating coyote reproductive seasonality.

Accordingly, deslorelin induced hormone synthesis and/or estrous-like behavior in treated females prompted out-of-season appetitive behavior in certain males. Was the male response provoked by a reflexive surge in testosterone, or were the males simply acting habitually? Also, the treatment effect (albeit seemingly short-acting) appeared to negatively influence normal sexual behavior months later during the winter breeding season. If verified, this consequence suggests a potential method for future manipulation of courtship and pre-ovulatory socio-sexual behavior; and by extrapolation, revealing possible roles these behaviors might play in pair-bond formation or stability.

Finally, despite artificial constraints, the captive animals in this study revealed that for reproductive success individuals must exercise spatial-temporal coordination of efforts and function. As antithetical examples; elaborate courtship and affiliative behaviors appeared before full emergence of steroid hormones, yet paradoxically, were abrogated by premature stimulation with GnRH. Similarly, males responded to out-of-season sexual cues of artificially stimulated females, yet later on, the fertile females rebuffed their mates' sexual advances. Meanwhile, pseudopregnant coyotes acted as though they were expecting pups. Free-roaming coyotes of course are not so constrained, nor are they subjected to pharmaceutical manipulations. Reproductive biology is, nevertheless, an intricate sometimes enigmatic fusion of physiology and behavior; perhaps even more so in species with complex mating systems such as the coyote.

APPENDICES

## APPENDIX A

PROGESTERONE ENZYME IMMUNOASSAY (EIA)<sup>1</sup>**PURPOSE AND PRINCIPLE**

The purpose of this assay is for the quantitative measurement of progesterone in the serum of coyotes (*Canis latrans*). EIA methodology utilizes antibody specific for a target antigen and bound to a solid surface (microtiter wells). To each well, enzyme-labeled antigen (progesterone) and unknown antigen (coyote serum) are added. The antigens compete for the available antibody sites coating the surface of the well. After incubation, unbound material is washed from the well and a chromagen solution (tetramethylbenzidine) is added. The subsequent color development is stopped by the addition of sulfuric acid and then measured by a photometer. Because the intensity of color development is directly related to the amount of bound enzyme-labeled antigen, the quantity of progesterone in an unknown sample is inversely proportional to the intensity of color that develops.

**SPECIMENS AND HANDLING**

An acceptable specimen for this assay is 0.5ml serum prepared from venous whole blood. The serum should be separated from the whole blood as soon as possible after clot formation. Gross hemolysis or lipemia should be avoided. The use of serum-separator gel (SST) is acceptable. Serum should be stored in a tightly sealed polypropylene micro-centrifuge tube and may be frozen at  $-20^{\circ}\text{C}$  or lower for up to two years prior to testing. Specimens must be clearly identified with the animal's unique

identification number, date collected (and time if appropriate), research project identifier and type of specimen (serum). Repeated thawing and freezing should be avoided.

### REAGENTS, SUPPLIES AND EQUIPMENT

1. Progesterone EIA. DSL-10-3900. Diagnostic Systems Laboratories, Inc., Webster, Texas 77598-4217. The kit contains:
  - a. Microtiter wells coated with goat anti-rabbit IgG.
  - b. Progesterone standards containing concentrations of approximately 0, 0.3, 1.3, 7.5, 30 and 80 ng/ml (refer to vial labels for exact concentrations).
  - c. Progesterone controls containing high and low concentrations (refer to vial labels for exact concentrations).
  - d. Anti-serum containing rabbit anti-progesterone.
  - e. Enzyme conjugate (progesterone conjugated to horseradish peroxidase).
  - f. Conjugate buffered diluent.
  - g. Tetramethylbenzidine (TMB) chromogen solution.
  - h. Buffered saline Wash Concentrate B.
  - i. 0.2M sulfuric acid stopping solution.
2. Sterile de-ionized water.
3. Benchmark microplate reader. Catalog #170-6850. Bio-Rad Laboratories, Hercules, California 94547.
4. Microplate Manager / PC (version 4.0) software. Bio-Rad Laboratories, Hercules, California 94547.
5. Jitterbug Microplate incubator/shaker. Model #130000 – 12vac. 2A. Boekel Industries, Inc., Philadelphia, Pennsylvania 19106.
6. Eppendorf adjustable pipettes. Series 2100 research. 10-100 $\mu$ l and 100-1000 $\mu$ l. Brinkman Instruments, Inc., Westbury, New York 11590-0207.
7. Nunc-Immuno 8 channel washer. Nalge Nunc International, Naperville Road, Illinois 60563-1796.
8. 17x100mm polystyrene tubes with caps. Falcon 2057. Becton Dickinson and Company, Lincoln Park, New Jersey 07035.
9. 5ml disposable glass serological pipets. VWR Scientific Products, So. Plainfield, New Jersey 07080.
10. 2.0ml micro-centrifuge tubes. Catalog #20170-098. VWR Scientific Products, So. Plainfield, New Jersey 07080.
11. Pipet tips. Catalog #53503-784 and #53503-769. VWR Scientific Products, So. Plainfield, New Jersey 07080.
12. Clear self-adhering laminating sheets.
13. One-gallon polyethylene (Nalgene) bottle with spigot. Nalge Nunc International, Naperville Road, Illinois 60563-1796.



14. Laboratory timer.
15. Aluminum foil.
16. Disposable absorbent towels or wipes.

### PROCEDURE

1. The reagent kit should be stored at 2-8°C until testing. Prior to use, all reagents should be brought to room temperature (22-28°C). Reagents expire three weeks after opening.
2. Prepare the wash solution by diluting Wash Concentrate B with 1.5 liters of sterile de-ionized water in a Nalgene bottle. The working wash solution may be stored at 22-28°C for up to one month.
3. Bring all specimens to room temperature.
4. **15–20 minutes prior to use:** In a Falcon tube dilute the **enzyme conjugate** solution with conjugate diluent (1:50). Dilute only enough enzyme conjugate solution for the number of wells that are to be used (all standards, controls and specimens are run in duplicate). Cover the tube with a cap and wrap the tube in aluminum foil. To protect it from light, place the covered tube in the dark until use. Preparation of the conjugate should account for the time it will take to pipet all standards, controls and specimens into the wells (allow 5-10 minutes for this step).
5. In a rack, arrange unknown specimens in the order in which they are to be run. Fill in a microtiter plate worksheet (see example below) with the identification of each standard, control and unknown to be tested.
6. Pipet **50µl** of the standards, controls and unknowns into each well according to the completed worksheet – **all samples are to be run in duplicate**.
7. Add **100µl** of the dilute **enzyme conjugate** solution to each well. Mix by tapping the plate gently for 5-10 seconds.
8. Add **100µl** of the **progesterone antiserum** to each well. Cover the plate with self-adhesive film.
9. Incubate the plate at room temperature (22-28°C) while shaking at 500-700rpm (press “MIX” and “1” for a rpm of approximately 625) – for **one hour**. **Note:** to minimize exposure of the wells to extraneous light, the darkened cover of incubator / shaker must be in place during all incubations. Also, do not incubate / shake with only one plate in the chamber – if necessary, use an empty balance plate.
10. Remove and discard the film covering the plate. Aspirate and **wash each well 5 times** with the working wash solution. Blot dry by inverting the plate on a disposable absorbent towel.
11. Add **100µl** of the **TMB chromogen** solution to each well. Cover the plate with a fresh piece of film.
12. Incubate and shake at room temperature and 500-700rpm for **30 minutes**.
13. After incubation, remove the plastic film and add **100µl** of the sulfuric acid to each well.
14. Read the absorbance of the solution in the wells within 30 minutes.

- Turn on plate reader allowing it to warm up for a couple minutes before reading a plate.
- Open the MICROPLATE MANAGER 4.0 program in the PC.
- Open file "C:\ mpm \proges~1 \plate1.mpm". This is a template that includes the standards, controls and unknowns in the format shown below on the plate worksheet example.
- Make any necessary changes to the format of the standards or unknowns (including controls) and save as "C:\ ..... \plate1.mpm". Close plate file.
- Open FILE → NEW READING → NEW ENDPOINT PROTOCOL. This window allows remote control of the plate reader from the PC.
- Select READING PARAMETERS → DUAL ; MEASUREMENT FILTER → 450; REFERENCE FILTER → 655.
- Select PICK TEMPLATE. Again open "C:\ mpm \proges~1 \plate1.mpm".
- After the addition of the sulfuric acid, check that the plate is clean and dry. Place the plate in the plate reader chamber and close the lid.
- Select RUN in the ENDPOINT PROTOCOL box.

Plate worksheet (example):

Filename: Progesterone / 5523.mpm

Assay: Progesterone Lot: 05301 Date: 9/12/01 By: DAC

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std A	Std E	5523 1-9-01	5523 2-27-01								
B	Std A	Std E	5523 1-9-01	5523 2-27-01								
C	Std B	Std F	5523 1-16-01	5523 3-6-01								
D	Std B	Std F	5523 1-16-01	5523 3-6-01								
E	Std C	Cntrl I	5523 2-13-01	5523 3-13-01								
F	Std C	Cntrl I	5523 2-13-01	5523 3-13-01								
G	Std D	Cntrl II	5523 2-20-01	5523 3-22-01								
H	Std D	Cntrl II	5523 2-20-01	5523 3-22-01								

- Review the graphs and charts that will automatically appear after the absorbance has been read.
  - Review the STANDARD CURVE graph for appropriate fit of the standards to the regression line or curve. Significant outliers bring into question the

- suitability of that standard to be used as a reference for any unknowns with similar concentration values.
- b. The UNKNOWN CONCENTRATION REPORT will list the %CV for each pair duplicated samples. Samples with a %CV of greater than 10 should be retested.
16. Save the “template” with the filename that has been designated at the top of the worksheet (for example: “C:\mpm \ progesterone \ 5523.mpm”). This will allow future access to any report or absorbance readings that are attached to that template and the samples analyzed.
  17. Remove plate from reader. Check that the chamber is clean and dry. Turn power off as soon as possible to prolong the life of the lamp.

### **INTERPRETATION AND REPORTING**

The UNKNOWN CONCENTRATION REPORT generated by MICROPLATE MANAGER contains the concentration values for all controls and unknown samples.

Compare the values of the controls to the acceptable range of concentration values printed on the label of the control’s vial. The controls must come within their acceptable range or the run will be considered invalid. Documentation of the controls’ ranges must be recorded in the laboratory notebook. Note: Controls are made from the same stock reagent as the standards. Therefore, it is permissible to use standards as controls. This is desirable in cases where the unknown samples may greatly exceed the concentration levels of the reagent controls.

Any samples within range of an invalid standard may not be reported. Such samples must be retested; also, any specimen with a %CV >10 should be retested.

Any concentration values that fall above the highest standard (with absorbances below the high standard) should be reported as “greater than 80ng/ml” or diluted 1:1 with Standard A (0ng/ml) and retested. In the later case, the subsequent concentration value will be multiplied by 2 before reporting.

Any concentration value that falls below the lowest standard (with an absorbance greater than the low standard) should be reported as “none detected”.

Also in the laboratory notebook, document the identification number of each sample as seen in MICROPLATE MANAGER and the associated specimen identification as seen on the worksheet. Place the worksheet in the appropriate envelope in the back of the laboratory notebook. Include in the laboratory notebook any comments or notes that are appropriate for that run (*e.g.*, “run accepted”, problems with standards, controls or equipment, etc.).

Computer file backup should be performed daily. All template files should be transferred to a diskette.

## VALIDATION

See Appendix F, Assay Validation.

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<sup>1</sup> Standard operating procedure (SOP) created: September 2001 by Debra A. Carlson  
Reviewed: May 2002 by Debra A. Carlson  
Reviewed: April 2004 by Debra A. Carlson

## APPENDIX B

### ESTRADIOL ENZYME IMMUNOASSAY (EIA)<sup>2</sup>

#### PURPOSE AND PRINCIPLE

The purpose of this assay is for the quantitative measurement of estradiol in the serum of coyotes (*Canis latrans*). EIA methodology utilizes antibody specific for a target antigen and bound to a solid surface (microtiter wells). To each well, enzyme-labeled antigen (estradiol) and unknown antigen (coyote serum) are added. The antigens compete for the available antibody sites coating the surface of the well. After incubation, unbound material is washed from the well. Streptavidin-horseradish peroxidase is added which binds to the estradiol-biotin fixed to the well surface. Again the plate is washed and a chromagen solution (tetramethylbenzidine) is added. The subsequent color development is stopped by the addition of sulfuric acid and then measured by a photometer. Because the intensity of color development is directly related to the amount of bound enzyme-labeled antigen, the quantity of estradiol in an unknown sample is inversely proportional to the intensity of color that develops.

#### SPECIMENS AND HANDLING

An acceptable specimen for this assay is 0.5ml serum prepared from venous whole blood. The serum should be separated from the whole blood as soon as possible after clot formation. Gross hemolysis or lipemia should be avoided. The use of serum-separator gel (SST) is acceptable. Serum should be stored in a tightly sealed polypropylene micro-centrifuge tube and may be frozen at  $-20^{\circ}\text{C}$  or lower for up to two

years prior to testing. Specimens must be clearly identified with the animal's unique identification number, date collected (and time if appropriate), research project identifier and type of specimen (serum). Repeated thawing and freezing should be avoided.

### REAGENTS, SUPPLIES AND EQUIPMENT

17. 3<sup>rd</sup> Generation Estradiol EIA. DSL-10-39100. Diagnostic Systems Laboratories, Inc., Webster, Texas 77598-4217. The kit contains:
  - a. Microtiter wells coated with rabbit anti-estradiol IgG.
  - b. Estradiol standards containing concentrations of approximately 0, 1.5, 5, 15, 50, 150, and 500 pg/ml (refer to vial labels for exact concentrations).
  - c. Estradiol controls containing high and low concentrations (refer to vial labels for exact concentrations).
  - d. Estradiol-biotin conjugate concentrate.
  - e. Estradiol-biotin conjugate diluent.
  - f. Streptavidin-enzyme conjugate concentrate.
  - g. Streptavidin-enzyme conjugate diluent.
  - h. Tetramethylbenzidine (TMB) chromogen solution.
  - i. Buffered saline Wash Concentrate B.
  - j. 0.2M sulfuric acid stopping solution.
18. Sterile de-ionized water.
19. Benchmark microplate reader. Catalog #170-6850. Bio-Rad Laboratories, Hercules, California 94547.
20. Microplate Manager / PC (version 4.0) software. Bio-Rad Laboratories, Hercules, California 94547.
21. Jitterbug Microplate incubator/shaker. Model #130000 – 12vac. 2A. Boekel Industries, Inc., Philadelphia, Pennsylvania 19106.
22. Eppendorf adjustable pipettes. Series 2100 research. 10-100 $\mu$ l and 100-1000 $\mu$ l. Brinkman Instruments, Inc., Westbury, New York 11590-0207.
23. Nunc-Immuno 8 channel washer. Nalge Nunc International, Naperville Road, Illinois 60563-1796.
24. 17x100mm polystyrene tubes with caps. Falcon 2057. Becton Dickinson and Company, Lincoln Park, New Jersey 07035.
25. 5ml disposable glass serological pipets. VWR Scientific Products, So. Plainfield, New Jersey 07080.
26. 2.0ml micro-centrifuge tubes. Catalog #20170-098. VWR Scientific Products, So. Plainfield, New Jersey 07080.
27. Pipet tips. Catalog #53503-784 and #53503-769. VWR Scientific Products, So. Plainfield, New Jersey 07080.
28. Clear self-adhering laminating sheets.

29. One gallon polyethylene (Nalgene) bottle with spigot. Nalge Nunc International, Naperville Road, Illinois 60563-1796.
30. Laboratory timer.
31. Aluminum foil.
32. Disposable absorbent towels or wipes.

### PROCEDURE

18. The reagent kit should be stored at 2-8°C until testing. Prior to use, all reagents should be brought to room temperature (22-28°C). Reagents expire three weeks after opening.
19. Prepare the wash solution by diluting Wash Concentrate B with 1.5 liters of sterile de-ionized water in a Nalgene bottle. The working wash solution may be stored at 22-28°C for up to one month.
20. Bring all specimens to room temperature and mix well after thawing.
21. In a rack, arrange unknown specimens in the order in which they are to be run. Fill in a microtiter plate worksheet (see example below) with the identification of each standard, control and unknown to be tested.
22. Pipet **100µl** of the standards, controls and unknowns into each well according to the completed worksheet – **all samples are to be run in duplicate**. Cover the plate lightly while proceeding through the next step.
23. In a Falcon tube, prepare the working Estradiol-biotin conjugate solution by diluting the conjugate concentrate 1:50 with the Estradiol-biotin conjugate diluent. **Note:** this working solution should be prepared just prior to use and in an amount sufficient only for the number of wells in the run. To minimize enzyme degradation from light, wrap the tube in aluminum foil while dispensing.
24. Add **50µl** of the dilute **Estradiol-biotin conjugate** solution to each well. Cover the plate with self-adhesive film.
25. Incubate the plate **overnight** (18-20 hours) at **2-8°C**.
26. Remove and discard the film covering the plate. Aspirate and **wash each well 5 times** with the working wash solution. Blot dry by inverting the plate on a disposable absorbent towel
27. **Just prior to use**, prepare the working Streptavidin-enzyme conjugate solution in a clean Falcon tube by diluting the conjugate concentrate 1:50 with the Streptavidin-enzyme conjugate diluent. Prepare only the amount needed for the number of wells in the run. Protect the enzyme solution from light by wrapping the tube in aluminum foil and keep in the dark until use.
28. Add **100µl** of the working Streptavidin-enzyme conjugate solution to each well. Cover with a fresh piece of film.
29. Incubate and shake at room temperature (~25 °C) and 500-700rpm (press “MIX” and “1” for a rpm of approximately 625) for **30 minutes**. Note: to minimize exposure of the wells to extraneous light, the darkened cover of incubator / shaker must be in

- place during all incubations. Also, do not incubate / shake with only one plate in the chamber – if necessary, use an empty balance plate.
30. Remove and discard the film covering the plate. Aspirate and **wash each well 5 times** with the working wash solution. Blot dry by inverting the plate on a disposable absorbent towel.
  31. Add **100µl** of the **TMB chromogen** solution to each well. Cover the plate with a fresh piece of film.
  32. Incubate and shake at room temperature (~25 °C) and 500-700rpm for **30 minutes**.
  33. After incubation, remove the plastic film and add **100µl** of the sulfuric acid to each well.
  34. Read the absorbance of the solution in the wells within 30 minutes.
    - a. Turn on plate reader allowing it to warm up for a couple minutes before reading a plate.
    - b. Open the MICROPLATE MANAGER 4.0 program in the PC.
    - c. Open file “C:\ mpm \ estrad~1 \ plate1.mpm”. This is a template that includes the standards, controls and unknowns in the format shown below on the plate worksheet example.
    - d. Make any necessary changes to the format of the standards or unknowns (including controls) and save as “C:\ ... \ plate1.mpm”. Close plate file.
    - e. Open FILE → NEW READING → NEW ENDPOINT PROTOCOL. This window allows remote control of the plate reader from the PC.
    - f. Select READING PARAMETERS → DUAL; MEASUREMENT FILTER → 450; REFERENCE FILTER → 655.
    - g. Select PICK TEMPLATE. Again open “C:\ mpm \ estrad~1 \ plate1.mpm”.
    - h. After the addition of the sulfuric acid, check that the plate is clean and dry. Place the plate in the plate reader chamber and close the lid.
    - i. Select RUN in the ENDPOINT PROTOCOL box.
  35. Review the graphs and charts that will automatically appear after the absorbance has been read.
    - a. Review the STANDARD CURVE graph for appropriate fit of the standards to the regression line or curve. Significant outliers bring into question the suitability of that standard to be used as a reference for any unknowns with similar concentration values.
    - b. The UNKNOWN CONCENTRATION REPORT will list the %CV for each pair duplicated samples. Samples with a %CV of greater than 10 should be retested.
  36. Save the “template” with the filename that has been designated at the top of the worksheet (for example: “C:\ mpm \ estradiol \ 5523.mpm”). This will allow future access to any report or absorbance readings that are attached to that template and the samples analyzed.
  37. Remove plate from reader. Check that the chamber is clean and dry. Turn power off as soon as possible to prolong the life of the lamp.



Plate worksheet (example):

Filename: Estradiol / 5523.mpm

Assay: Estradiol Lot: 05162 Date: 9/30/02 By: DAC

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std A	Std E	5523 1-9-01	5523 2-27-01								
B	Std A	Std E	5523 1-9-01	5523 2-27-01								
C	Std B	Cntrl I	5523 1-16-01	5523 3-6-01								
D	Std B	Cntrl I	5523 1-16-01	5523 3-6-01								
E	Std C	5358 3-13-01	5523 2-13-01	5523 3-13-01								
F	Std C	5358 3-13-01	5523 2-13-01	5523 3-13-01								
G	Std D	5358 3-22-01	5523 2-20-01	5523 3-22-01								
H	Std D	5358 3-22-01	5523 2-20-01	5523 3-22-01								

### INTERPRETATION AND REPORTING

The UNKNOWN CONCENTRATION REPORT generated by MICROPLATE MANAGER contains the concentration values for all controls and unknown samples.

Compare the values of the controls to the acceptable range of concentration values printed on the label of the control's vial. The controls must come within their acceptable range or the run will be considered invalid. Documentation of the controls' ranges must be recorded in the laboratory notebook.

Any samples within range of an invalid standard may not be reported. Such samples must be retested. Also, any specimen with a %CV >10 should be retested.

Any concentration values that fall above the highest standard (with absorbances below the high standard) should be reported as “greater than 500pg/ml” or diluted 1:1 with Standard A (0 pg/ml) and retested. In the later case, the subsequent concentration value will be multiplied by 2 before reporting.

Any concentration value that falls below the lowest standard (with an absorbance greater than the low standard) should be reported as “none detected”.

Also in the laboratory notebook, document the identification number of each sample as seen in MICROPLATE MANAGER and the associated specimen identification as seen on the worksheet. Place the worksheet in the appropriate envelope in the back of the laboratory notebook. Include in the laboratory notebook any comments or notes that are appropriate for that run (*e.g.*, “run accepted”, problems with standards, controls or equipment, etc.).

Computer file backup should be performed daily. All template files should be transferred to a diskette.

## VALIDATION

See Appendix F, Assay Validation.

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<sup>2</sup>Standard operating procedure (SOP) created: October 2002 by Debra A. Carlson  
Reviewed: April 2004 by Debra A. Carlson

## APPENDIX C

RELAXIN ENZYME-LINKED IMMUNOASSAY (ELISA)<sup>3</sup>**PURPOSE AND PRINCIPLE**

The purpose of this assay is for the qualitative measurement of relaxin in the plasma of coyotes (*Canis latrans*). In canines, relaxin is synthesized in the placenta and is not detectable in the peripheral blood of non-pregnant females or males. Therefore, the detection of relaxin in a plasma sample is diagnostic of pregnancy.

In this ELISA assay, polyclonal anti-relaxin antibodies are fixed to the surface of microtiter wells. A second antibody conjugated to horseradish peroxidase is added to the well followed by an unknown sample. Any relaxin present in the unknown sample is bound to the well surface and “sandwiched” between the two antibodies. Unbound antibody-enzyme complexes and other extraneous material are washed from the well and a chromogenic substrate is added. Color development beyond the intensity of a negative control may be interpreted as a positive reaction for relaxin. Because of individual variability, the intensity of color development (even when measured by a photometer) can not be used to determine the exact date of gestation or predict the date of parturition. An exception to this might exist if specimens were collected often enough to estimate the day when a female converted from negative to positive.

**SPECIMENS AND HANDLING**

An acceptable specimen for this assay is heparinized plasma prepared from a minimum of 1ml venous whole blood. Gross hemolysis or lipemia should be avoided.

Serum or plasma in EDTA may not be used. Blood collection tubes containing either lithium heparin or sodium heparin are acceptable.

The plasma should be separated from the whole blood as soon as possible and transferred to a tightly sealed polypropylene micro-centrifuge tube. Prior to testing, specimens may be stored at 2-7°C for up to 48 hours or frozen at  $\leq -20^{\circ}\text{C}$  for up to six months. Specimens that will not be tested within 48 hours must be frozen.

Specimens must be clearly identified with the animal's unique identification number, date collected, research project identifier, and type of specimen (plasma).

### REAGENTS, SUPPLIES AND EQUIPMENT

1. ReproCHEK™ Canine Pregnancy Test Kit. Synbiotics Corporation, San Diego, California 92127. The kit includes:
  - a. Polyclonal antibody coated microtiter wells.
  - b. Bottle A - Antibody-enzyme conjugate diluent.
  - c. Bottle B - Antibody-enzyme conjugate.
  - d. Bottle C - negative control.
  - e. Bottle D – positive control.
  - f. Bottle E - Wash solution concentrate.
  - g. Bottle F - Chromogenic substrate.
2. Benchmark microplate reader. Catalog #170-6850. Bio-Rad Laboratories, Hercules, California 94547.
3. Eppendorf adjustable pipettes. Series 2100 research. 10-100 $\mu\text{l}$ . Brinkman Instruments, Inc., Westbury, New York 11590-0207.
4. Pipet tips. Catalog #53503-784 and #53503-769. VWR Scientific Products, So. Plainfield, New Jersey 07080.
5. 2.0ml micro-centrifuge tubes. Catalog #20170-098. VWR Scientific Products, So. Plainfield, New Jersey 07080.
6. Sterile de-ionized water.
7. Laboratory timer.
8. Disposable absorbent towels or wipes.
9. Wash bottle.

## PROCEDURE

1. Prepare the working wash solution by diluting the Wash Concentrate 1:10 with sterile de-ionized water. Mix well and transfer a portion to a smaller wash bottle. The remaining stock solution should be stored at 2-7°C until the expiration of the kit.
2. Bring all reagents, specimens and working wash solution to room temperature (21-25°C) prior to testing.
3. Arrange the specimens in a rack in the order in which they are to be tested and complete an assay worksheet (see example below). **It is recommended that not more than 10 unknown specimens be included in one run.** If the specimens have been frozen, mix well before testing. Check all specimens for fibrin formation prior to pipetting.
4. Mark wells with the identifier of the sample or control that will be dispensed.
5. To each well, add **1 drop** from **Bottle A**.
6. To each well, add **1 drop** from **Bottle B**.
7. Add **2 drops** from each control (**Bottle C** and **Bottle D**) to the respective wells.
8. Dispense **50µl** of each unknown sample into the respective well. Tap well holder lightly to mix contents of wells.
9. Incubate at room temperature for **10 minutes**.
10. Discard fluid from wells and blot dry.
11. Flush wells and **wash** with working wash solution – **5 times**. Rinse **2 more times** with de-ionized water. Invert wells over absorbent paper and blot dry.
12. To each well, add **3 drops** from **Bottle F**. Tap plate lightly.
13. Incubate at room temperature for **5 minutes**.
14. The results may be read visually or by a plate reader but **must be read immediately** after incubation. The intensity of color development in each test well is compared to the well containing the manufacturer's negative control.
15. If the wells are to be read on a photometer, a clean dry well must be added to the plate as a blank.
  - a. Turn the plate reader on a couple of minutes prior to the end of the last incubation period and allow the lamp to warm up.
  - b. After the self-diagnosis the plate reader will display the PLATE READING window. Press PAGE+ to get MAIN menu. Using CURSOR arrows select "Analy" and press ENTER.
  - c. Select D/S, Filter and press ENTER. Use the VALUE key to choose DUAL and press the SELECT key. Use the VALUE key to scroll through the choices. Select 655nm for the measurement filter, and 450nm for the reference filter. Use the SELECT key to confirm each choice.
  - d. Press ENTER or PAGE- to return to the MAIN menu. Select "Blanks" and press ENTER. Use CURSOR to select "Well" and press ENTER. Use the VALUE key to select the well in which the blank is to be placed. Press ENTER or PAGE- to return to MAIN menu.
  - e. Return to the PLATE READING window.

- f. Before placing the plate in the chamber of the reader, check that the wells and holder are dry. Set the plate in the chamber and close the door. Press START/STOP.
- g. A tape with the absorbance readings will be printed. Mark on the tape the identifier of each control, unknown and blank. Date and initial the tape and affix to the worksheet.

*Worksheet (example):*

**Qualitative Relaxin Assay (ReproCHEK™)**

Manufacturer: Synbiotics, Corp.  
San Diego, Calif. 92127

Kit # \_\_\_\_\_ Plate lot# \_\_\_\_\_  
Exp: \_\_\_\_\_

Date Performed	Date Collected	Animal Id#	Clover	Observed 1 <sup>st</sup> Tie	Specimen Quality	Optical Density	Result	Performed By

**INTERPRETATION AND REPORTING**

Every run must include the positive and negative controls provided in the kit. These controls verify that the test procedure was performed correctly and that the reagents have not been altered or contaminated. The negative control should be clear and the positive control should present a blue color. Failure of either control to perform properly will invalidate the entire run.



There are two acceptable methods of interpreting the reactions obtained in this relaxin assay:

1. Visual comparison. Any test well with a blue color of greater intensity than the negative control may be reported as positive.
2. Measurement by a plate reader (photometer). An optical density slightly greater than that measured for the manufacturer's negative control may be obtained for specimens from coyotes that are not pregnant (see validation charts below). These samples still appear negative to the eye, however when read on a plate reader the following optical density parameters should be used for reporting:
  - a.  $<0.030$  = Negative
  - b.  $0.030 - 0.050$  = Indeterminate
  - c.  $>0.050$  = Positive

Animals with indeterminate results should have another specimen collected to confirm their status as positive or negative. A specimen taken later in a pregnancy will produce a darker color of blue than one taken earlier near the time of implantation. In coyotes, a positive result may first be detectable between 22 - 25 days of gestation, but reliability is greater if the sample is collected after 28 days.

Although relaxin is synthesized in the placenta and may be expected to disappear after parturition, data collected during the validation of this assay for the coyote showed that the hormone can persist for several months in peripheral blood (similar evidence has also been reported for the domestic bitch). Therefore, results from this assay should not be considered a reliable indicator for the diagnosis of abortion in the coyote.

## VALIDATION

Following is data collected for validation of this assay in the coyote. Specificity of the assay was acceptable; there were no false positives (none of the males or non-pregnant females tested positive) or false negatives (all females who whelped or had documented abortions tested positive).

**Table C1.** Specificity of relaxin assay with coyote samples.

	<i>n</i> =	Positive result	Negative result
Males	9	0	9
Mated females	55	48	7
Non-bred females	7	0	7
Pregnant females	48	48	0

**Table C2.** Performance of internal (coyote) negative controls

	# Individuals	# Samples	Range (O.D.)
Control Males	7	7	0.001 – 0.018
Control Females	7	25	0.004 – 0.023
Manufacturer's Negative Control			0.003 – 0.010

**Table C3.** Sensitivity of relaxin assay with diestrous coyote samples.

	Day of gestation (back-calculated from the day of parturition) All samples after day 28 test positive					
	Day 23	Day 24	Day 25	Day 26	Day 27	Day 28
Min OD	0.020	0.009	0.049	0.097	0.024	0.100
Max OD	0.138	0.424	0.188	0.242	0.502	0.653
Median OD	0.043	0.056	0.107	0.140	0.275	0.497
% Pos	25	50	91	100	90	100
<i>n</i>	4	4	11	4	10	7



Among those coyotes who tested positive and subsequently whelped live pups, the day relaxin was first detectable was between 22-25 days gestation (back calculated from the day of parturition and based on a 60-63 day gestation).

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<sup>3</sup>Standard operating procedure (SOP) created: February 2001 by Debra A. Carlson  
Reviewed: May 2002 by Debra A. Carlson  
Reviewed: April 2004 by Debra A. Carlson

## APPENDIX D

MANUAL HEMATOLOGY ASSAYS:  
HEMATOCRIT, WHITE BLOOD CELL COUNT,  
WHITE CELL DIFFERENTIAL AND RED CELL MORPHOLOGY<sup>4</sup>**PURPOSE AND PRINCIPLE**

The measurements of such physiological parameters as hematocrit (the percentage of a given volume of whole blood that is represented by red blood cells) and white blood cell (WBC) count help facilitate the assessment of an animal's hematopoietic and immune functions.

In the presence of an elevated WBC count, differentiation of the types of leukocytes present on a peripheral blood smear can provide diagnostic evidence of the cause of the increase (*e.g.*, bacterial versus viral infection, inflammation, parasitic infection, allergic reactions, toxemia). While on the other hand, leukopenia (abnormally low WBC) may suggest suppression or disease of the bone marrow.

Anemia may result from iatrogenic (investigator or clinician induced) or physiological causes. Blood loss in excess of the animal's regenerative capacity will be reflected in a decreasing or abnormally low hematocrit. Certain pharmaceutical or biological agents may affect the production of only selected hematopoietic cell lines (for instance erythroid precursors) but not others (granulocytic / monocytic cells). In concert with measuring the hematocrit, visual evaluation of red blood cell (RBC) morphology will provide additional information regarding the animal's health. For example, the number of red cells being produced may not be critically low, however, inspection of the RBC morphology may reveal that the cells being produced appear pale (hypochromasia)

or small (microcytic). Such evidence suggests that the animal's overall oxygen carrying capacity may be diminished.

For a manual hematocrit determination, a micro-capillary tube is filled with anticoagulated whole blood then centrifuged at approximately 5,000rcf. To read the hematocrit, the micro-capillary tube is held against a chart with the interface between plasma and red cell mass aligned to the lines on the graph.

In the manual WBC count, anticoagulated whole blood is diluted 1:100 in a buffered ammonium oxalate solution then each side of a two chamber hemacytometer is loaded. The number of WBC in each chamber is counted and an average is calculated.

A smear is made from a drop of anticoagulated whole blood on a slide. The slide is stained with a polychromatic Wright's stain then examined under high power. One hundred WBC are counted and each leukocyte is categorized by cell type (based upon the cell's morphology and staining characteristics). Red cell morphology is examined through several fields of view then generalized and described. An estimate of the platelet count can also be done.

### **SPECIMENS AND HANDLING**

An acceptable specimen for hematology is anticoagulated whole blood collected in ethylenediaminetetraacetic acid (EDTA). Collection of the blood should be done in a way that minimizes hemolysis. Grossly lipemic samples should be avoided. Specimens must be protected from freezing and should not be refrigerated prior to testing. Clotted whole blood or blood collected in other anticoagulants is not acceptable.

The peripheral blood smear may be made from the EDTA anticoagulated specimen but should be prepared as soon as possible after specimen collection. All other testing should be performed within 24 hours of collection. Micro-clots discovered in specimens that were thought to be adequately anticoagulated may cause inaccurate assay results. Such specimens should be recollected.

### REAGENTS, SUPPLIES AND EQUIPMENT

33. Micro-hematocrit capillary tubes. Red band / heparinized, 1.1 – 1.2mm diameter. Cat#15401-628. VWR Scientific Products, So. Plainfield, New Jersey 07080.
34. Critoseal® plastic putty. Oxford Labware, Sherwood Medical, St. Louis, Missouri 63103.
35. Micro-hematocrit reading chart.
36. Micro-hematocrit centrifuge. International Equipment Co., Needham Heights, Massachusetts.
37. Unopette® for Platelet/WBC determination with 20ul pipettes. Cat#5855. Becton Dickinson and Co., Franklin Lakes, New Jersey 07417.
38. Bright-Line Hemacytometer, Hausser Scientific, Horsham, Pennsylvania.
39. Pre-cleaned glass micro slides. 25 x 75mm. Cat#48312-002. VWR Scientific Products, So. Plainfield, New Jersey 07080.
40. Microscope with a x40 dry lens and x100 oil lens.
41. Differential cell counter (Hematab™ I). Analytical Products, Inc., Belmont, California.
42. Wright's stain. Cat#9360-16. Ricca Chemical Co., Arlington, Texas 76012.
43. Buffer Solution (pH6.4-Giordano formula). Cat#1450-16. Ricca Chemical Co., Arlington, Texas 76012.
44. Distilled water.
45. Wash bottles.
46. Disposable wipes.
47. Alcohol wipes.
48. Lens paper.
49. Immersion oil.
50. Laboratory timer.
51. Wooden applicator sticks.
52. Blotting paper.
53. Covered petri dish.

## PROCEDURE

### *Micro-hematocrit (packed cell volume)—*

1. Mix EDTA anticoagulated whole blood gently and thoroughly.
2. Hold the micro-capillary tube so that the end with the red line is away from the specimen. While holding the capillary tube at a slight angle, allow blood to fill the tube via capillary action.
3. Place finger over the end and withdraw the capillary tube when it is approximately 2/3 full.
4. Wipe excess blood off the outside then press the distal end of the capillary tube into the Critoseal® putty a couple of times to seal the end.
5. Micro-hematocrit should be performed in duplicate. Place two filled capillary tubes opposite each other in the micro-hematocrit centrifuge head. Fasten the inner lid securely but do not over-tighten.
6. Close the outer lid and set the timer dial on the centrifuge for 4 minutes.
7. After the head has come to a complete stop, remove the capillary tubes and check each for leakage (as evidenced by a reduced fill of the capillary tube). Tubes that have leaked may give inaccurate results.
8. Keep the capillary tubes upright until they are read. To read; hold the tube against the micro-hematocrit chart. Align the interface between the putty and the red cell mass to the zero line. Align the top of the plasma layer to the 100 line. The hematocrit (as percentage) correlates to the line that corresponds to the interface between the red cell mass and plasma. **Note:** if the layer containing leukocytes and platelets (buffy coat) is visible, read between the red cell mass and the buffy coat. The duplicate capillary tubes should have the same result  $\pm 1$  point.

### *White blood cell count—*

1. Mix the specimen gently but thoroughly (avoid introducing air bubbles into the specimen).
2. Remove a Unopette reservoir from the jar and immediately replace the lid (to prevent evaporation of the preservative/humidifying solution inside the jar).
3. Specialized pipettes come with the Unopette system. Pierce the sealed port of the reservoir with the lid on the pipette (taking care not to bend or break the pipette while doing this).
4. Remove the lid from the pipette. While holding the pipette at a slight upward angle, insert the end into the specimen. Lower the hub and allow the pipette to fill by capillary action. **Note:** the pipette will draw 20 $\mu$ l of sample for dilution – any air bubbles accidentally introduced will significantly affect the results.
5. Cover the hole in the hub with a finger and withdraw the pipette from the specimen. Wipe the outside of the pipette to remove extraneous blood. **Note:** care must be taken not to touch the end of the pipette with the wipe. Such contact will wick blood from the pipette and lead to erroneous results.

6. Squeeze reservoir with one hand and insert pipette through port until pipette hub is seated. Release reservoir allowing the negative pressure to pull the sample from the pipette into the reservoir.
7. Rinse the pipette several times with the diluent. Mix gently by swirling the diluted blood within the reservoir.
8. Allow the reservoir to rest for a couple of minutes (the diluted sample is stable in the reservoir but testing should not be delayed past 60 minutes). If more than one specimen is to be tested, label each reservoir with the animal's unique identifier.
9. Meanwhile, check that the hemacytometer and cover slip are clean and dry. Moisten the blot paper inside the petri dish.
10. Remove the pipette from the reservoir, invert it and re-seat the hub. Discharge several drops of the diluted sample on to an absorbent wipe. Hold the tip of the pipette so it is lightly touching the groove of the hemacytometer chamber (but not the cover slip). Discharge the sample carefully, checking that the effluent does not contain air bubbles that may interfere with the filling of the chamber.
11. Charge the chamber completely without over-filling. Then charge the second chamber in the same way.
12. Set the hemacytometer in the petri dish and cover. Allow the hemacytometer to rest for a couple of minutes while the cells settle within the chamber. To avoid desiccation (and thus distortion of the cell distribution) begin the count within 5 minutes of charging the chambers.
13. With a x40 power dry lens (x400 magnification) count the number of leukocytes present in each of the four outer large squares. Repeat with the second chamber and divide the total number by 8 to obtain the average number of leukocytes per square millimeter ( $\text{mm}^2$ ).
14. Clean the hemacytometer by flushing well with distilled water. Dry with lens paper only to prevent scratching of the chamber or coverslip surfaces.

*White cell differential and red cell morphology—*

1. Only unused and pre-cleaned glass slides should be used for the preparation of a peripheral blood smear.
2. Mix the whole blood specimen gently and thoroughly.
3. Hold two wooden applicator sticks together in one hand and dip them into the upright blood sample. If the sticks are touching, a drop of blood will be suspended between them. Touch the sticks on the slide approximately 1cm medially from the frosted end (if present).
4. Hold another slide at an angle to the first and touch the slides just at the edge of the drop of blood. Let the blood run along the edge until it has extended across the width of the slide. Move the second slide across the first, drawing the blood across the glass and forming the smear.
5. Allow the blood film to air dry. Label the slide with the animal's unique identifier and the date of the specimen.

6. Place the slide on the staining rack. Cover the slide with Wright's stain. Be careful not to dislodge the blood film with undue force from the bottle. Let the stain sit on the slide undisturbed for 2 minutes.
7. Carefully add the buffer in a volume approximately equal to the stain. To mix, gently blow on the slide (when sufficiently mixed a greenish sheen will appear). Let the slide sit undisturbed for 4 minutes.
8. Aim a steady but gentle stream of distilled water at one end of the slide. The water will float the stain off the surface of the slide and the stain will begin to fall off the opposite end and sides. At this point, the slide may be picked up and the remaining stain may be gently flushed off the smear (again be careful not to dislodge any areas of the blood film).
9. Set the slide on its end on absorbent paper. Residual water will drain off and the slide should be allowed to thoroughly air dry.
10. After drying, an alcohol wipe may be used to remove extraneous stain from the off-side of the slide.
11. **Differential:** Using a x100 power oil lens (x1000 magnification) and one drop of immersion oil, count 100 WBC. Select an area of the smear where the cells are free of distortion (neither over-crowded nor stretched too thin). Each leukocyte is identified and categorized by cell type (polymorphonuclear granulocyte or neutrophil, lymphocyte, monocyte, eosinophil and basophil). Immature cells, inclusion bodies, vacuoles and other unusual features should be noted.
12. **Erythrocyte morphology:** Under the same power, examine several fields of view. Make note of the general palor of the red cells, their size and shapes. Also note if nucleated red cells are seen, and if so, how many.
13. **Platelet estimate:** A rough estimate of the platelet population should be made and characterized as "decreased", "adequate" or "increased". Any unusual morphological characteristics or the presence of clumping should be noted.
14. To store the smears; first lay the slide face-down on an absorbent wipe and allow the excess oil to be wicked of the surface – do not wipe the slide. The slides may then be kept in a covered box (do not allow them to touch) and stored out of direct light.

### INTERPRETATION AND REPORTING

The WBC count is reported as follows:

$$\text{avg. \# of leukocytes / mm}^2 \times \text{dilution (1:100)} \times \text{conversion factor (10)} = \text{\# of leukocytes / mm}^3$$

The hematocrit is reported as the percentage of the red cell mass in whole blood.

For the WBC differential, each category of WBC type is reported as the percentage seen in 100 WBCs. The observation of unusual characteristics is described and noted as a comment.

Red cell morphology may also be noted as a comment or may be further described by semi-quantitative descriptors such as: “rare”, “occasional”, “few”, “moderate”, “many” or “1+”, “2+”, “3+” and “4+”.

The platelet population estimate is described as “decreased”, “adequate” or “increased”. Unusual morphological characteristics are described in a comment.

#### **VALIDATION**

See Table D1 on next page.

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<sup>4</sup>Standard operating procedure (SOP) created: February 2000 by Debra A. Carlson  
Reviewed: May 2002 by Debra A. Carlson  
Reviewed: April 2004 by Debra A. Carlson



**Table D1.** Comparison of manual hematology results to automated CBC and differentials (reported as % of all leukocytes viewed) performed at Logan Regional Hospital clinical laboratory. Samples collected and performed on February 18, 2000.

Note: automated differentials are based on 1000 cell counts and calibrated to human blood cell morphology (for example: reporting normal coyote PMN as basophils; also coyote RBC were reported to have low MCV and MCH, MCHC was normal).

	5608		5628		5333		5633		5627		5358	
WBC x 10 <sup>3</sup> /mm <sup>3</sup>	18.5	16.9	11.6	9.9	12.4	10.0	9.0	9.2	12.5	11.5	9.0	6.7
HCT %	40	39.4	42	41.5	42	42.6	42	41.6	41	42.0	39	37.3
HGB g/dl		13.4		14.4		14.7		14.7		14.3		13.3
PLT x 10 <sup>3</sup> /μl		423		427		343		557		431		342
Bands	17		21		16		17		21		9	
Segs	59	1.3	47	5.0	60	1.0	42	2.0	49	78.4	75	2.8
Lymphs	15	10.9	18	20.3	10	14.4	31	21.3	16	13.7	3	13.2
Monos	8	4.1	4	3.6	7	3.7	7	2.9	10	3.2	9	2.3
Eos	1	1.5	10	6.1	7	4.3	3	4.1	4	4.7	4	3.3
Baso		82.2		65.0		76.6		69.7				78.4
Plt est	Adeq Occ giant plt		Adeq Occ giant plt		Adeq Occ giant plt		Adeq Occ giant plt		Adeq		Adeq Occ giant plt	
RBC morph	N/N	2+ micro 2+ hypo	N/N	2+ micro 2+ hypo	Norm- cytic 1+ poly- chrom	1+ micro 1+ hypo	Norm- cytic 1+ poly- chrom	2+ micro 2+ hypo	Norm- cytic 1+ poly 6 NRBC	2+ micro 2+ hypo	3+ cren- ated Norm- chrom	2+ micro 2+ hypo
Other	1+ react lymph		3+ react lymph		1+ react lymph				1+ react lymph		3+ react lymph	
Method	<u>Man</u>	Auto	<u>Man</u>	Auto	<u>Man</u>	Auto	<u>Man</u>	Auto	<u>Man</u>	Auto	<u>Man</u>	Auto

## APPENDIX E

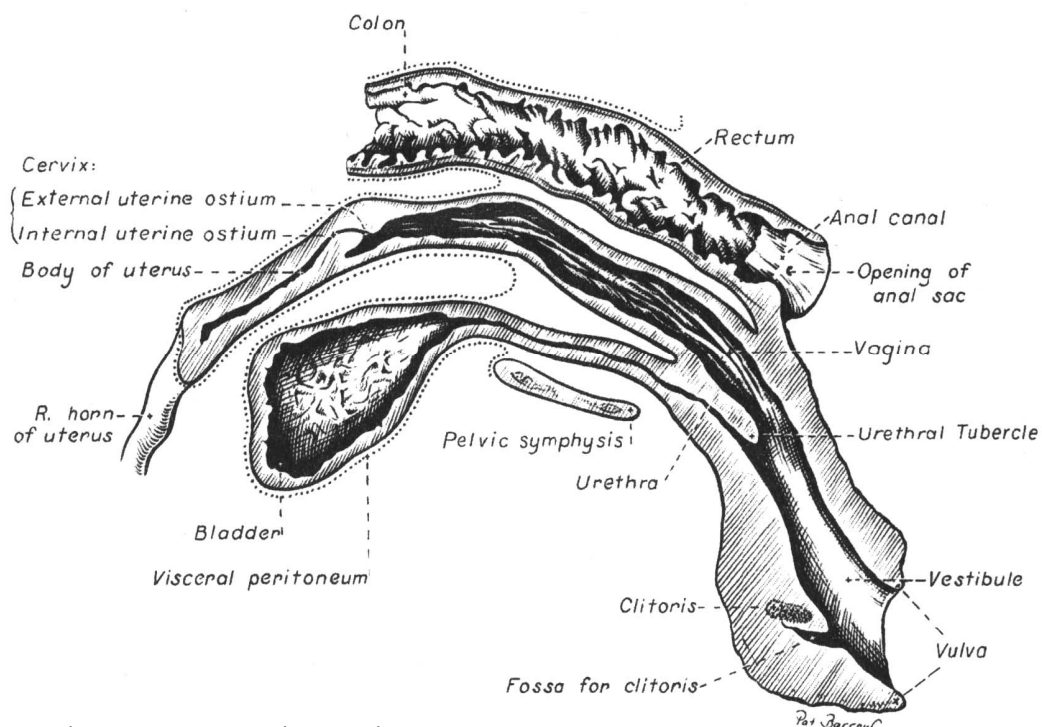
### VAGINAL CYTOLOGY<sup>5</sup>

#### **PURPOSE AND PRINCIPLE**

In canines, the wall and epithelial lining of the vagina experience profound changes during the estrous cycle. The cellular changes can be easily visualized on smears made of stained exfoliated cells. A sample of the epithelial lining is obtained using a sterile cotton swab pre-moistened with sterile saline. The cells are transferred to a glass slide, stained with a modified Wright-Giemsa stain then viewed under dry magnification (x400) and categorized.

#### **SPECIMEN COLLECTION AND HANDLING**

A sterile swab is passed through the vulva and vestibule and into the vaginal vault. After entering the vulva, the swab is pressed along the caudal wall so that it is not accidentally pushed into the clitoral fossa (Fig. E1). As it is advanced dorsally it should continue to be held to this attitude so that the urethra will also be passed without trauma. Past the urethra, the vagina will arch cranially and the swab will pass into the vagina proper. Rarely, there may be little or no discharge and a dry swab will not pass easily. In this event, the swab should be moistened with sterile normal saline thus easing its passage through the tract.



**Figure E1.** Diagram representing canine reproductive tract (Evans 1993).

At certain times of the estrous cycle, the vaginal musculature may resist passage of the swab (also some females will be more tense than others and unwilling to relax). To avoid traumatic injury the swab must never be forced. Repositioning the female, lubricating the swab with saline, or withdrawing the swab and re-entering the vagina are methods of successfully obtaining an acceptable sample with minimal discomfort.

Once the swab has reached the pelvic canal it should be rotated several times then withdrawn. It is then rolled along the length of a glass slide, making 2-3 rows. Rolling will deposit the cells on the slide without shearing or distorting them. However, if the slides have been exposed to very cold temperatures, the cells will not stick to the glass.

Holding the back of the slide against one's hand will warm it sufficiently to allow the cells to be transferred.

### REAGENTS, SUPPLIES AND EQUIPMENT

1. Cotton-tipped applicators (sterile single use). Cat#C15053-006. Allegiance Healthcare Corp., McGaw Park, Illinois 60085.
2. Pre-cleaned glass micro slides. 25 x 75mm. Cat#48312-002. VWR Scientific Products, So. Plainfield, New Jersey 07080.
3. Diff-Quik® stain. Jorgensen Laboratories, Loveland, Colorado 80538.
  - a. Methanol (cat#J-322A-1).
  - b. Eosin (cat#J-322A-2).
  - c. Thiazine (cat#J-322A-3).
4. Distilled water.
5. 0.9% sterile normal physiological saline.
6. Microscope with x40 power dry lens.

### PROCEDURE

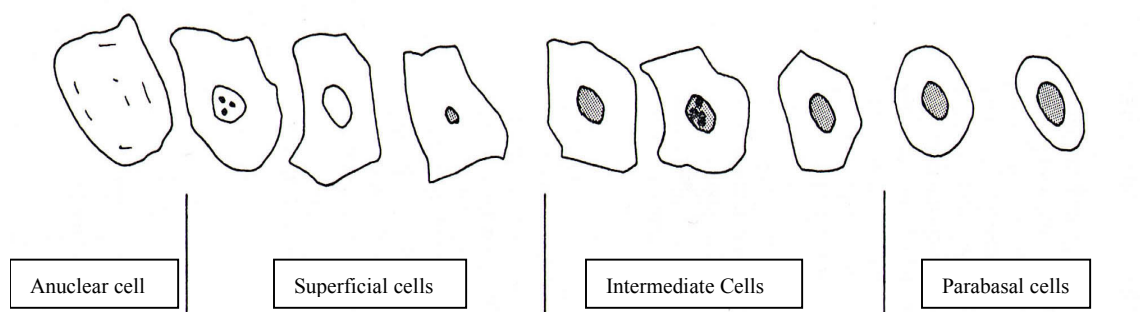
1. Allow slides to air dry at room temperature (21–25°C) before staining.
2. Stains should be filtered periodically to remove any foreign material that may become dislodged as the slides are stained.
3. Dip the slide into jar #1 (methanol is light blue) for 5-6 seconds. Do not agitate up and down. Remove slide and briefly touch the end on absorbent toweling or wipe.
4. Dip the slide into jar #2 (eosin is red) for 5-6 seconds. Remove and again wick excess stain off the slide.
5. Dip the slide into jar #3 (thiazine is a dark purple/blue) for 5-6 seconds.
6. Rinse the slide under a gentle stream of distilled water. Caution: The force of a direct stream of water may dislodge the cells from the slide. Direct the stream above the cells and allow the water to flow over the smear.
7. To air dry, set the slide on its edge on absorbent toweling.
8. Examine the smear under dry magnification (x400). Examine several areas along all rows. Identify and grade the type of epithelial cells seen.

### INTERPRETATION AND REPORTING

The epithelial cells seen on the smear will be categorized as; “parabasal”, “small intermediate”, “large intermediate”, “nucleated superficial” or “anucleated

superficial” (Fig. E2). Their relative presence in the general population will be graded as “rare”, “occasional”, “few”, “moderate” or “many”.

In addition, the presence of mucus, red blood cells, leukocytes, and spermatozoa should be described and graded. A comment may be included noting the conspicuous presence or absence of amorphous material.



**Figure E2.** Schematic representation of vaginal epithelial cells based on classification by Wachtel (Christie et al. 1972)

## REFERENCES

- Christie, D.W., J.B. Bailey, and E.T. Bell. 1972. Classification of cell types in vaginal smears during the canine oestrous cycle. *British Veterinary Journal* 128: 301-310.
- Evans, H.E. 1993. *Miller's anatomy of the dog*. 3rd ed. WB Saunders Co., Philadelphia, Pennsylvania.

<sup>5</sup>Standard operating procedure (SOP) created: February 2000 by Debra A. Carlson  
 Reviewed: May 2002 by Debra A. Carlson  
 Reviewed: April 2004 by Debra A. Carlson

## APPENDIX F ASSAY VALIDATION<sup>6</sup>

### PURPOSE AND PRINCIPLE

Validation of the performance of the assays includes the following evaluations where possible: physiological appropriateness, inter-assay variation, intra-assay variation, recovery and parallelism.

Evaluation of physiological appropriateness is based on general knowledge of the species' hormone secretion patterns. For example, specimens from a spayed female are expected to have low concentrations of estradiol and progesterone; alternatively in an intact female, progesterone is expected to increase concurrent with a decline in estradiol (in estrus and peri-ovulation) or decrease around the time of parturition. Samples from males may also be used as sources of low (or absent) hormone concentrations.

Inter-assay variation evaluations include at least three samples. Each sample is from a different individual and generally represents low, medium or high concentrations of hormone. The three samples are tested (in duplicate) in three assay "runs" and the standard deviation (SD) and coefficient of variation (CV) are calculated and reported for each individual.

Intra-assay evaluations also include three samples, each from separate individuals and representing low, medium and high concentrations. Each sample (in duplicate) is tested three times, but in one run; the mean, variance, SD and CV is calculated and reported for each individual.

Recovery evaluates the possible presence of interfering substances in a coyote specimen. A selected standard is diluted in coyote sera; the analyte in question having already been measured in the coyote specimen. It is therefore possible to predict the total amount of analyte that should be measured in a sample containing part reagent standard and part coyote sera. Several different dilutions are made (containing 20%, 40%, 60%, 80% and 100% reagent standard). Measurable “recovery” of the standard is reported as a percentage and compared to the quantity expected at the time of dilution. Some variability is expected due to pipetting and other errors inherent in handling.

For parallelism, three samples each from different coyotes will be serially diluted with the reagent “zero” standard. A reagent control or standard (other than zero) of known hormone concentration will be similarly diluted. The dilutions of each specimen (neat, 1:2, 1:4, 1:8, 1:16 and 1:32) reflect dilutions that may need to be made of unknown samples when hormone concentrations fall outside the assay’s standard curve. Results of the coyote samples are compared to the slope of the control dilutions.

The validation procedures described above have variable suitability, depending on the assay method to which they are applied. Definitive thresholds are therefore difficult to predict but the results will be reported for those assays selected to be performed on site. Procedural changes are possible and may be incorporated, as the analyst deems appropriate in order to improve the performance of an assay. Documentation of similar assay validation from reference laboratories will be obtained if available.

## **SPECIMENS AND HANDLING**

Refer to the specific analyte SOP found elsewhere in this manual.

## REAGENTS, SUPPLIES AND EQUIPMENT

Refer to the specific analyte SOP found elsewhere in this manual.

### PROCEDURE

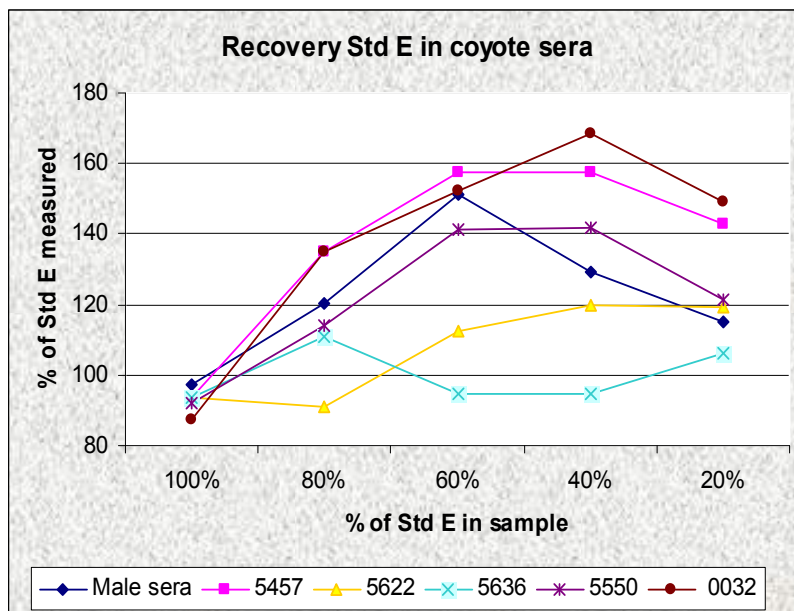
Samples for validation are assayed according to the SOP of each analyte. Refer to the appropriate assay found elsewhere in this manual.

### RESULTS AND INTERPRETATION

#### *Progesterone (EIA)*

1. Inter-assay (intra-lot) mean %CV = 9.6%
2. Intra-assay mean %CV  $\leq$  10%
3. Inter-lot mean %CV = 23.4%
4. Recovery:

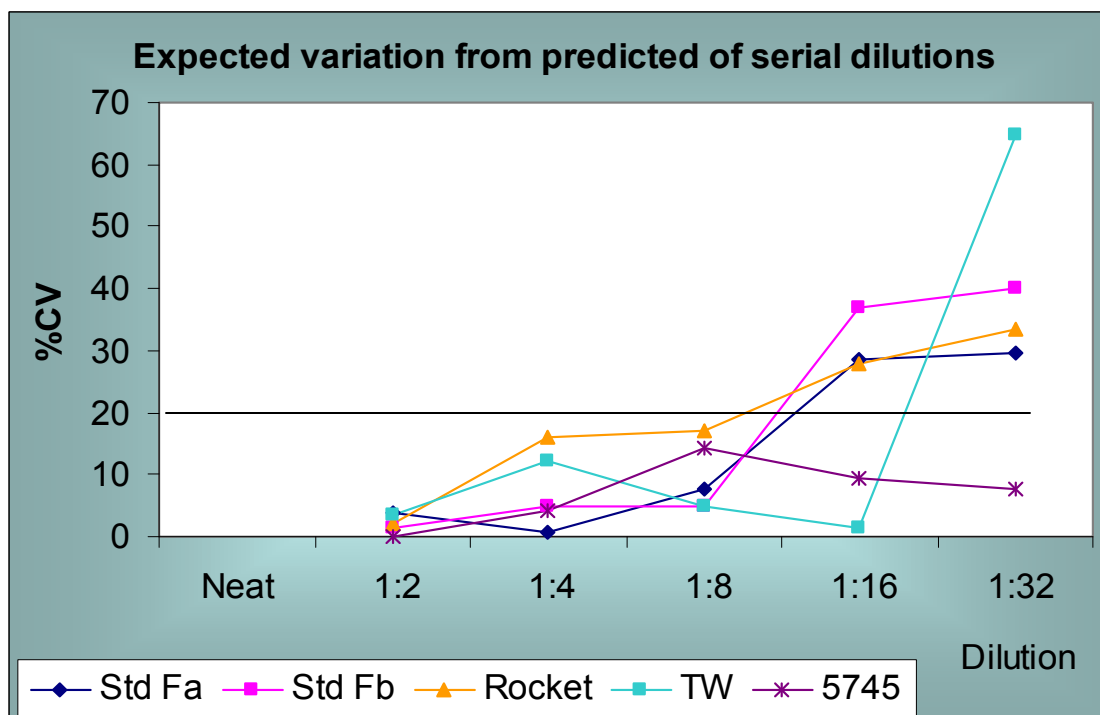
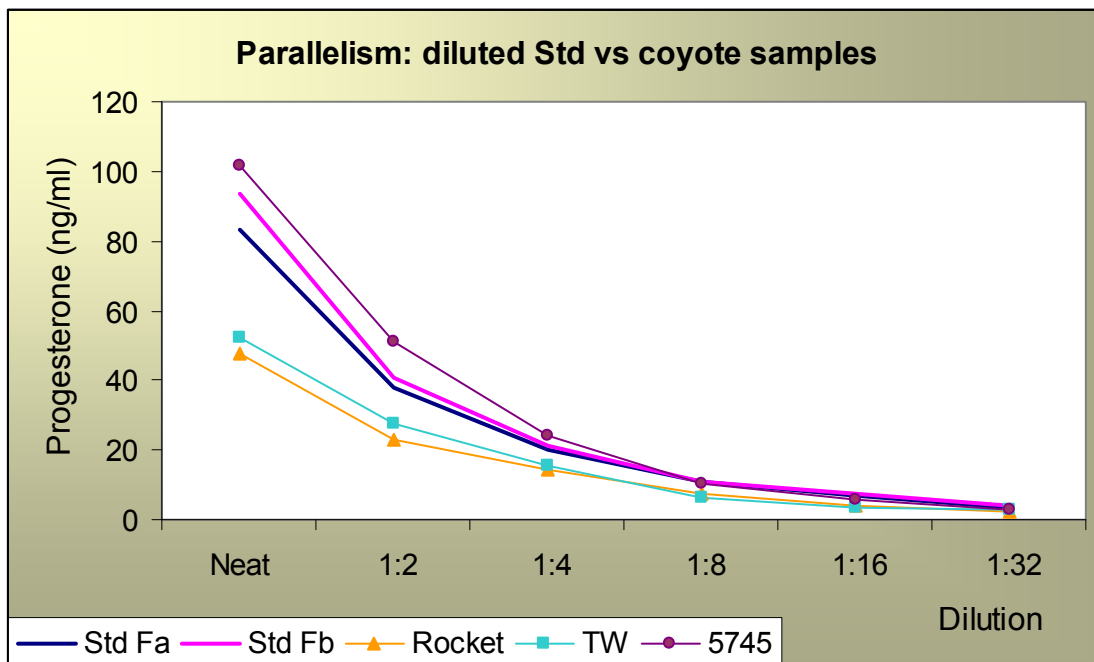
Mean recovery (%) 6 trials	
Level	Mean
100%	93.0
80%	117.7
60%	134.8
40%	135.3
20%	125.6



**Figure F1.** Recovery of progesterone reagent standard in coyote sera. No apparent evidence of interfering substances in coyote sera detected.



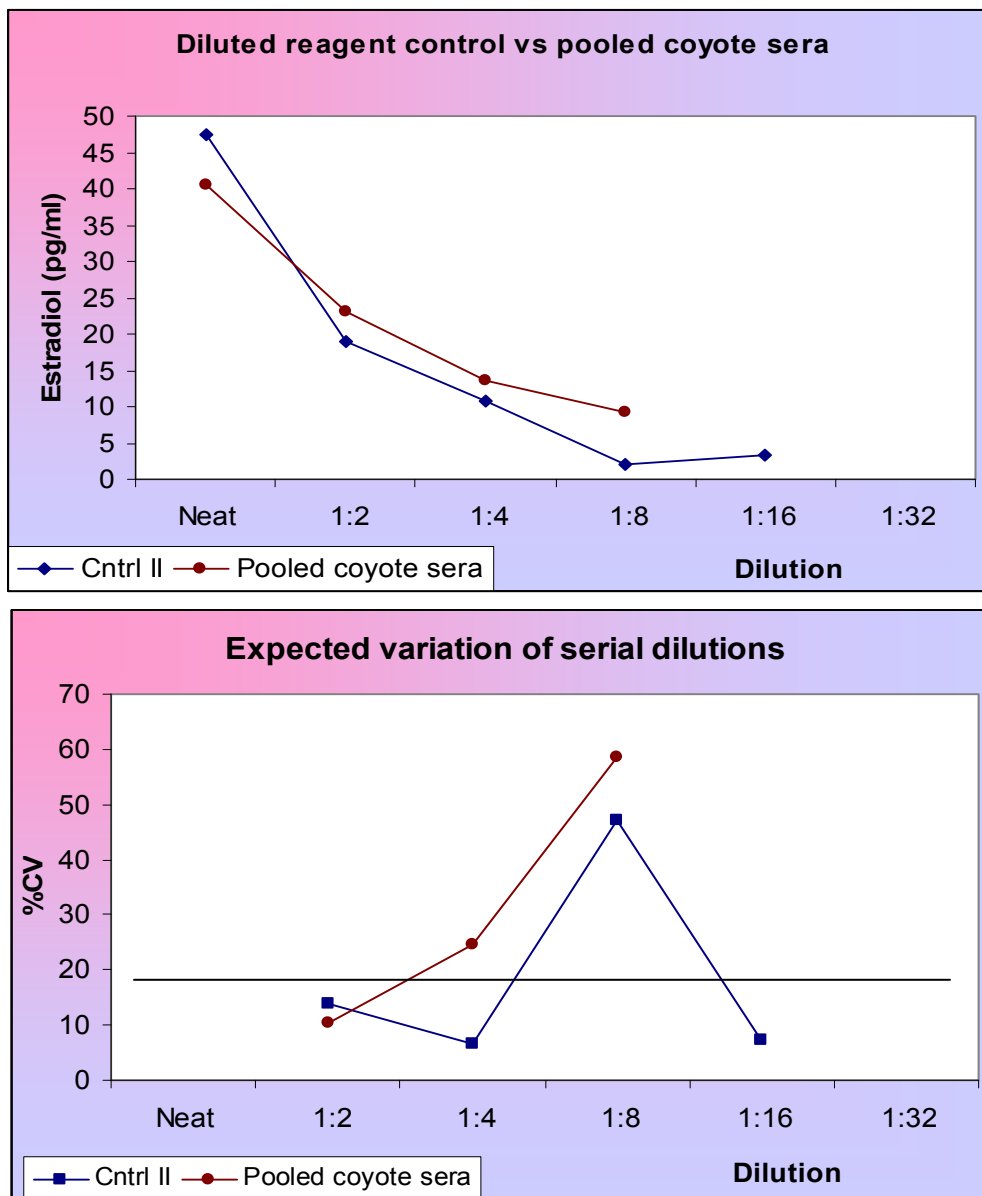
## 5. Parallelism (linearity):



**Figure F2.** Comparison of diluted coyote sera to diluted reagent progesterone standard (parallelism or linearity). No significant effect from interfering substances detected. Serial dilutions  $\leq 1:8$  appear to be acceptable ( $< 20\%$  CV).

*Estradiol (EIA)*

1. Inter-assay (single lot only) mean %CV = 11.2%
2. Intra-assay mean % CV  $\leq$  10%
3. Parallelism:



**Figure F3.** Comparison of diluted coyote sera to diluted reagent estradiol standard (parallelism or linearity). No significant effect from interfering substances detected. Serial dilutions 1:2 appear to be acceptable (< 20% CV).

## CURRICULUM VITAE

Debra Anne Carlson  
2008

**ACADEMIC and RESEARCH INTEREST**

Reproductive biology of wild carnivores

**EDUCATION**

- |      |  |
|------|--|
| 1979 | Bachelor of Science, Medical Technology<br>University of Utah<br>Salt Lake City, Utah                              |
| 2008 | Doctor of Philosophy, Wildlife Biology<br>Department of Wildland Resources<br>Utah State University<br>Logan, Utah |

**ACADEMIC APPOINTMENTS**

- |           |   |
|-----------|---|
| 2003      | Doctoral candidate<br>Department of Forest, Range, and Wildlife Sciences<br>Utah State University<br>Logan, Utah          |
| 1999-2005 | Graduate Research Assistant<br>Department of Forest, Range, and Wildlife Sciences<br>Utah State University<br>Logan, Utah |
| 1988-1997 | Clinical Faculty<br>School of Allied Health Sciences<br>Weber State College<br>Ogden, Utah                                |
| 1978-1980 | Teaching Assistant<br>Department of Biology<br>University of Utah<br>Salt Lake City, Utah                                 |

**PROFESSIONAL ACTIVITIES – EDUCATION**

- 1990-1999 Pathology Resident clinical orientation  
Blood Bank and Pediatric Transfusion Services  
Department of Pediatric Pathology  
University of Utah, School of Medicine  
Salt Lake City, Utah
- 1990-1993 Instructor: Pediatric immunohematology and transfusion  
School for Blood Bank Specialists  
Intermountain Health Care, Blood Services  
Salt Lake City, Utah
- 1986-1997 Nursing education: Blood administration and component  
usage  
Primary Children's Medical Center  
Salt Lake City, Utah

**PROFESSIONAL ACTIVITIES – ORAL PRESENTATIONS**

- 2007 Reproductive biology of the coyote (*Canis latrans*);  
integration of behavior and hormones.  
PhD defense seminar, Utah State University, Logan, Utah
- 2005 An experimental challenge to the reproductive seasonality  
of a wild canid; the monestrous coyote (*Canis latrans*).  
The Animal Behavior Society, 2005 Annual Meeting  
Snowbird, Utah
- 2002 Comparison of reproductive hormone patterns in pregnant  
and pseudo-pregnant coyotes.  
Defenders of Wildlife, Carnivores 2002  
Monterey, California
- 2002 Defining estrus in the coyote by examination of vaginal  
cytology and the associated reproductive hormones.  
The Wildlife Society, 9<sup>th</sup> Annual Conference  
Bismarck, North Dakota
- 2002 Investigation in reproduction of the coyote (*Canis latrans*).  
PhD candidate, project seminar, Utah State University  
Logan, Utah

- 2001 Detection of relaxin for the diagnosis of pregnancy in the coyote.  
The Wildlife Society, 8<sup>th</sup> Annual Conference  
Reno, Nevada
- 2000 Correlation of breeding behavior, reproductive endocrine patterns, and vaginal cytology in captive female coyotes (*Canis latrans*).  
Defenders of Wildlife, Carnivores 2000  
Denver, Colorado
- 1995 Interpreting lab values: CBC's, Chemistry, Blood Bank, and etc.; a discussion based on a specific disease process.  
Center for Pediatric Continuing Education, Pediatric Oncology Workshop, Salt Lake City, Utah
- 1994 Transfusion Considerations and other Blood Bank Issues.  
Pediatric Bone Marrow Transplant Symposium  
University of Utah and Primary Children's Medical Center  
Salt Lake City, Utah
- 1992 Paroxysmal Cold Hemoglobinuria: A Case Study.  
Utah Society for Medical Technology, Spring Seminar  
Salt Lake City, Utah
- 1995 Goldsby, R.E. *et al.* Comparing effects on temperature of early versus late leukodepletion in platelet transfusions.  
37<sup>th</sup> Annual Meeting, American Society of Hematology  
Seattle, Washington

#### ORIGINAL JOURNAL PUBLICATIONS – IN PREPARATION

- Carlson, D.A. and Gese, E.M. Relaxin as a diagnostic tool for pregnancy in the coyote. *Animal Reproduction Science*.
- Carlson, D.A. and Gese, E.M. Reproductive biology of the coyote (*Canis latrans*): integration of mating behavior, reproductive hormones, and vaginal cytology. *Journal of Mammalogy*.
- Carlson, D.A. and Gese, E.M. Influence of exogenous GnRH on reproductive seasonality and mating behaviours of a monoestrous carnivore. *Animal Behaviour*.

- Carlson, D.A. and Gese, E.M. Integrity of mating behaviours following oestradiol manipulation in a monogamous carnivore. *Animal Behaviour*.

### ADMINISTRATIVE APPOINTMENTS

1998-1999	Technical Consultant, Blood Bank Associated Regional & University Pathologists, Inc. Salt Lake City, Utah
1998	Quality Assurance Consultant Myriad Genetics, Inc. Salt Lake City, Utah
1987-1997	Laboratory Supervisor, Blood Bank and Transfusion Service Primary Children's Medical Center Salt Lake City, Utah
1987-1997	Laboratory Supervisor, Immunology Primary Children's Medical Center Salt Lake City, Utah

### SERVICE APPOINTMENTS

1993-1999	Inspector, Inspection and Accreditation Program American Association of Blood Banks Arlington, Virginia
1993-1999	Inspector, Laboratory Accreditation Program College of American Pathologists Northfield, Illinois
1988-1999	Transfusion Review Committee Primary Children's Medical Center Salt Lake City, Utah

### OTHER RELATED ACTIVITIES

2004	Wildlife Capture and Immobilization Clinic Kathy Quigley, DVM Utah State University & USDA/NWRC Logan, Utah
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2004	Field technician volunteer Utah Division of Wildlife Resources
2003	Wildlife Handling and Chemical Immobilization Clinic Wildlife Veterinary Resources, Mark R. Johnson, DVM The California Wolf Center Julian, California
2002	Field technician volunteer Idaho Department of Fish and Game
1990 – 1999	Bird of Prey and Wild Bird Rehabilitation and Education Wild Redux Holliday, Utah
1986 – 1996	Docent volunteer Utah's Hogle Zoo Salt Lake City, Utah

#### **MEMBERSHIP in PROFESSIONAL SOCIETIES**

2005-present	American Society of Mammalogists
2005-present	Animal Behavior Society
2001-2003	The Wildlife Society
1991-1999	American Association of Blood Banks
1979-1999	American Society of Clinical Pathologists

#### **PROFESSIONAL REGISTRATION**

1990	Specialist in Blood Banking, #SBB-3661 American Society of Clinical Pathologists
1979	Medical Technologist, #MT-127592 American Society of Clinical Pathologists