ASPECTS OF REPRODUCTIVE ENDOCRINOLOGY IN THE RED WOLF (CANIS RUFUS)

A Thesis

Presented to

The Faculty of Graduate Studies

of

The University of Guelph

by

SUSAN LORENE WALKER

In partial fulfillment of requirements

for the degree of

Master of Science

August, 1999

ABSTRACT

ASPECTS OF REPRODUCTIVE BIOLOGY IN THE RED WOLF (CANIS RUFUS)

Susan Lorene Walker
University of Guelph. 1999

Advisor:
Dr. K. Goodrowe
Co-Advisors:
Dr. C. Gartley
Dr. A. King
Dr. J. Leatherland

Reproductive endocrinology of the red wolf (Canis rufus) was studied in captive animals by fecal enzyme immunoassay. In cycling females, estrogen metabolites increased through proestrus, reached maximal values during estrus and declined concurrent with rising progestin metabolite values. Progestin metabolite levels remained elevated until mid- to late luteal phase and then gradually declined to nadir levels. There was no significant difference between pregnant and ovulatory non-pregnant hormone profiles. Acyclic animals maintained basal ovarian hormone concentrations throughout the breeding season. During the periovulatory period, serum and fecal hormone profiles exhibited comparable trends.

In male red wolves, changes in testosterone metabolite concentrations were consistent with photoperiod synchronization. Testosterone metabolite levels began to increase prior to the breeding season (November-December), and reached peak levels coincident with estrus in late winter (late February).

It was concluded that sex determination through fecal steroid ratios of progestin/testosterone and testosterone/estrogen is possible only from late December to early May.

ACKNOWLEDGMENTS

I would like to thank my supervisor Dr. Karen Goodrowe for her invaluable guidance, advice, support and encouragement. You have been a wonderful teacher and mentor. Thank-you.

I would like to thank my family and friends for being so patient and understanding over the past 2 years. For my mother, thank-you for always being there when I needed encouragement. Keener, thank-you for your patience and support.

I am grateful to Will Waddell and Sue Behrns for allowing me to play such an active role in the red wolf SSP. Thank-you for always making me feel so comfortable. Without your dedication to the survival of this endangered carnivore, there would have been no research.

Great appreciation is extended to the staff at the Burnet Park Zoo, Great Plains Zoo, Knoxville Zoo, Mill Mountain Zoo, Oglebay's Good Zoo, Point Defiance Zoo & Aquarium and Red Wolf Breeding Facility, Racine Park Zoo, Ross Park Zoo, Trevor Zoo and Western North Carolina Nature Center. Without your commitment the knowledge gained in this study would not have been possible. You have all played a crucial role on the road to understanding the basic reproductive physiology of the red wolf.

I would like to express my gratitude to Dr. Cathy Gartley and Dr. Allen King. Your guidance and advice was invaluable. To Dr. John Leatherland, Dr. James Raeside and Rick Renaud, thank-you for teaching me and allowing me to tap into your vast knowledge of steroids and endocrinology. Special thanks to Dr. John Leatherland and Dr. Suzanne MacDonald for statistical assistance and to Astrid Bellem, Dr. Janine Brown and Coralie Munro for assistance with the assays.

A very special thanks to members both past and present in reproductive physiology laboratory at the Toronto Zoo, Helen Bateman, Astrid Bellem, Maria Franke, Dr. Margery Hay, Merryn M^{c}Ininch, Gabriela Mastromonaco, Leanne Othen, Dave Ryckman and

Kristen Young. You have all provided me with helpful advice, support and laughter, all of which has allowed for the completion of this study.

Financial support was provided by the Morris Animal Foundation, the Zoological Society of Metropolitan Toronto and Natural Sciences and Engineering Research Council of Canada.

DECLARATION OF WORK PERFORMED

I declare that with the exception of the following, all work covered in this thesis was performed by myself.

Fecal samples were collected by animal care staff at the following institutions: Burnet Park Zoo, NY; Great Plains Zoo, SD; Knoxville Zoo, TN; Mill Mountain Zoo, VA; Oglebay's Good Zoo, WV; Racine Park Zoo, WI; Point Defiance Zoo & Aquarium and Red Wolf Breeding Facility, WA; Ross Park Zoo, NY; Trevor Zoo, NY and Western North Carolina Nature Center, NC. Blood sample collection was preformed by staff at the Point Defiance Zoo & Aquarium and Red Wolf Breeding Facility, WA. Assistance with fecal steroid extraction was provided by G. Mastromonaco and D. Ryckman during the Fall and Winter of 1998. Fecal steroid HPLC fractionation was performed by R. Renaud (University of Guelph, Guelph, Ontario). Enzyme immunoassay antibodies and HRP conjugate stocks were provided by C.J. Munro (University of California, Davis, CA). Luteinizing hormone antibody, label and standard was supplied by Dr. J.L. Brown (National Zoological Park Conservation and Research Centre, Front Royal, VA). Assay buffers were prepared by A. Bellem, G. Mastromonaco, L. Othen, D. Ryckman, K. Young or myself. Validation and analysis of serum progesterone values were conducted by staff at the Gynecologic Female Treatment Clinic, Tacoma, WA, February and March (1991-1997). Validation of radioimmunoassys for serum estradiol and LH was performed by A. Bellem (National Zoological Park Conservation and Research Centre, Front Royal, VA).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AC	Nonovulatory or acyclic female
AI	Artifical insemination
ANOVA	Analysis of variance
AAZPA (or AZA)	American Association of Zoological Parks and Aquariums
CL	Corpus luteum/corpora lutea
E2	Fecal estrogen metabolites
$E2_s$	Serum estradiol
EDTA	Ethylenedinitrilo-tetraacetic acid
EIA	Enzyme immunoassay
FSH	Follicle stimulating hormone
GAM	Goat anti-mouse gamma globulin
GnRH	Gonadotropin-releasing hormone
hCG	Human chorionic gonadotropin
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
LH	Lutenizing hormone
NMS	Normal mouse serum
NP	Ovulatory nonpregant female
NSB	Non-specific binding
P	Pregnant female
P4	Fecal progesterone metabolites
$P4_s$	Serum progesterone
PDZA	Point Defiance Zoo and Aquarium
RIA	Radioimmunoassay

RWSSP©	Red Wolf Species Survival Plan©
T	Fecal androgen metabolites
USFWS	United States Fish and Wildlife Service

INTRODUCTION

In the last century extinction of animal species has been occurring at an unparalleled rate (Flesness, 1989). Global biological diversity is eroding largely due to the spread of mankind and our 'innovative' technological advancements. As a species, mankind has altered ecosystems through: 1) habitat destruction and fragmentation, 2) introduction of non-native species, 3) pollution and 4) over-exploitation of natural resources (Wilson, 1989; Tudge, 1992). It has now become increasing clear that the world in which we live is "poised on the edge of an unprecedented biological disaster" (Hutchins & Wiese, 1991).

The challenge to artificially preserve biological diversity is a daunting task. However, success possibly can be achieved through the dedicated cooperation of numerous individuals and organizations. One such organization is the American Association of Zoological Parks and Aquariums (AZA). The foundation of their conservation efforts is based on Species Survival Plans (SSP©): breeding programs which act to coordinate the preservation of both a species and its habitat (Wiese & Hutchins, 1994). Each SSP©, under the direction of a species coordinator, is responsible for managing the demographics and genetics of an endangered species' population in a captive environment. The cooperative actions of institutions that house the species ensure the genetic integrity of the population. Captive breeding programs serve as a genetic reservoir for future reintroductions and maintenance of healthy, stable captive and wild populations through the infusion of new genes. It is well recognized that the tightly managed captive population in no way serves as a substitute for wild populations.

Over the past 2 decades, assisted reproductive technologies, such as artificial insemination, embryo transfer, long-term storage of germplasm or non-invasive endocrine monitoring have begun to play an ever increasing role in conservation programs. These technologies allow for flexibility in a breeding program, escaping restrictions such as limited holding space and transportation costs, while still presevering the genetic diversity

of the population. However, with only 20 years of experience and an ever increasing number of animals in need of captive propagation programs (Soulé et al., 1986), there is still much to learn before these technologies become practical and repeatable on a large scale. One important lesson that has been acknowledged is that what is effective for one species often may be totally unreliable for another, even in a given taxon (Goodrowe, 1997). Therefore, to apply these biotechnologies, basic research concerning reproductive norms must be conducted to improve our fundamental knowledge of a given species.

In situ and ex situ conservation efforts for the red wolf (Canis rufus) have led to non-invasive endocrine studies in our laboratory at the Toronto Zoo. The goal of this study is to non-invasively acquire basic knowledge concerning this endangered canid's reproductive biology. The knowledge gained will help provide a resourceful strategy for more effective management of captive and wild red wolf populations.

REVIEW OF LITERATURE

Reproduction in Canids

Female Reproductive Biology

General Overview

In the female domestic dog (Canis familiaris), sexual maturity ranges from 5 to 24 months of age (Wildt et al., 1981; Feldman & Nelson, 1987; Concannon, 1991) with the average age of puberty onset being dependent on breed (Christiansen, 1984). Fertility progressively declines once a bitch is ≥7 years of age (Feldman & Nelson, 1987). The number of ova present in the ovaries of a newborn bitch has been estimated at 700,000. By puberty, this number declines to 250,000, and at 5 years of age there is a dramatic drop to 30,000. By age 10, only a few hundred ova remain (Anderson & Simpson, 1973). Beyond 6-8 years of age, there are progressive reductions in litter size, increases in the interestrus interval, congenital birth defects, and problems at parturition (Feldman & Nelson, 1987). Although bitches typically continue to cycle throughout their life-span (as they do not experience a menopause; Feldman & Nelson, 1987), the recommended age for breeding is between 2 and 6 years of age (Feldman & Nelson, 1987).

Captive female gray wolves (Canis lupus) have been reported to conceive at 10 months of age (Medjo & Mech, 1976; Zimen, 1976); however, most do not breed until at least 22 months of age (Seal et al., 1979). Mech (1987) reported signs of reproductive activity, including mammary development and pairing behaviour, in wild wolves during their first breeding season. However, most do not breed until they are 4-5 years of age (Mech, 1991). Captive gray wolves may live to 16 years of age and free ranging wolves have been reported to live as long as 13 years (Sheldon, 1992). The reproductive age in wild gray wolves has been estimated to extend to 11 years of age (Mech, 1988). Captive female red wolves (Canis rufus) also are capable of reproducing during their first breeding season; however, breeding is generally more common during their second season (Nowak 1972; Parker, 1988, Waddell, 1995). Fecundity of the captive red wolf peaks at

approximately 7 years of age and declines to senescence at 12 years (Sparks database, Waddell, 1996). Coyotes (Canis latrans) also are capable of reproducing in their first year (Kennelly & Johns, 1976); however, the percentage that does so is variable, ranging between 10% and 70% (Gier, 1975). Longevity in the wild coyote is estimated to be 14-16 years (Sheldon, 1992).

Domestic dogs are monoestrus, ovulating one to two times a year at intervals of 5-13 months (Concannon et al., 1989). Several studies indicate that, as a group, domestic dogs cycle on average every 7 months, and are nonseasonal breeders (Christie & Bell 1971; Stabenfeldt, 1977; Felman & Nelson, 1987; Concannon, 1991). It has been demonstrated that domestic dogs housed outdoors cycle year round (Anderson, 1970), as do dogs housed under constant lighting conditions of 12 hours light:12 hours dark (Concannon, 1986a). With the exception of some fox-like canids (Sheldon, 1992), and the bush dog (Speothos venaticus; Bekoff, 1975; Porton et al., 1987), wild canids generally are classified as monoestrus (Sheldon, 1992). However, unlike their domestic relatives, they have well-defined breeding seasons: from February to March, for the red wolf (Carley 1979; Waddell, 1995), and January to April in the coyote (Hamlettt, 1938; Fowler, 1986) and gray wolf (Sheldon, 1992; Busch, 1995). A trend shared among these species is that animals from southern latitudes tend to breed earlier than their northern conspecifics (Mech, 1970; Paradiso & Nowak, 1971; Ensely, 1986; Fuller, 1989a; Fuller, 1989b; Waddell, 1995).

Ovarian Cycle

The classical stages of the canine estrous cycle are anestrus, proestrus, estrus, and metestrus (Heape, 1900). In recent years, the term diestrus has largely replaced the term metestrus in the bitch (Olson et al., 1984a, Concannon, 1991). In wild canids for which there is relevant information, the estrous cycle follows the general trends observed in the domestic bitch (Möller, 1973; Kennelly & Johns, 1976; Seal et al., 1979; Stellflug et al.,

1981; Seal et al., 1987; Pauiraj et al., 1992, Wasser et al., 1995; Monfort et al., 1997; Velloso et al., 1998).

Anestrus is the period of apparent reproductive quiescence following diestrus (Concannon, 1991). In the domestic dog, anestrus ranges from 2-10 months, with an average of 4 months (Feldman & Nelson, 1987; Concannon, 1991).

Proestrus is a period of heightened follicular activity (Feldman & Nelson, 1987). In response to increasing levels of estrogen, proestrus is marked by a discharge of serosanguinous fluid through the vagina, vaginal cornification and bleeding, progressive increases in vulval edema, and pheromonal attraction of males (Feldman & Nelson 1987; Concannon, 1991). During the majority of proestrus, the bitch refuses to permit mounting by turning and growling at the male. Near the end of proestrus the bitch may permit mounting, but usually prevents intromission by lying or sitting down (Concannon, 1991). Proestrus in the domestic dog can be as brief as 1-3 days or as long as 3 weeks; the average duration is 9 days (Christie & Bell, 1972; Feldman & Nelson, 1987; Concannon 1983). Physical signs of proestrus in wild canids, such as vaginal bleeding and swelling, appear to be longer in duration compared to their domestic cousin, ranging in duration from 1-6 weeks in gray wolves (Seal et al., 1979) to as long as 2-3 months in coyotes (Kennelly & Johns, 1976; Stellflug et al., 1981). However, studies indicate that the duration of proestrus for the maned wolf (Chrysocyon brachyurus; mean 10.6 days, Velloso et al., 1998) and raccoon dog (Nyctereutes procyonoides; mean 7.6 days, Valtonen et al., 1977) fall within the range described for the domestic dog.

Estrous behaviour within the genus Canis is characterized by reflex or spontaneous stiffening and deviation of the tail, firmly standing to permit mounting, and a reflex lordosis-like presentation of the vulva (Concannon et al., 1979b; Seal et al., 1979; Beach et al., 1982; Pauiraj et al., 1992, Monfort et al., 1997). In the domestic dog estrus may be as short as 3 days or last for several weeks, but it is generally 4-12 days in length, with an average of 9 days (Concannon, 1991). The duration of estrus in wild canids appears to be

highly variable: gray wolf (3-15 days; Seal et al., 1979) coyote (mean of 10.2 days; Kennelly and Johns, 1976) silver fox (Vulpes vulpes, 1.5-5 days; Lindeberg et al., 1993) raccoon dog (mean of 4 days; Valtonen et al., 1977) dhole (Cuon alpinus, 14-39 days; Pauiraj et al., 1992) bush dog (1-12 days; Porton et al., 1987) African wild dog (Lycaon pictus, 6-9 days; Monfort et al., 1997). Characterization of red wolf behaviours associated with courtship and breeding are currently under investigation (T. Wagner, personal communication).

Estrous behaviours in domestic dogs are a response to a decline or withdrawal of estrogen, and are facilitated by a rise in progesterone (Concannon et al., 1979b, Beach et al., 1982; Feldman & Nelson, 1987). This theory is supported by the administration of estradiol alone followed by either the absence or administration of progesterone to ovariectomized domestic bitches. Behavioural estrus was not displayed until after the administration of estradiol was halted, and the onset of estrus was more synchronous when progesterone was administered at the time of estrogen withdrawal (Concannon & Hansel, 1975). Estrous behaviours, including copulation, also have been observed during declining estrogen and rising progesterone concentrations in serum samples from gray wolves (Seal et al., 1979) and fecal samples from African wild dogs (Monfort et al., 1997) and maned wolves (Velloso et al., 1998).

In the domestic dog it has been reported that intromission and mating may not occur until a few days after the onset of estrous behaviour. During estrus, the vulva must progress through a turgid phase to become soft and flaccid, therefore no longer providing a difficult barrier for male penetration (Feldman & Nelson, 1987). Mating is usually 5 to 45 minutes in duration and usually results in a copulatory tie, during which the male dismounts and stands beside or behind the bitch, with the penis locked in the vagina. The lock withstands tugging and pulling, and is terminated by detumescence of the penis (Concannon, 1991). The copulatory tie that is common among domestic canids also has been observed in the maned wolf (Rodden, 1995), gray wolf (Busch, 1995), red wolf (S.

Behrns personal communication), coyote (Sheldon, 1992), raccoon dog (Valtonen et al., 1977) and the dhole (Pauiraj et al., 1992). The tie appears to be slightly shorter in duration in wild canids, perhaps as mechanism for survival in the wild (Pauiraj et al., 1992; Sheldon, 1992; Busch, 1995)

Diestrus begins once the bitch stops accepting the male (Evans & Cole, 1931; Olson et al., 1984a) and is considered to last until the subsidence of the effects of luteal phase progesterone. Diestrus can be characterized by mammary gland development or progesterone measurement in the nonpregnant or pseudopregnant bitch. There is no acute luteolytic mechanism in the dog, and the transition from diestrus to anestrus is gradual (Concannon, 1991). The luteal phase in a pregnant bitch ends with parturition (Concannon, 1986b).

Follicular and Hormonal Changes

It has been suggested that developing follicles can occur in bitches of all ages, breeds and stages of the estrous cycle (Durrant et al., 1998). Throughout much of anestrus, the ovaries of the domestic dog contain slow but continuously developing follicles, with most becoming atretic (Feldman & Nelson, 1987). Follicles that develop parallel to gonadotropin stimulation mature and attain the ability for estrogen synthesis and secretion (Feldman & Nelson, 1987). During the onset of proestrus, follicular atresia continues to overtake the majority of vesicular follicles. However, some remain viable and continue to enlarge. A characteristic feature of the ovary at the onset of proestrus is the presence of several follicles 1.0-1.5 mm in diameter. During proestrus, antral follicles increase in size and by late proestrus reach 3-4 mm in diameter. At the onset of estrus, follicles appear much as they did in late proestrus except that they measure 3-5 mm in diameter (Anderson & Simpson, 1973). In the domestic bitch preovulatory follicular development has been detected as early as 11 days before ovulation, with the most rapid follicular maturation occuring 2 to 3 days before ovulation (Wildt et al., 1977).

Although anestrus has been referred to as the quiescent phase of the canine ovarian cycle, studies suggest that neither the ovary nor the pituitary gland are completely quiescent during anestrus (Olson et al., 1982). Fluctuations have been identified in both pituitary hormones and ovarian steroids (Feldman & Nelson, 1987).

Serum estradiol or estrogen levels in the domestic dog fluctuate throughout anestrus, usually ranging between 5-20 pg/ml (Concannon, 1991). Surges in estrogen (in the range of 30-50 pg/ml) also have been observed (Olson et al., 1982), and are assumed to be derived from waves of follicle development (Feldman & Nelson, 1987). During early proestrus, estrogen concentrations range from 10-20 pg/ml (Concannon & Lein, 1989). Peak concentrations of estrogens range from 50-100 pg/ml and are attained in late proestrus or early estrus, generally 0-3 days before the preovulatory surge in luteinizing hormone (LH; Edquist et al., 1975, Concannon et al., 1975; Hadely , 1975; Nett et al., 1975; Wildt et al., 1979a; Chakraborty, 1987, Concannon, 1991). Estrogen levels decline during estrus to concentrations of 5-20 pg/ml (Concannon, 1991), and represent the final follicular maturation process prior to ovulation (Feldman & Nelson, 1987). Concentrations of serum estrogens in the gray wolf and coyote during late anestrus, proestrus and the estrogen surge have been reported to fall within ranges reported for the domestic dog (Seal et al., 1979, Stellflug et al., 1981). Changes in estrone concentrations reported throughout most of the domestic dog ovarian cycle tend to parallel those of estradiol (Wildt et al., 1979; Chakraborty, 1987).

Plasma progesterone concentrations in bitches increase during proestrus from baseline values of 0.4-0.6 ng/ml to values of 0.8-1.2 ng/ml at the time of peak estradiol levels (Concannon, 1986a, Concannon et al., 1977a). Progesterone increases rapidly above 1.0 ng/ml during the LH surge as estrogen concentrations fall (Wildt et al., 1979; Chakraborty, 1987; Concannon et al., 1977a). Progesterone concentrations are ~ 4-8 ng/ml at the time of ovulation, which occurs ~ 2 days after the LH surge (Wildt et al., 1979; Concannon, 1986a).

Plasma/serum LH concentrations have been reported to be low and variable during anestrus, and low during proestrus and estrus except during the preovulatory surge (Smith & MacDonald, 1974; Concannon et al., 1975; Nett et al., 1975; Concannon et al., 1977a; Chakraborty, 1987). Generally, LH serum concentrations during anestrus are well below LH peak values, representing only 10-20% of peak values (Concannon, 1991). However, LH pulses of 2-25 ng/ml have been detected at intervals of 2-8 hours throughout anestrus. Inter-pulse levels were 0.2-1.2 ng/ml. Just prior to the onset of proestrus, pulse intervals increase to 60-90 minutes and mean LH levels are elevated to 3 ng/ml (Concannon et al., 1986; Concannon, 1989; Concannon et al., 1989). During proestrus, LH levels are low and pulses are nondectecable, possibly due to estrogen negative feedback. LH concentrations increase to peak levels, ranging from 8-50 ng/ml during the preovulatory surge (Concannon, 1991).

Unlike LH, follicle stimulating hormone (FSH) does not have wide fluctuations in peripheral concentrations. This hormone varies only slightly throughout anestrus before declining with the onset of proestrus, presumably due to negative feedback effects of estradiol and inhibin. Follicle stimulating hormone then rises with the LH peak (Reimers et al., 1978; Olson et al., 1982, Concannon, 1993). Mean levels of FSH during anestrus may surpass levels observed during the preovulatory surge, and concentrations have been reported to be 50-100% of those of the periovulatory peak (Olson et al., 1982; Concannon, 1991). Unlike that of LH, the pulsatile nature of FSH secretion during anestrus has not been clearly demonstrated, probably because of the relatively small increases in magnitude and the longer half-life of this hormone (Concannon, 1993).

Compared to most domestic and laboratory animals, there is a paucity of information regarding regulation of gonadotropin secretion or control of ovarian activity by gonadotropins in the female dog (Concannon, 1991). It is likely a complex interaction between environment, age, ovarian status, general health, and other yet unidentified factors that dictate roles for FSH and LH (Feldman & Nelson, 1987).

Studies have demonstrated a pulsatile release of LH secretion in the bitch (Hegstad et al., 1993; Jeffcoate, 1993; Hoffmann & Schneider, 1993). It has been suggested that anestrus may be naturally terminated by an increase of this episodic release, resulting in an elevation of mean LH levels shortly before proestrus (Concannon et al., 1986; Concannon, 1989). However, the mechanisms involved in altering the LH secretion pattern remain unknown (Concannon, 1991). A minor, but significant increase in estradiol has been observed shortly before the onset of proestrus (Jeffcoate, 1993). Such elevations in estradiol may result in an increased rate of pulsatile LH secretion and/or may also be the result of increased LH secretion. Together, both mechanisms could be involved in a brief positive feedback for the initiation of proestrus (Concannon, 1993). This theory can be supported by data that demonstrate the induction of proestrus by injections of LH alone (Concannon, 1993) and by administration of estrogen alone (Bouchard et al., 1993).

As demonstrated in other species, gonadotropin-releasing hormone (GnRH) routinely causes the release of LH and FSH in the domestic canid (McRae et al., 1985; Vanderlip et al., 1987; Van Haaften et al. 1992; Concannon, 1993). The administration of a GnRH agonist for 14 days in an anestrus bitch stimulated LH and FSH secretion and resulted in the successful induction of estrus and the production of two pups (Concannon, 1993). It is possible that elevated LH concentrations at the end of anestrus involve an increase in sensitivity to GnRH in addition to an increase in pulse frequency (Concannon, 1993). It also has been reported that the sensitivity of the LH response to GnRH was higher during late anestrus than during early anestrus (Van Haaften et al., 1992). The cause of the increase in sensitivity remains unknown (Concannon, 1993).

Concannon (1993) suggests that both FSH and LH are folliculotrophic hormones. The administration of FSH alone or LH alone can cause follicular growth and induce proestrus (Concannon, 1993). However, alterations in LH, not FSH secretion, may be more significant in the initiation of a functional follicular phase, as FSH appears to be elevated throughout most of anestrus and LH concentrations are usually low except near the

end of estrus LH secretion. This assumes that the biopotency of FSH does not increase during late anestrus. Therefore, either the start of a follicular phase does not require FSH, or follicles acquire responsiveness to FSH at the end of anestrus, perhaps due to increased concentrations of LH and estradiol (Concannon, 1993).

Prolactin also may play a role in the initiation of the ovarian cycle in the domestic bitch. Suppression of prolactin with dopamine agonists causes an extensive shortening of interestrous intervals (Okkens et al., 1985a; van Haaften et al., 1989) and induction of estrus in females with a prolonged anestrus (Arbeiter et al., 1988; Jochle et al., 1989). However, no obvious alterations in prolactin concentrations have been observed in late anestrus (Olson et al., 1982), suggesting modifications in prolactin receptors may be involved (Concannon, 1993).

As with other species, endogenous opioids appear to modulate LH release in the ovarian cycle of the domestic dog (Concannon & Temple, 1988). The responsiveness to naloxone, an opioid antagonist, late in anestrus was elevated compared to the proestrus phase. The authors suggest that there is an endogenous opioid tone inhibiting LH release during late anestrus and a possible decline in opioidergic tone or decline in non-opioid inhibitory mechanism which may causes the onset of proestrus.

Ovulation

It has been suggested that changes in sexual behaviour can be related to ovulation (Christiansen, 1984). Holst & Phemister (1971) indicated that ovulation occurred 24-48 hours after the bitch first allowed mating. Later, studies demonstrated that ovulation may occur over several days following the beginning of standing estrus (Wildt et al., 1978). Penton et al. (1991) used circulating progesterone and LH to determine time of ovulation and elucidate its relation with behaviour. Their results indicate that the time of ovulation bore little relation to alterations in sexual behaviour. It also has been demonstrated that signs of proestrus, such as vulval swelling or serosanguinous discharge, may occur 3 weeks to 3 days prior to the LH peak, while first acceptance of a male can occur as early as

4 days before or as late as 6 days after the LH surge (Concannon, 1983, Concannon, 1986a). In relation to behavioural estrus, the time of ovulation in the coyote is highly variable. It may occur as early as the 1st day of estrus or as late as the 9th (Kennelly & John, 1976). This suggests that the timing of ovulation is more precisely determined by endocrine changes rather than by observations of behavioural or clinical changes.

Declining estradiol concentrations with increasing progesterone concentrations are associated with the LH surge in the domestic bitch (Smith & MacDonald, 1974; Concannon et al., 1975, Concannon et al., 1977a; Concannon et al., 1979b; Wildt et al., 1979; Olson et al., 1982). The pre-ovulatory LH surge has been demonstrated to occur concurrently with elevations in progesterone concentrations; the two coincide on the same day or may differ by only a single day (Concannon et al., 1977a; Penton et al., 1991). This phenomenon also has been demonstrated in gray wolves, in which the initial increase in progesterone concentrations from baseline values occurred during the LH surge (Seal et al. 1979).

In the domestic dog the preovulatory LH surge represents a 20-40 fold increase in LH levels and a 2-4 fold increase in FSH levels. The LH surge may last for 1-4 days and generally occurs near the onset of estrus, 0-3 days after the estrogen peak of late proestrus (Smith & MacDonald, 1974; Nett et al., 1975; Concannon et al., 1975, Concannon et al., 1977a, Wildt et al., 1978). Similar to the domestic dog, the gray wolf exhibits a preovulatory LH surge which last for 3 days immediately following the estrogen peak (Seal et al., 1979).

Ovulation, which is spontaneous in the bitch, has been reported to occur 1-3 days following the LH surge (Wildt et al., 1978), although others reported little variation in the 2 day interval between the LH surge and ovulation in the bitch (Phemister et al., 1973; Concannon et al., 1977a, Concannon et al., 1989). Duration of ovulation has been variably recorded as transpiring over only a few hours (Holst & Phemister, 1971; Phemister et al., 1973; Concannon et al., 1977a; Anderson & Simpson, 1973), 12-72

hours (Graf, 1978) or even over several days (Wildt et al., 1977; Wildt et al., 1978). Perhaps the discrepancy regrading the duration of ovulation is related to the technique used (histologic or laprascopic). Unlike most species, the ovary of the domestic bitch is completely surrounded with a fatty bursa which inhibits the direct examination of the ovary (Wildt et al., 1977).

Follicles which fail to ovulate following the LH surge undergo atresia (Feldman & Nelson, 1987). The number of ova released is partially dependent upon the breed of the bitch. Based on litter size, smaller breeds tend to ovulate fewer ova (2-10) than larger breeds (5-20 ova; Feldman & Nelson, 1987). An approximation of the number of ova released and corpora lutea (CL) formed following an ovulatory surge of LH was determined through examination of ova recovered after flushing either the oviduct or uterus at different intervals post-ovulation. The number of CL identified on the ovary seldom agreed with the number of ova collected, with the average numbers of ova and CL being 5.4 (range 2-9) and 7.6 (4-9), respectively (n=10 dogs; Penton et al., 1991).

The timing of the LH surge can be supported by studies in which ovariectomized bitches received estradiol-releasing implants and increasing doses of injected estradiol benzoate. Ceasing injections, estradiol concentrations fell and patterns of LH secretion were investigated with or without the simultaneous administration of progesterone. The decline in estradiol caused preovulation-like surges in LH which were further facilitated by the administration of progesterone (Concannon et al., 1977a, Concannon et al., 1979a). These temporal hormonal relationships have lead to the idea that in the bitch, elevated estradiol concentrations maintain a negative feedback effect on LH secretion. Further, the preovulatory LH surge is initiated by a failure of an additional estradiol increase or decline and is facilitated through an elevation in progesterone levels (Concannon et al., 1979b). Ovariectomy at any stage in the reproductive cycle results in a rapid and lasting increase in LH and FSH concentrations. Estradiol appears to be the major, and perhaps only, ovarian hormone involved in this negative feedback (Concannon & Hansel, 1975; Concannon et

al., 1979a). The administration of increasing estradiol concentrations to ovariectomized bitches, even when above normal peak values, suppresses LH levels (Concannon et al., 1979a). In contrast, the administration of progesterone, or progesterone agonists does not reduce LH in ovariectomized bitches (McCann et al., 1987). As with LH, FSH secretion in either ovariectomized or intact bitches is inhibited following the administration of estradiol but not by the administration of the progestagen megestrol acetate (Colon et al., 1993; Concannon, 1993). The ability of progesterone to synergize with estradiol and produce a negative feedback suppression of LH has been investigated in the anestrous bitch. Basal LH levels maintained at low values by endogenous estrogen were not further reduced through the administration of megestrol acetate (Colon et al., 1993).

Administration of LH or human chorionic gonadotropin (hCG) causes pro-estrual follicles to luteinize or ovulate (Concannon, 1993). However, FSH, in addition to LH, may serve as an ovulatory hormone. In the absence of a distinct detectable LH surge and associated with a near-normal FSH surge, spontaneous fertile ovulations have been observed in bitches induced with an agonist of GnRH following proestrus (Concannon, 1989; Concannon, 1993). Factors regulating FSH concentrations in the periovulatory period are likely to be similar to those which regulate LH (a decrease in the estradiol:progesterone ratio), except that, as in other species, ovarian inhibin promotes the suppression of FSH secretion during proestrus (Concannon, 1993). Although is has been demonstrated that GnRH stimulates gonadotropin release in dogs (MacRae et al., 1985; Vanderlip et al., 1987; Van Haaften et al. 1992; Concannon, 1993), whether a preovulatory surge in GnRH secretion triggers the surge release of LH and FSH has not been investigated in the domestic dog (Concannon, 1993).

Pregnancy and Pseudopregnancy

Once released, oocytes (~120 μm in diameter; Holst & Phemister, 1971) pass through the bursal cavity into the proximal portion of the oviduct within a few hours (Anderson & Simpson, 1973). The bitch ovulates primary oocytes (Evans & Cole, 1931;

Anderson & Simpson, 1973; Tsutsui, 1975), which may require 2-5 days for the completion of meiotic maturation (Tsutsui, 1989). The formation of polar bodies to complete meiosis occurs in the mid- and distal portions of the oviduct (Holst & Phemister, 1971; Anderson & Simpson, 1973; Phemister et al., 1973). On average, oocyte maturation is believed to occur within 2-3 days post ovulation and, therefore 4-5 days after the LH surge (Concannon et al., 1989). Significant oocyte maturation can transpire within 2 days (Mahi & Yanagimachi, 1976); however, oocytes may remain fertile for an additional 2-3 days (Tsutsui & Shimizu, 1975) since fertile matings have occured 7-8 days after the LH peak (Concannon et al., 1989). Fertile matings earlier than 2 days before or 9-10 days after the LH peak are rare and result in reduced litter sizes (Concannon, 1986a, Concannon et al., 1989). Fertilization takes place in the distal portion of the oviduct (Anderson & Simpson, 1973). Ova may remain quiescent in the oviduct of different individuals for differing periods of time prior to fertilization suggesting that individual variation may occur with respect to the timing of fertilization (Tsutsui, 1975, Penton et al., 1991). The embryo of the bitch remains in the oviduct for ~9-10 days following ovulation, then enters the uterus either in the morula or early blastocyst stage (Anderson, 1927; Penton et al., 1991). Free floating blastocysts are present in the uterus between days 8 and 20, and during this period edematous areas appear in the endometrium which represent future implantation sites (Holst & Phemister, 1971). Implantation in the dog has been reported to occur 13-20 days after ovulation (Griffiths et al., 1939; Thatcher, 1997).

Gestation length in the domestic dog from fertile mating to parturition has been reported from 55-68 days; however, gestation length recorded from the preovulatory LH peak to parturition is 64-66 days (Concannon et al., 1983, Concannon et al., 1989). In wild canids it has been reported that gestation length tends to increase with adult size, ranging from 48-58 days in the smaller fox-like canids (Sheldon, 1992) to 58-65 days in the larger wolf-like canids (gray wolves, 60-65 days, Seal et al, 1979; Lentfer & Sanders, 1973; maned wolves, 65 days, Brady & Ditton, 1979; Vellero et al., 1998; dhole, 62.7

days, Pauiraj et al., 1992; coyote, 58-61 days; Gier 1975). Litter size in the domestic canid ranges from 1-23 pups; in most breeds the average litter size is between 4 and 8 pups (Christiansen, 1984). Reported litter sizes in the gray wolf (1-13, average 6; Sheldon, 1992), coyote (2-12, average 4-7; Gier, 1975), and red wolf (2-8, average 3-4, Riley & McBride, 1972; Parker 1988) are similar to that of the domestic dog. In general, litter size in wild canids is reported to increase with increased adult weight (Moehlman, 1992).

Post-implantation pregnancy can be detected by palpation (Concannon, 1983) and/or ultrasound (Bondestam et al., 1983). To date there are no readily available endocrine methods for the early diagnosis of pregnancy in the domestic bitch. However, relaxin is detectable in plasma by radioimmunoassay after the third week of gestation (Steinetz et al., 1987; Steinetz et al., 1989) and total urinary estrogens are elevated 21 days after mating (Richkind, 1983). Both have been suggested as possible pregnancy diagnostic tools and just recently a relaxin assay became commerically available (Repro Chek, Synbiotics).

The long luteal phase of the nonpregnant ovarian cycle has been termed a physiological pseudopregnancy (Stabenfeldt & Shille, 1977). Unlike the ferret or cat, which experience a pseudopregnancy, the long luteal phase in dogs occurs spontaneously and is not dependent on mating-induced ovulation or activation of luteal function. Overt or clinical pseudopregnancy varies with breed and individual and is characterized by extensive mammary gland development and behavioural changes typical of pregnancy and lactation (Concannon, 1991).

Endocrinology

Little or no significant difference has been reported in circulating plasma progesterone levels for pregnant and nonpregnant bitches (Hadely, 1975; Nett et al., 1975; Austad et al., 1976; Reimers et al., 1978; Concannon et al., 1989; Onclin & Verstegen, 1997). However, the presence of secondary increases in progesterone following implantation have been suggested (Smith & MacDonald, 1974; Concannon et al., 1977b).

Concannon (1991) outlined that during pregnancy, progesterone concentrations reach peak levels of 15-80 ng/ml ranging from days 12-30, slowly decline to values of 2-10 ng/ml by day 45, and less than 1 ng/ml by days 60-110. Estradiol values reach values of 5-20 pg/ml in the early luteal phase, followed by a slight increase before returning to low variable concentration during anestrus (Concannon, 1991). Most studies report that mean estradiol or total estrogen levels are not significantly different during the last half of pregnancy when compared to the nonpregnant bitch (Edqvist et al., 1975; Hadely, 1975; Nett et al., 1975; Austad et al., 1976; Graf, 1978; Reimers et al., 1978). Estrone levels follow similar patterns to those of estradiol, except that a pregnancy-specific increase in estrone levels has been suggested (Chakraborty, 1987). It also has been reported that mean levels of serum sex steroids were higher in the nonpregnant bitch than in pregnant bitches during weeks 1 through 6, and weeks 1 through 3 for progesterone and estradiol, respectively (Chakraborty, 1987). With the exception of a few studies, differences in progesterone and estrogen concentrations between nonpregnant and pregnant bitches may not be obvious as pregnancy is associated with: 1) an increase in blood volume diluting concentrations in serum and plasma, and 2) a possible increase in clearance rate due to an elevated metabolism (Concannon et al., 1977b). However, pregnancy related differences in both progesterone and estrogen fecal metabolites have been found in the domestic dog (Gudermuth et al., 1998) and the maned wolf (Wasser, 1995; Vellero, 1998).

Luteal Maintenance and Life Span

Secretion of progesterone and maintenance of luteal tissues during pregnancy are dependent on pituitary secretion of luteotrophic hormones since pregnancy can be terminated at any stage by hypophysectomy (Concannon, 1980). The two main luteotrophic factors involved in regulation of the canine CL are LH and prolactin (Okkens et al. 1986; Concannon et al., 1987; Okkens et al., 1990). It has been suggested that LH and prolactin may exert their activities through different mechanisms (Hoffman & Schneider, 1993). The removal of LH, using anti-LH serum, leads only to a temporary decrease in

progesterone. The removal of prolactin, using bromocriptine, results in an irreversible decrease in progesterone concentrations (Concannon et al., 1987).

Luteotrophic control of the dog CL appears to shift from the first to second half of the luteal phase (Olson et al., 1989). Corpora lutea are more resistant to luteolytic factors during the early luteal vs. late luteal phase (Concannon, 1989). Observed decreases in progesterone levels following hypophysectomy were more pronounced during late rather than early stages of diestrus in the nonpregnant bitch (Concannon, 1980; Okkens et al., 1986). Following administration of a LHRH antagonist, it was determined that the CL in the pregnant dog may be independent of pituitary luteotrophins up until ~Day 10 of pregnancy and dependent upon pituitary luteotrophins after Day 22 (Vickery et al., 1987). The luteotrophic hormone requirements of the early developing CL have yet to be determined (Concannon, 1995).

Pregnancy-specific increases in prolactin (~25 days after the LH surge) have been reported in the domestic dog (Knight et al., 1977; Concannon et al., 1978; De Coster et al., 1983; McCann et al., 1988; Concannon et al., 1989, Onclin & Vertegen, 1997). It has been suggested that prolactin is the main luteotrophic factor during the second half of pregnancy, but its mode of action remains unknown (Onclin & Verstegen, 1996). The low prolactin concentrations observed during the luteal phase of the nonpregnant bitch suggests that its role in the nonpregnant bitch is less significant than in pregnancy (Onclin & Verstegen, 1997).

Progesterone may be the only ovarian steroid required for maintenance of pregnancy, since synthetic progestins can maintain pregnancy in the ovariectomized bitch (Sokolowski, 1971; Concannon et al., 1977b). However, progesterone alone may not be the only hormone necessary for all aspects of pregnancy, as mammary enlargement did not occur in ovariectomized bitches during the maintenance of pregnancy by administration of a synthetic progestagen (Steinetz et al., 1989). The concentrations of progesterone during pregnancy appear to be in excess, as bitches treated with doses of prostaglandins (which

produce an incomplete luteolysis) have been able to maintain pregnancy despite reductions in progesterone levels to ≤ 2 ng/ml for several days (Concannon & Hansel, 1977; Vickery & McRae, 1980; Jackson et al. 1982). Corpora lutea remain the main source of progesterone and are required for maintenance of the entire pregnancy (Concannon et al., 1977b). Ovariectomy at any stage results in the termination of pregnancy (Sokolowski, 1971; Anderson & Simpson, 1973). Histochemical studies of the dog placenta suggest that there is little if any de-novo placental steroidogenesis (Kiso & Yamauchi, 1984).

Estrogens do not increase dramatically during pregnancy. This appears to be critical, since abnormally high estrogen levels can terminate a fully established post-implantation pregnancy (Concannon, 1977b). However, the presence of estrogens is essential during pregnancy, as they contribute to the synthesis and/or availability of progesterone receptors (Concannon et al., 1989).

To date, no luteolytic mechanism has been demonstrated in the dog (Hoffman & Schneider, 1993). Mechanisms allowing luteolysis may be: 1) reduced responsiveness to luteotrophic stimuli and/or 2) a decrease in luteotrophic support. Studies have demonstrated concentrations of LH and prolactin may increase in the serum of pregnant and nonpregnant bitches during late diestrus. Therefore, luteal regression likely occurs independently of the availability of LH and prolactin (Olson et al., 1984a; Chakraborty, 1987; Hoffman & Schneider, 1993). It also has been demonstrated that prolactin and LH receptor formation remain constant during diestrus, making endogenous control of luteolysis difficult to explain (Fernandes et al., 1987; Hoffman & Schneider, 1993).

As with some other species, the CL of pregnant and nonpregnant bitches are responsive to the luteolytic effects of prostaglandin (PG) F-2α. Experiments have demonstrated that the CL of pregnant dogs in the late luteal phase may be more sensitive to PG-induced luteolysis than during early luteal phase (Concannon & Hansel, 1977; Paradis et al., 1983). However, dogs in general appear to be less sensitive to the luteolytic effects of exogenous PGF-2α than most domestic species. Prolonged administration of relatively

high doses, often resulting in severe side effects, is required to acheive luteolysis (Concannon et al., 1989). In many species, the uterus may be involved in regulating luteal regression (Olson et al. 1989); however, hysterectomy does not influence luteal function in the dog (Olson et al., 1984c; Okkens et al., 1985b).

In the nonpregnant bitch there appears to be no acute luteolytic mechanism (Concannon, 1995). There is a slow regression of the CL resulting in an undramatic decline of progesterone to near basal levels 60-90 days after ovulation (Concannon et al., 1989). In the bitch, it has been suggested that pregnancy is normally terminated by an acute prepartum luteolysis and abrupt fall in progesterone over the 24 hour period prior to parturition. The mechanisms involved are speculated to involve the maturation of the fetal pituitary adrenal axis, effects of fetal glucocorticoids on placenta/uterus, and release of luteolytic levels of PGF-2α into the circulation (Concannon et al., 1988, Concannon, 1995). The mechanism by which endogenous PGF-2α depresses progesterone concentrations is unknown (Concannon et al., 1989).

Artificial Insemination

As canids are generally monoestrus, detection of the time of peak fertility is critical to breeding management. As mentioned previously, for the domestic dog, oocyte maturation is believed to occur within 2-3 days of ovulation and 4-5 days after the LH surge (Concannon et al., 1989). Dog sperm have been reported to remain fertile for up to 6-7 days in the female reproductive tract (Doak et al., 1967; Concannon et al., 1983); however, the average is probably only 2-3 days, since fertility decreases in breedings which occur more than 1 day prior to the LH surge (Holst & Phemister, 1974; Holst & Phemister, 1975).

Natural mating of the bitch usually results in a conception rate of greater than 90% (Holst & Phemister, 1974; Farstad, 1984; Johnston, 1995). Vaginal artificial insemination (AI) using fresh semen can give comparable results to natural matings when the timing is right and the semen quality is high (Linde-Forsberg & Forsberg, 1989). The pregnancy

rate from frozen semen varies considerably (25%-80%; Olar, 1985; Linde-Forsberg & Forsberg; 1989; Farstad, 1984; Farstad & Anderson-Berg; 1989; Fontbonne & Badinand, 1993; Linde-Forsberg & Forsberg, 1993; Wilson, 1993) and is dependent upon a number of factors, such as site of semen deposition, timing of insemination, semen quality, sperm dose and frequency of insemination (Wilson, 1993). Repeated inseminations using frozen semen have been reported to result in higher conception rates compared to single inseminations (Farstad & Anderson, 1989; Linde-Forsberg & Forsberg, 1989; Linde-Forsberg & Forsberg, 1993). Investigators also have reported significantly lower pregnancy rates with vaginal insemination than with intrauterine deposition of frozen thawed semen (Anderson, 1972; Anderson, 1974; Smith & Graham, 1984; Olar, 1985). Therefore, a high AI success rate with frozen-thawed semen requires deposition of semen into the uterine body (Fontbonne & Badinand, 1993) by either cervical cannulation or surgical insemination. Post-thaw longevity of frozen spermatozoa is usually short-lived; therefore, insemination must be timed so that the ova present in the uterus or oviduct are mature. The peak time for AI in the domestic bitch with frozen-thawed sperm is 4-5 days after the LH peak (Battista et al., 1988; Concannon & Battista, 1989).

There are very few accounts of successful assisted reproduction in wolves. Seager et al. (1975) reported a successful pregnancy in a gray wolf using frozen-thawed semen. Timing for the 4 vaginal inseminations was based on vaginal cytology and bleeding. A single litter of red wolf pups also has been produced using fresh semen, obtained by electroejaculation, and surgical deposition into the uterine horns (Waddell & Platz, personal communication). Timing for the 2 surgical inseminations was determined through daily vaginal cytology and serum progesterone concentrations.

Male Reproductive Cycle

Endocrinology and Spermatogenesis

Pituitary gonadotropins are the major controlling mechanisms in spermatogenesis and androgen production (Eik-Nes, 1962; DePalatis et al., 1978; Kawakami et al., 1997).

In most species, regulation of FSH and LH is controlled by secretions of gonadal steroids, peptides (e.g. inhibin) and ultimately, GnRH (England, 1997). As with other species, GnRH stimulates the release of LH (Jones & Boyns, 1976; Jones et al., 1976) and a subsequent rise in serum testosterone levels within the male domestic dog (Lacoste et al. 1988; Knol et al., 1993; Purswell & Wilcke, 1993). Knol et al. (1993) were able to demonstrate a dose dependant relationship between GnRH and LH response. However the lack of relationship between GnRH and testosterone response, suggests that GnRH does not directly effect Leydig cell function in the male dog. Follicle stimulating hormone levels also have been shown to increase following the administration of a GnRH analogue; however, the response was not as dramatic as was observed for LH (Purswell & Wilcke, 1993). These authors suggest that the slow elevation of FSH over a 72 hour period may be due to a delayed reponse to the GnRH analogue or merely a normal diurnal variation. To date, very little information is available regarding FSH in the male or female dog (Concannon, 1993; Puswell & Wilcke, 1993).

In the domestic dog, castration elevates the mean concentration of plasma LH, but does not effect the rhythm of LH secretion (DePalatis et al., 1978). Luteinizing hormone secretion is episodic in the male dog with peaks occuring at intervals of 1.5 to 5 hours, followed by surges in testosterone 60 minutes later (DePalatis et al., 1978). In the domestic dog there is little information regarding the effects of gonadal steroids on suppressing spermatogenesis and sexual behaviour (England, 1997). Studies that have been conducted, using exogenous progesten and androgens, have demonstrated minimal effects upon semen quality and libido (Wright et al., 1979; Taha, 1980; Paramo et al., 1993). A recent study suggests that the sensitivity of the pituitary-hypothalamus to progestogen feedback in the male domestic dog may be different from that of other species. Progestins may have a direct action upon sperm quality by acting on the epididymal phase of development (England, 1997). In contrast, androgens have an indirect action upon sperm quality, causing the suppression of gonadotrophs (England, 1997). It also has been

demonstrated that LH secretion is inhibited more by estradiol than by testosterone (Jones et al., 1976; Winter et al., 1982) and that aromatization of androgens to estrogens in the brain regulates LH secretion (Aman, 1986).

One cycle of the seminiferous epithelium in the domestic dog has a duration of 13.6 days (Foote et al., 1972), while spermatogenesis ranges from 52-70 days (Ghosal et al., 1983; Shille & Stabenfeldt, 1986). The transit time of sperm cells through the epididymis has been estimated at 14 days (Austin & Short, 1972). The duration of spermatogenesis and epididymal transport time have also been estimated in the coyote and closely resemble that of the domestic dog (Kennelly, 1972). One cycle of the seminiferous epithelium was 13.6 days, arrival in the caput epididymis ranged from 37-40 days, and epididymal transport time was estimated at 14 days.

Seasonality

Male domestic dogs are sexually active year-round (Concannon 1991), producing sperm throughout the year with unchanged efficiency (Aman, 1986). However, Kuroda & Hiroe (1972) have reported that sperm production in the domestic dog declines during the summer. Falvo et al. (1980) also suggest the possiblity of ancestral seasonality in male mongrel dogs. Measurements of serum LH and testosterone indicated an alteration in the annual cyclic pattern of LH secretion and a significant rise in testosterone during late August/early September.

In contrast, most non-domestic canid species experience strict reproductive seasonality [red fox, Lloyd & Englund, 1973; blue fox (Alopex lagopus), Smith et al., 1987; Mondain-Monval et al., 1988; gray wolf, Mitsuzuka, 1987; raccon dog, Yongjun et al., 1994; coyote, Hamlett, 1938; Green et al. 1984; red wolf, Waddell, 1995]. There is a period, corresponding to the mating season, when spermatogenic and androgenic functions of the testes reach a maximum. Beyond this period, regression of the testes renders the male infertile. Typically full sexual capability in the male is achieved some weeks before females are receptive (Lincoln, 1989). For the female red wolf, which is annually

monestrus, receptivity towards the male is between February and March (Carley, 1979, Riley & McBride, 1972, and Waddell, 1995), a period similar to that observed in the gray wolf (Gensh, 1968, Seal et al., 1979 and Seal et al., 1987) and the coyote (Gipson et al., 1975, Hamlett, 1938 and Stellflug et al., 1981). Seasonality in sperm production has been documented through the collection of viable sperm from January to April in the gray wolf (Mitsuzuka, 1987; Seager et al., 1974), and from November to April in the coyote (Gipson et al., 1975, Green et al., 1984 and Hamlett 1938). In a study by Gipson et al. (1975), one red wolf x coyote hybrid actively produced mature sperm from November 5th-March 22nd. For all species, including the red wolf (W.Waddell, personal communication), mature sperm have been collected weeks prior to and following the suspected estrus period of the female.

Seasonal changes in the testes are dictated by the hypothalamus and the anterior pituitary gland through gonadotropin secretions. Many species have a mechanism that responds to specific cues from the environment, including changes in day length, food supply and temperature to regulate hypothalamic activity (Lincoln, 1989). Wild canids such as the gray wolf are known to reproduce seasonally. The timing of reproduction in the gray wolf varies with latitude, occurring later at higher latitudes (Seal et al., 1983; Seal et al. 1987). Fluctuations in the secretory capacity of testosterone and LH in response to LHRH stimulation were closely related to temperature and photoperiod cycles, suggesting that spermatogenesis and androgenesis are seasonally regulated (Seal et al., 1987). It also has been documented that the cape hunting dog (Cunningham, 1905) and maned wolf (Ewer, 1973) all shift their breeding season 6 months when translocated across the equator. All of these events are consistent with photoperiod synchronization. It has been well documented that the photoperiodic message within most animals is conveyed by the pineal hormone, melatonin (Bartness & Goldman, 1989; Morgan & Mercer, 1994). However, despite evidence of photoperiod influence within the canid family, it has been suggested by Asa et al. (1987) that the gray wolf may rely on a system other than the pineal for

controlling seasonal reproduction. Pinealectomy and superior cervical ganglionectomy in male and female prepubertal wolves did not alter the age at which puberty was attained. In addition, animals were monitored for a minimum of 3 years post surgery for serum levels of tesosterone/LH and progesterone/estradiol, in males and females, repectively. All wolves exhibited normal reproductive cyles when compared with sham- and unoperated animals. It also has been suggested that a prolactin rhythm may be involved in the regulation of seasonal breeding (Mirarchi et al., 1978; Schulte et al., 1981; Bubenik et al., 1985). Prolactin levels can be altered by the administration of melatonin (Adam & Atkinson, 1984). Kreeger et al. (1990) demonstrated that pinealectomy of gray wolves does not alter the prolactin circannual rhythm, while, melatonin feeding significantly depressed prolactin levels. Together, the results obsereved by Kreeger et al. (1990) and Asa et al. (1987) suggest that in the gray wolf, melatonin may be a primary messanger mediating the effect of photoperiod on reproductive events, but that the main source of melatonin is not the pineal gland.

For many species, changes in the endocrine activity of the testes in relation to cues from the environment have been observed by measuring testicular content/volume and/or serum levels of testosterone (Audy et al., 1985; Ben Saad & Bayle, 1985; Lincoln, 1989; McGuckin & Blackshaw, 1991ab; Atkinson & Gilmartin, 1992; Wieser et al., 1992; Garshells et al., 1994; Johnson et al., 1994; Kaplan & Mead; 1994). Seasonal changes in testicular size also have been recorded for some non-domestic canids including the gray wolf (Mitsuzuka, 1987) coyote (Hamlett, 1938; Gipson et al., 1975; Green et al., 1984) and blue fox (Smith et al., 1984; Smith et al., 1985). Goodrowe et al. (1998) also have recorded the testis size and combined testicular weights of red wolves. This study found that there was a decreasing trend from February and March, with patterns beyond this time period remaining unknown. In addition to testicular measurements in wild canids, seasonal variations in fecal testosterone metabolite concentration in the African wild dog (Monfort et al., 1997) and serum testosterone concentrations in the blue fox (Smith et al., 1985) have

been documented. The results in both studies indicated that peak testosterone concentrations were related to peak mating activity. It should be noted that although peak testis size and testosterone concentrations can be correlated with the breeding season, circulating testosterone levels may not always be a reliable index of normal spermatogenesis as testosterone concentrations fluctuate throughout the day (DePalatis et al., 1978). Similar testosterone concentrations can be found in domestic dogs with impaired and normal spermatogenesis (Feldman & Nelson, 1987).

Stress and Reproductive Function

All stressors, whether physical or emotional, are perceived by the central nervous system (CNS). The intimate relationship of the pituitary with the CNS suggests that pituitary hormones play a central role in an animal's strategy to cope with stress, dictating which of the physiological systems must respond to maintain homeostasis. Under acute stress, the sympathetic-adrenal medullary system is activated, resulting in the release of catecholamines and a rapid increase in arousal and muscular activity. Chronic stress stimulates the release of adrenocorticotrophic hormone (ACTH) from the pituitary, activating the adrenal cortex to release various steroids, which in turn allows for energy availability over prolonged periods (Moberg 1985).

Indicators such as behaviours and endocrine physiology frequently are used to define levels of 'stress' experienced by an animal. However, behavioural expression varies both between and within species and there are inconsistencies with regards to what is recorded by observers as stress-related behaviours. On the other hand, increases in plasma, fecal, urine and saliva concentrations of corticosteroids in wild and domesticated species have been successfully used as physiological indices for stress (Franzmann et al., 1975; Wildt et al., 1984; Hamilton & Weeks, 1985; Moberg, 1985; Wildt et al., 1986; Miller et al., 1991; Carlstead et al., 1992; Vincent et al., 1992; Morton et al. 1995; Graham & Brown, 1996; Monfort et al., 1997). Futhermore, the non-invasive nature of monitoring

fecal and urine steroid concentrations makes them particularly inviting for use in wildlife species. Longitudinal monitoring can occur without manipulation of subject animals thereby eliminating their exposure to potentially 'stressful' restraint episodes for blood sampling and allowing for a more accurate picture of endocrine status (Lasley & Kirkpatrick, 1991).

Because the neuroendocrine system is an essential component of reproduction, it is logical to assume that any response of this system to stress may influence fertility (Moberg, 1985). In the reproductive process, stress primarily affects those steps where neuroendocrine control is essential (i.e. ovulation). Therefore, a high priority for captive breeding programs is to interpret if stress impacts on a female's reproductive physiology during the peri-copulatory period and pregnancy. Although both male and female fertility may be compromised by stress, the female appears more vulnerable. Her reproductive success is centered upon a series of carefully orchestrated neuroendocrine events. If one of these events is disrupted, reproductive failure can occur (Moberg, 1985).

The negative impact which acute and chronic stress have upon the reproduction of serveral animals has been extrensively reviewed (Ramaley, 1981; Collu et al., 1984; Moberg, 1985; Liptrap 1993; deCantanzaro & MacNiven, 1992). Numerous studies have shown that environmental stressors can influence ovulation and estrous cycles in mammals. The incidence of estrus in ewes is reduced upon exposure to severe cold, wind and rain (MacKenzie et al., 1975) and dairy cows subjected to high temperature and humidity exhibit an abnormal estrous cycle (Stott & Williams, 1962). Social stress from overcrowding shortens estrous cycles in domestic bovines (MacMillan and Watson, 1971; Wagon et al., 1972). Stress associated with social status in talapoin and marmoset monkeys influences the physiological mechanisms essential for ovulation (Bowman et al., 1978; Abbott et al., 1981). The stress associated with transportation can alter the length of the estrous cycle or delay ovulation in cattle, sheep and swine (Lamond, 1962; Braden et al., 1964; Nalbandov 1964). In the rat, surgical stress and chronic restraint have been

shown to alter ovulation patterns (Schwartz, 1964; Nequin & Swartz, 1971) and delay estrus (Euker and Riegle, 1973), respectively. A new management routine can result in a drop in the ovulation rate of ewes (Doney et al. 1973, Doney et al., 1976). Together the combined evidence of these studies supports the idea that the estrous cycle is vulnerable to the influences of a variety of physical and emotional stressors (Moberg, 1985).

Although most experimental evidence indicates that folliculogenesis and ovulation are the phases of reproduction most vulnerable to stress, implantation and development of the embryo may be equally at risk (Moberg, 1985). Heat stress reduces implantation success and embryo development in ewes (Dutt, 1963) and dairy cows (Putney et al., 1988; Liptrap 1993; Ulberg, 1967). Young sows stressed by boar harassment at the time of mating exhibit higher levels of embryo mortality (Rich et al., 1986). Physical restraint (Euker & Riegle, 1973), human handling (Runner, 1959), exposure to predators (deCantanzoaro, 1988) and exposure to strange males and their odours (Parkes & Bruce, 1962) have been shown to depress pregnancy rates in rats. Overcrowding mice results in a general failure of reproduction either through imperfect (Christian & LeMunyan, 1958), delayed (Dickson, 1964) or blocked implantations (Weibold et al., 1986) in mice.

Mechanisms of Stress Impact

A physiological mechanism by which stress may influence fertility is through a disruption in gonadotropin regulation and action by influencing the hypothalmic-pituitary-adrenocortical axis (Moberg, 1985). Experiments have shown stress-induced elevations of cortisol or corticosterone for a variety of species, such as exposure to loud noise in rats (Zondek & Tamari, 1960), handling in monkeys (Elvidge et al., 1976), restraint in gerbils (Fenske, 1986), white-tailed deer (Wesson et al., 1975), impala, zebra and giraffe (Morton et al., 1995), repeated venipunctures in rabbits (Fenske, 1981), and social dominance in captive gray wolves (McLeod et al., 1996), red foxes (Hartley et al., 1994) and African wild dogs (Creel et al., 1996).

In stress related research, the administration of exogenous ACTH or corticosteroids has been used to evaluate physiological responses to stressors. It has been demonstrated that the administration of either ACTH or cortisol can block the preovulatory release of LH in cattle (Stoebel & Moberg, 1982), swine, (Barb et al., 1982) and monkeys (Moberg et al., 1982). Injections of ACTH can disrupt estrous cycles in sheep (Doney, 1976), and rats (Hagion et al., 1969) and menstrual cycles in baboons (Rowell, 1970). Exogenous ACTH or corticosteroids can also disrupt implantation and fetal development in sheep and rats (Velardo, 1957; Howarth & Hawk, 1968).

Elevations of corticosteroids frequenty are used as a measure of stress but there also are stressful conditions in which corticosteroid secretion is unaltered or the effects of stress-induced adrenal secretions are minimal (Mason, 1968). Hensleigh & Johnson (1971) demonstrated that adrenalectomy in pregnant rats did not reverse the adverse effects of heat stress. However, removal of the ovaries, followed by progesterone and estrogen replacement, eliminated any effects of such stress on fetal development. They suggested that the stressor may have a direct effect on pituitary-gonadal function, resulting in pregnancy disruption, and that the effects of adrenal secretions were only minor.

The adrenal axis also secretes sex steroids. There is extensive evidence that these steroids can have a profound effect on pituitary responsiveness to GnRH, primarily decreasing sensitivity to GnRH (Moberg, 1985). Androgens, androstenedione and dehydroepiandosterone, which are released from the adrenal during stress events (Fuller et al., 1984) can expel fertilized eggs from the female's reproductive tract (Harper, 1967; Harper 1969). These androgens are believed to act primarily through their conversion to estrogens (Fenske, 1986; deCantanzaro & MacNiven, 1992). Elevations of exogenous androgens and estrogens have demonstrated a complete disruption of early pregnancy in a variety of mammalian species (Deansley, 1963; Stone 1964; Smith & Biggers, 1968; deCatanzaro et al., 1991) and have been found to have adverse effects on the migration of fertilized ova. It also has been demonstrated that large doses of exogenous androgens and

estrogens cause acceleration of ova transport and smaller doses result in the retention of ova in the fallopian tube and subsequent degeneration (Parkes & Bellerby, 1926; Whitney & Burdick, 1936; Burdick & Whitney, 1937; Greenwald, 1965; Chang & Yanagimachi, 1965). Coinciding evidence suggests that administration of ACTH can increase endogenous estrogen levels in sheep (Strott et. al., 1975) and androgen levels in the domestic dog (Wasserman et al, 1978).

The viability of pregnancy is dependant on a specific ratio of estrogen to progesterone. These two hormones act synergistically to control the rate of embryonic travel and development through the fallopian tubes and the precise time of implantation (Smith & Biggers, 1968; Roblero, 1979; Liptrap, 1993). Any disruption of this balance can result in pregnancy loss (Gidley-Baird, 1981; Gidley-Barid et al., 1986). Therefore any disturbance in the maternal hormone enviroment by stress-induced elevation of androgens and estrogens could contribute to the early demise of pregnancy.

Red Wolf

Taxonomy

Within the order Carnivora, the family Canidae consists of approximately 35 species that are categorized into 15 genera. The genus Canis consists of 6 species, including varying species of jackals, the coyote, the gray wolf and the red wolf (Sheldon, 1992).

The first recorded description of the red wolf by Batram (1791) was in the southeastern state of Florida. However, it would not be until 1851 that the first valid scientific name for the red wolf (C lupus rufus), commonly known then as the "red Texan wolf", would be published by authors Audubon and Bachman (1851). The authors suggested that all wolves found throughout the southern United States (US) were only variations or subspecies of the commonly known gray wolf (C lupus). During the late 1800's and early 1900's significant revisions were made regarding the taxonomy of the phenotypically different "red Texan wolf". Bailey (1905) recognized the red wolf as its

own distinct species and renamed the "red Texan wolf" C rufus. Years later, Goldman (1944), who examined a large number of canid specimens in the US, determined that the red wolf found in Texas posed similar characteristics, including key cranial and dental features, to other canids in the southeastern US. Goldman therefore assigned all of the wolves of the southeastern US to one species (C rufus) and recognized three subspecies: C r rufus, the western Texas form; C r gregoryi, the central Mississippi Valley red wolf; and C r floridanus, the eastern subspecies. Goldman's classifications were generally well accepted until 1967 when authors Lawrence and Bossert (1967) suggested, based on skull measurements of several North American Canis, that measurements from red and gray wolves overlapped and that the red wolf should be considered only a subspecies of the latter. Paradiso and Nowak (1971) suggested Lawrence and Bossert's sample size was too small and by sampling a larger number of skulls they came to the conclusion that the red wolf was indeed a distinct species. However, this would not be the end of the debate regarding the taxanomic status of the red wolf.

Although some authorities consider the red wolf to be a distinct species and others consider it to be only a subspecies of the gray wolf there is a third and controversial suggestion that the red wolf is a hybrid, or cross-breed of the coyote and the gray wolf. To date, the issue regarding the evolution and taxanomic validity of the red wolf as a distinct species is still widely debated and extends far beyond the scope of this review (Atkins & Dillon, 1971; Dowling et al., 1992; Elder & Hayden, 1977; Ferrell et al., 1978; Kurten & Anderson, 1980; Nowark, 1972; Nowark, 1979; Nowark, 1992; Phillips & Henry, 1991; Roy et al., 1994a; Roy et al., 1994b; Wayne & Jenks, 1991; Wayne, 1992; Wayne & Gittleman; 1995). Despite taxonomic squabbling, the red wolf, as it exists today in captive populations is now considered by most authorities to be a true species. However, the red wolf's place in the evolutionary ladder of the Canidae family may always remain uncertain.

Historical Perspective

Very little is known about the natural history of the red wolf. Based on physical appearance it is intermediate in size between the larger gray wolf (C lupus), found in the northern and western ranges of North America, and the smaller coyote (C latrans), a historically western canid. The red wolf's most distinguishing features are its cinnamon coloured pelage, long ears and long legs. It has been suggested that the species' long slender legs were an adaptation to long distance running and pursuing prey in coastal prairies and river bottom swamps where it was especially abundant.

The red wolf was formerly distributed north to the Ohio River Valley and Pennsylvania, south to Florida and west to central Texas (Carley, 1979; Nowak, 1972, Nowak 1996). It is believed that human persecution played a major role in the steady reduction of the species' range that would eventually confine them to the coastal regions of southwestern Louisiana and southeastern Texas (Carley, 1979; McCarley & Carley; 1979, Nowak, 1972; Riley & McBride; 1972). In the early 1900's, increasing human population and subsequent changes in land use resulted in an encroachment on red wolf habitat and a decline of prey species. Red wolves were forced to approach man and agricultural lands. Deeply rooted fears and a gross misunderstanding of wolves led to indiscriminate killing and predator control programs that would eventually lead to the removal of the red wolf from its former range. It has been suggested that this was the final blow that eliminated C r floridanus around 1920 (USFWS, 1990; Goldman, 1944). Deforestation in eastern Texas and Oklahoma between 1920 and 1930, allowed for an eastern surge by coyotes. The coyote was a much more adaptable and opportunistic species than the larger, more easily caught red wolf (Pimlott & Joslin, 1968). As red wolves were being extirpated from their former habitats, coyote populations were expanding and spreading into these now uninhabited areas (Paradiso & Nowak, 1971). Human interference had created a situation that permitted interbreeding between coyotes and red wolves and as a result further diluted the population of pure red wolves (Gipson et al., 1974). Today C r gregoryii is believed to

be the only "pure" red wolf subspecies remaining, as it is believed that C r rufus became extinct in the late 1960's (Carley, 1975).

It was not until 1962 that the scientific community was informed by McCarley (1962) that the red wolf was in danger of extinction. The red wolf population continued to be persecuted by man and the ever-expanding coyote population threatened to overwhelm the species unless dramatic actions were taken (USFWS, 1990).

Recovery Plan and Reintroduction

In 1967, the red wolf was listed as an endangered species under provisions of the Endangered Species Preservations Act of 1966 and in 1973 was one of the first species to receive attention under the US Endangered Species Act. The initial recovery program that was established for the red wolf was based on information which indicated that a pure population of red wolves still existed in southeast Texas and adjacent areas of Louisiana (Pimlott & Joslin, 1968). However, field work demonstrated that a hybrid "coyote-wolf" swarm had formed and was spreading eastward (Carley, 1975). As a consequence of this finding, the recovery program was redirected from an objective of local preservation to one of planned extirpation of the species in the wild. The decision to remove the last red wolves from the wild could only be justified through the development of a long-range objective to eventually return the species to its native range.

In the fall of 1973, a formal recovery plan was completed and all remaining free-ranging red wolves were to be captured and placed in a captive breeding/certification program. To identify "pure" red wolves indicators included skull x-rays, skull length, brain to skull ratio, weight, total length, hind foot length, ear length and shoulder height (USFWS, 1990). Canids determined to be possible wolves were placed in the breeding/certification program or released with radio collars on public or private lands where landowners gave permission. Canids determined not to be red wolves were euthanized. The captive-breeding/certification program was organized through a cooperative agreement between the US Fish and Wildlife Service (USFWS) and the

Metropolitan Park District of Tacoma at the Point Defiance Zoo and Aquarium (PDZA) in Tacoma, Washington. The specific goals of the program were to: 1) determine the purity of wild wolves 2) increase the number of genetically pure red wolves in captivity and 3) maintain the gene pool for the reintroduction of the species in the wild and for distribution among zoo populations (USFWS, 1984). From the fall of 1973 to July 1980, over 400 canids were examined through the recovery program. From this pool only 43 canids met the morphological standards used to identify red wolves (Carley, 1975; McCarley & Carley, 1979). Final proof of the genetic integrity of the animals was determined by breeding experiments and examining first and second generations for evidence of hybrid litters (USFWS, 1990). In the end 14 individuals, were certified as pure red wolves. However, because of unsuccessful breedings of certain offspring, 2 genetic lines have been lost and the founding population of red wolves today is based on 12 individuals (Waddell, 1999). The last certified red wolf was removed in 1979 and the species was declared extinct in the wild by 1980 (USFWS, 1990).

In 1984, the American Association of Zoological Parks and Aquariums (AAZPA) approved the red wolf for a species survival plan (SSP©) which would involve 4 participating facilities and 63 animals. As captive husbandry techniques were refined and reproduction increased, preparations to initiate a reintroduction program progressed (Waddell, 1996). By 1987, the first reintroduction attempt was conducted at Alligator River National Wildlife Refuge in northeastern North Carolina. As of 1998, there are a minimum of 75-80 red wolves considered to be free ranging, 175 animals remaining in captivity and 33 institutions participating in the red wolf SSP© (Waddell, 1999).

RATIONALE

The red wolf recovery program is considered to be one of the Endangered Species Acts' success stories (Waddell & Henry, 1996). Since the first reintroduction of the red wolf to its former range, wild populations have continued to grow and reproduce successfully on their own. However, although red wolves have proven themselves in the wild, the entire red wolf population is founded on only 12 individuals (Waddell, 1999) and as discussed at a recent population habitat viability assessment (PHVA) meeting, red wolf hybridization with coyotes is evident among wild populations (W. Waddell, personal communication). Therefore, the tightly controlled genetic management of the captive population acts as a buffer, ensuring the future of wild populations in terms of genetic integrity and availability of individuals for future reintroduction areas.

In recent years, the growth of the captive population has slowed due to restricted reproduction as a result of a limited number of holding areas. This presents a problem for the long-term maintenance of the captive population. With limited holding areas, and an ever decreasing number of breeding age animals, the conservation of the genetic integrity of the population is at risk. Upon recommendation by the red wolf recovery team, assisted reproductive technologies such as artificial insemination and sperm banking are being investigated to help overcome this problem and to allow for more flexibility in the breeding program. By using these technologies, genetically valuable individuals could reproduce even if they are located in different areas of the country, are behaviourally incompatible or even if they are deceased. Assisted reproductive technologies have the potential to become valuable management tools, ensuring a genetically diverse population in the future.

Repeated attempts at artificial inseminations (AI) using fresh and frozen-thawed sperm have resulted in little success in red wolves. A factor impeding the potential success of AI may be the frequent restraint episodes used to acquire blood samples for hormone analysis and timing AI. It has been demonstrated in several domestic species that stress

may alter reproductive success (Liptrap, 1993). Therefore, an alternative non-invasive approach for monitoring ovarian status was desired. In addition to females, knowledge of the basic reproductive characteristics of the male red wolf is limited with the exception of three studies conducted to define semen characteristics (Koehler et al., 1994; Goodrowe et al., 1998; Koehler et al., 1998). As sperm banking is a major focus of the breeding program, a detailed endocrine evaluation defining normal annual reproductive parameters may prove to be advantageous.

In response to concerns of the red wolf recovery program, our laboratory proposed to conduct endocrine studies that would not only increase the knowledge regarding the basic reproductive physiology of both males and females but potentially enhance reproductive success. Previous studies have shown that long-term evaluation of steroid metabolite concentrations in feces is effective for evaluating ovarian, testicular and adrenal activity in numerous carnivores including the mink (Mustela vison; Möstl et al., 1993), cheetah (Acinonyx jabatus; Brown et al., 1994; Graham et al., 1995; Brown et al., 1996a), domestic cat (Felis catus; Czekala et al., 1994; Graham et al., 1995; Brown et al., 1996b; Jurke et al., 1997), clouded leopard (Neofelis nebulosa; Brown et al., 1995) maned wolf (Chrysocon brachyurus; Wasser et al., 1995; Velloso et al., 1998), and African wild dog (Lycaon pictus; Monfort et al, 1997). Compared to blood sampling, fecal monitoring permits longitudinal non-invasive studies of steroid excretion and allows for a more complete characterization of reproductive events for a given species.

The hypothesis of this study was that ovarian and testicular steroids could be quantitatively and qualitatively measured in the feces of the female and male red wolf, respectively. The first objective was to develop a non-invasive technique for monitoring ovarian and testicular steroidogenic activity in canids by validating an extraction protocol to measure fecal progestin, estrogen and testosterone metabolites by enzyme immunoassay (EIA).

The second objective was to establish a database of endocrine norms for both males and females. In general, a detailed examination of the normal patterns and their range of variation would allow for the recognition of disturbances in these patterns in future analyses. These longitudinal analyses could potentially diagnose cases of infertility, test effectiveness of contraceptive treatments or determine influences of external factors such as husbandry, nutrition and environment on reproductive function. In females, by describing endocrine changes during pregnancy and pseudopregnancy, early pregnancy detection or insight into failed conception might be possible. From a husbandry perspective, the ability to detect pregnancy would facilitate the requirement of special care for pregnant animals. In males, longitudinal monitoring would allow for investigation of the relationship between changes in fecal testosterone concentrations and photoperiod.

The third objective was to elucidate the relationship between fecal estrogen and progesterone metabolites during the periovulatory period. If fecal steroid patterns were similar to those observed in serum, fecal monitoring could be used as a reliable alternative to blood sampling for indexing ovarian function and timing AI.

The fourth objective was to develop a non-invasive technique to monitor adrenal activity in red wolves through the validation of an extraction protocol to measure cortisol metabolites by EIA. Monitoring cortisol as a measure of 'stress' may allow future investigations to determine stressful situations for the wolves. Removing or limiting these stressful situations may improve reproductive efficiency.

The fifth objective was to evaluate the potential use of fecal steroid analysis in gender determination. This technique would facilitate the tracking and reproductive monitoring of animals in the wild. Each of these goals have great potential as valuable tools towards the management of red wolf population and would improve the reproductive success for this species.

METHODS AND MATERIALS

Fecal Study Animals and Sample Collection

Animals

Intact adult (n=16, age range 4-13 years) and immature (n=1, yearling) female red wolves were maintained in facilities across the United States during the 3 year study period (1996-1998). The facilities and their relative geographical locations were: Burnet Park Zoo, NY, 43.0°N 76.1°W; Great Plains Zoo, SD, 43.5°N 96.7°W; Knoxville Zoo, TN, 36.0°N 83.9°W; Oglebay's Good Zoo, WV, 40.1°N 82.6°W; Point Defiance Zoo & Aquarium and Red Wolf Breeding Facility, WA, 47.2°N 122.5°W; Racine Park Zoo, WI, 42.7°N 87.8°W; Ross Park Zoo, NY, 42.1°N 75.9°W; Western North Carolina Nature Center, NC, 35.6°N 82.6°W.

Intact adult (n=5, age range 4-8 years) and immature (n=1, yearling) male red wolves were maintained during the the 1 year study peroid at the following institutions: Burnet Park Zoo, NY; Knoxville Zoo, TN, 36.0°N 83.9°W; Mill Mountain Zoo , VA, 37.3°N 80.0°W; Point Defiance Zoo & Aquarium and Red Wolf Breeding Facility, WA; Trevor Zoo, NY, 41.8°N 37.7°W.

Depending on space availability and red wolf SSP© breeding recommendations, wolves were housed either individually, with potential partners or in a family unit. All wolves were housed outdoors in chainlink pens (no less than 5000 square feet) which included a dry den structure. Since all animals were considered potential release candidates, a 'hands-off' policy was implemented to avoid wolf habituation to humans. Therefore, wolves were subjected to a minimal number of disturbances other than daily husbandry routines. All wolves were fed 1-2.5 pounds of a high quality commercial dry dog food 6 times a week and were fasted 1 day per week. During the whelping period, a female's food intake was monitored and additional food was provided if warranted. All wolves had ad libitum access to fresh water.

Fecal Collection

Fecal samples from females were collected during the breeding season, mid-December until the end of May (1996, 1997, 1998), on average 4 times per week from each individual during daily husbandry routines. Fecal samples from males were conducted on average 4 times a week over a 1 year period beginning mid-November, 1997.

The entire fecal sample was retrieved into a zip-lock plastic bag, labeled with the date and animal's studbook number, shipped frozen to the Toronto Zoo and then stored at -20°C until steroid extraction. In cases where a study individual could not be isolated from other wolves, fecal samples were marked with small 0.5 cm x 0.5 cm pieces of coloured surveyors tape (Hanson Co., Franklin Park, IL) or by locally purchased green vegetable food colouring. The study animal would be fed a small meatball made from ground meat which contained the labeling substance. The labeling substance was subsequently excreted enabling for animal identification.

Serum Study Animals and Sample Collection

Animals

Blood samples were obtained from intact female red wolves (n=12; age range 3-11 years) housed at the Red Wolf Breeding Facility in Graham, Washington from 1992-1997. From 1992-1997 wolves were monitored for rising progesterone levels to time AI. The husbandry and housing of all animals were the same as described above.

Blood Collection

To obtain blood samples, wolves were allowed/directed to retreat into their dens or a holding/shifting area. The entrance was blocked with a net to prevent the animal from escaping. Personnel familiar with the wolves then had controlled access into the confined area. A noose (5 foot pole, Ketch-All Company, San Deigo, CA) was slipped around the wolf's neck and the animal secured. The wolves were manually restrained and blood samples taken from the cephalic vein from late February through early March. The process

was repeated every 1 to 2 days until blood progesterone levels were found to be above 5.5 ng/ml. On average, each wolf was restrained 11 times. Blood samples (3-6 mls) were placed into red top vacutainers® (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) and allowed to coagulate for ~ 15-20 mins. Samples were then centrifuged for 10 minutes at 3500 rpm and the resultant serum fraction was removed and stored in cryogenic vials (Fisher, Fair Lawn, NJ) at -20°C until hormonal analyses.

Fecal Steroid Hormonal Analyses

Fecal Steroid Extraction

Progesterone, Estrogen and Testosterone metabolites

Method 1

Fecal progestin (P4), estrogen (E2) and testosterone (T) metabolites were extracted from all samples according to a method described by Graham et al. (1995). To ensure uniformity of hormonal metabolites within fecal samples, thawed samples were thoroughly mixed within their plastic storage bags. A wet weight of 0.5 g of feces was combined with 4.0 ml methanol, 0.5 ml distilled water and 1.0 g aluminum oxide in a clean 15 ml glass tube (VWR/Canlab, Missisauaga, ON). Samples were then futher mixed manually with a metal stir rod. A teflon-lined cap was then screwed onto the tube and the sample was vortexed for 30 seconds. Samples were mixed at room temperature for 1 hour on a rotator and then centrifuged for 10 min at 3500 rpm. The methanol fraction was decanted into a labeled 5 ml polypropylene tube (VWR/Canlab, Missisauga, ON) and stored at -20°C until hormonal analysis.

Method 2

As part of the assay validation process, exogenous P4 and E2 were extracted from fecal samples according to methods modified from Graham et al. (1995). The method was identical to that of method 1 with the exception that aluminum oxide was deleted.

Method 3

As part of the assay validation process, in addition to methods 1 and 2, exogenous steroids (P4 and E2) were extracted from fecal samples using a modified method from Shideler et al. (1993). Once frozen samples were thawed, 0.5 g wet feces were combined with 2.5 ml of extraction buffer (aqueous solution of 0.1M phosphate buffer containing 0.149 M NaCl, 0.1% BSA, 10% methanol and 0.2% Tween) in a 5 ml polypropylene tube. The mixture was homogenized by hand with a stir rod then capped and vortexed for 1 min. Tubes were placed on a rotating shaker overnight (~ 18 h) at room temperature. Following centrifugation for 10 min at 3500 rpm, the supernatant was transferred into a labeled 5 ml polypropylene tube, sealed with a cap and stored at -20°C until analysis.

Cortisol

The protocol for extraction of cortisol differed from that of ovarian and testicular steroids as it has been demonstrated that the removal of the aluminum oxide from the extraction process greatly improved corticosteroid extraction efficiency in another carnivore species (black-footed ferret: Young, 1998). To ensure sample uniformity, thawed fecal samples were thoroughly mixed within the sample bags. For extraction, 0.5 g wet feces were added to 2.5 ml of 90% methanol in a 5 ml polypropylene tube. The mixture was then mixed with a stir rod, capped and vortexed for 1 min. The sample tubes were then placed on a rotator and shaken for 2 h followed by centrifugation for 10 min at 3500 rpm. The supernatant was transferred to a clean, labeled polypropylene tube, capped, and stored at -20°C. Prior to hormone analysis, 20 µl of extract was aliquotted into 12 x 75 mm glass culture tubes (VWR/Canlab, Missisauga, ON), submersed in a 34°C waterbath and dried down under a light flow of nitrogen gas. Samples, accounting for the 1:35 dilution required for hormonal analysis, were then reconstituted in 700 µl enzyme immunoassay (EIA) buffer (0.1 M phosphate buffer, 0.1% bovine serum albumin, pH 7.0).

Enzyme Immunoassays

Fecal extracts were analyzed for steroid hormone concentrations by EIA as previously described (Munro and Stabenfeldt, 1985; Munro et al., 1991) and as adapted for use in our laboratory with wood bison and black footed-ferrets (Matsuda et al., 1996; Young, 1998; Othen, 1999). Feces collected from female red wolves (n=17) were quantified for P4 and E2 concentrations while those collected from males (n=6) were analyzed for T metabolites. As a pilot study, fecal cortisol metabolite concentrations were measured in 2 female red wolves; one female that endured repeated restraint episodes for blood sampling to time for AI and one female that was not restrained and became pregnant naturally. Randomly selected male and female fecal samples (n=960) were analyzed for P4, E2 and T metabolites for the gender determination study.

Progesterone

The polyclonal antiserum raised in rabbits to progesterone (R4861) was provided by C.J. Munro (University of California, Davis). The cross-reactivities for the antibody (Appendix II A) were provided by Munro and Stabenfeldt (1985).

A primary antibody stock of 1:20 was prepared by dilution in 50 mM sodium bicarbonate coating buffer (pH 9.6) and stored at -20°C until required. One day prior to running the assay, the primary antibody stock was further diluted in coating buffer to a working stock of 1: 6,000. Microtiter plates (96 wells) were coated with 50µl/well of working antibody stock and tapped slightly to settle the antibody. Plates were covered with an acetate plate sealer (VWR/Canlab, Missisauga, ON) to prevent evaporation and incubated overnight (14 - 18 h) at 4°C in a styrofoam container. The first column on these plates was not used because of high variability in antibody binding.

Progesterone controls, standards and sample extracts were prepared in glass culture tubes by dilution in EIA buffer immediately prior to running the assay. The progesterone standard curve ranged 2-fold from 9.75-2500 pg/well, plus '0' wells consisting of EIA buffer only. Progesterone control stock was prepared by extracting feces, using the

protocol of Schideler et al. (1993), from a pregnant lowland gorilla. The two internal controls were prepared by diluting the control stock 1:10 and 1:100. Based on ~50% binding from parallelism results, sample extracts were diluted 1:7.

After overnight incubation, the microtiter plates were rinsed 5 times with a wash solution (0.15mM NaCl, 0.05% Tween 20) in a Dynatech Ultrawash II mircoplate washer to remove unbound antibody. Plates were then patted dry to ensure the removal of excess wash solution. After washing, standards, controls and samples were loaded in 50 µl/well aliquots onto the plate. Diluted extracts and controls were pipetted in duplicate and standards in triplicate; a pair at the beginning of the plate and a third aliquot at the end to account for any time lag or drift across the plate. Once all known and unknown samples had been added, 50 µl progesterone-horseradish peroxidase (progesterone-HRP; working stock 1:60,000 dilution in EIA buffer; primary stock supplied by C.J. Munro, University of California, Davis) was added to each well. Two wells in the first column of the plate served as blanks for calibration purposes and were loaded with HRP only.

After loading, plates were covered with an acetate plate sealer and incubated for 2 h at room temperature. After incubation, unbound material was removed from the wells by washing 5 times with wash solution and patting the plates to dryness. One hundred µl of freshly prepared substrate solution [0.4 mM azino-bis (3-ethyl-benzthiazoline-6-sulfonic acid), pH 6.0 and 1.6 mM H_2O_2 in 0.05 M citrate buffer, pH 4.0] was then pipetted into each well. Plates were sealed with acetate plate sealers and slowly shaken on a flat rotator at room temperature for approximately 15-30 minutes. The optical density of each well was read using a Dynatech 700 plate reader (test filter 405 nm and reference filter 630 nm) interfaced with a MacIntosh computer. Mid-study, this plate reader was updated with a Dynex Technologies MRX plate reader interfaced with a PC computer. Standards, controls and unknown values (pg/well) obtained from reading across plates on both plate readers were comparable.

Estradiol

Fecal E2 was quantified with an EIA, using a polyclonal antibody (R4972) raised in rabbits and HRP conjugate supplied by C.J. Munro (University of California, Davis). The antibody cross-reactivities (appendix II A) were supplied by C.J. Munro (University of California, Davis).

The working antibody and HRP conjugate were diluted 1:10,000 and 1:50,000 in coating and EIA buffer, respectively. Standards were serially diluted 2-fold 1.95-500 pg/well in EIA buffer, plus '0' wells consisting of EIA buffer only. Using EIA buffer fecal samples were diluted 1:4, while internal controls were diluted 1:10 and 1:50 from the control stock. The estrogen control stock was prepared from the supernatant of extracted feces (method of Schideler et al. 1993) from a pregnant lowland gorilla. With two exceptions, the estradiol EIA was identical to the progesterone EIA: 1) following the initial wash to remove unbound antibody, plates were coated with EIA buffer 50µl/well and then incubated for 2 h at room temperature before any samples were loaded onto the plates and 2) 20 µl/well of the standards, samples and controls were loaded onto the plates.

Testosterone

The polyclonal testosterone antibody (R156/7), HRP conjugate and cross-reactivities of the antibody (appendix II A) were supplied by C.J. Munro (University of California, Davis). The antibody and HRP conjugate were diluted 1:10,000 and 1:15,000 in coating and EIA buffer respectively. Standards were prepared 2-fold, 4.8-1250 pg/well in EIA buffer, plus '0' wells consisting of EIA buffer only. Fecal samples were diluted 1:40, while internal controls were diluted 1:65 and 1:650 from stocks, all in EIA buffer. Testosterone control stock was prepared from the supernatant collected after extracting feces from male red wolves during the breeding season by extraction method 1. With one exception, the testosterone protocol is identical to the progesterone EIA; following the initial wash to remove unbound antibody, 50µl of EIA buffer were aliquoted to each well

and the plates were incubated for 30 minutes at room temperature before the addition of standards, controls, samples and HRP.

Cortisol

The cortisol polyclonal antibody (R4972), HRP conjugate and antiserum cross-reactivities (appendix II A) were supplied by C.J. Munro (University of California, Davis). The antibody and HRP conjugate were diluted 1:8500 and 1:50,000 in coating and EIA buffer respectively. Standards 3.9-1000 pg/well were prepared 2-fold in EIA buffer, plus '0' well consisting of EIA buffer only. Fecal samples were diluted 1:35 with EIA buffer, while internal controls were diluted, in EIA buffer, 1:2 and 1:4 from stocks. The control stock was prepared from a neat urine sample collected from a single male wood bison during the fall rut. This assay was conducted in a similar manner to that of the progesterone assay except plates were incubated for 1 hour at room temperature following the loading of the standard, samples, controls and HRP.

Assay Validation

Parallelism

Females

Fecal samples taken pre, during and post breeding season (6 wolves, 10 fecal samples each) were extracted (method 1) to evaluate parallelism of P4, E2 and T metabolites in the feces to a standard curve. Pooled fecal extracts (20 µl from each extract) were serially diluted 2-fold in EIA buffer to yield a range of dilutions from neat to 1:2048. The dilutions were analyzed against progesterone, estradiol and testosterone standard curves.

Fecal samples from 2 female wolves that were undisturbed (20 fecal samples each) and from 2 female wolves that were subjected to restraint episodes for blood sampling (20 fecal samples each) were used to evaluate parallelism with a cortisol standard curve. Fecal samples were extracted using the methods of Young (1998) as described earlier. The

resulting extracts were pooled (20 µl from each extract), serially diluted 2-fold neat to 1:2048 and analyzed against a cortisol standard curve.

Males

Male fecal samples (2 wolves, 50 fecal samples each) collected from November to March were extracted (method 1) to evaluate parallelism of P4, E2 and T metabolites in the feces to a standard curve. Fecal extracts were pooled (20 µl from each extract), serially diluted from neat to 1:2048 and analyzed against a progesterone, estradiol and testosterone standard curve.

In all cases, the mean percentage bindings observed from the standard curves were plotted against their expected logarithmically transformed concentrations (pg/well) on the x-axis. Mean percentage bindings observed from the diluted pooled extracts were plotted 2-fold against the transformed concentrations on the x-axis.

Recovery - Progesterone and Estradiol

To compare extraction methods 1, 2, and 3, the percent recoveries of exogenous estradiol and progesterone from fecal extracts were examined. The fecal extracts were produced by thoroughly mixing together 4 entire wet fecal samples obtained from anestrus female wolves (n=4). The pooled fecal sample was then divided, and for each method (1, 2 and 3) six 0.5 g extractions were made. The resultant supernatants from each extraction method were pooled together, producing 3 separate pools (~27 mls each, one for each extraction protocol). One ml aliquots from each of the pools were spiked with increasing amounts of exogenous progesterone and estradiol standards to obtain expected concentrations, 0 (unspiked), 19.5, 39, 78, 156, 312, 625, 1250 and 0, 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250 pg/well, respectively. Sample duplicates were then analyzed for the appropriate steroids as described above to obtain the observed pg/well value.

Extraction Efficiency - Progesterone and Estradiol

Feces collected from anestrus adult female wolves (n=4) were pooled as described above, and used to assess methods 1, 2 and 3 for exogenous steroid extraction efficiency

from fecal samples. Increasing levels of steroid standards were added to 0.5 g aliquots of pooled wet feces prior to extraction with methods 1, 2 and 3. The expected spiked concentrations of progesterone and estradiol were 0, 19.5, 39, 78, 156, 312, 625, 1250 and 0, 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250 pg/well, respectively. Sample duplicates of the fecal extracts were assayed for the appropriate steroids as previously described above to obtained the observed pg/well value.

Extraction Efficiency - Testosterone

An endogenous T source was used to assess extraction efficiency from fecal samples. The endogenous source was obtained from adult male (n=2) fecal samples during the breeding season (late February to early March). The fecal samples were extracted (method 1) and the supernatants were pooled together and analyzed for T concentration (pg/well). Because of the low T concentration in the pooled extract, three different volumes of the pooled extract were placed in glass tubes, dried down under a light flow of nitrogen gas and resuspended in an equal amount of EIA buffer allowing for expected concentrations of 155, 519 and 950 pg/well.

Feces collected from anestrus adult female wolves (n=4) were pooled and used to assess the endogenous testosterone extraction efficiency from fecal samples. Aliquots of 0.5 g of pooled wet feces were added to the 3 spiked glass tubes containing the different levels of expected concentrations along with 1 unspiked tube and allowed to sit at room temperature for 24 h prior to extraction (method 1). Sample duplicates of the extracts were assayed for the testosterone as described above to obtained the observed value pg/well.

Extraction Efficiency - Cortisol

An endogenous cortisol source was obtained from a series of fecal samples collected from manually restrained, adult females (n=4). Samples were extracted using the cortisol extraction protocol, pooled together, then serially 2-fold diluted and analyzed on the cortisol assay to determine expected concentrations of 40, 103, 167 pg/well.

Feces collected from anestrous adult female wolves (n=4) were pooled and used to assess the endogenous cortisol extraction efficiency from fecal samples. Aliquots of 0.5 g of pooled wet feces were spiked with the expected concentrations and along with 1 unspiked sample, allowed to sit at room temperature for 24 h prior to extraction using the cortisol extraction method. Sample duplicates of the fecal extracts were analyzed for cortisol metabolites as previously described above to obtained the observed pg/well value.

In all examples, the calculation of percent extraction efficiency or recovery was equal to amount observed minus the background (unspiked sample) divided by amount expected.

High Performance Liquid Chromatography

Fecal extracts (n=7) chosen for high performance liquid chromatography (HPLC) included: 1 during the follicular phase and 1 during the luteal phase from two ovulatory nonpregnant female wolves, 1 elevated cortisol sample from a female wolf and 2 elevated T concentrated samples from two male wolves.

Steroids in fecal extracts were recovered for separation on HPLC by solid phase extraction with Sep-Pak C_{18} cartridges. Cartridges were primed with 5 ml methanol followed by 5 ml distilled water. Fecal extracts were dried under nitrogen flow, resuspended in 5% methanol/water and allowed to percolate through the cartridge. The cartridge was washed with 5 ml distilled water and the steroids were eluted with 5 ml diethyl ether (unconjugated steroids) and 5 ml methanol (conjugated steroids), successively. After evaporation of the ether under nitrogen flow, the residue (unconjugated fraction) was dissolved in 100 µl acetonitrile for HPLC. Separation of steroids was accomplished by injecting a sample (25 µl for progesterone and estrogen; 50 µl for testosterone and cortisol) on a HPLC system consisting of two Water Model 510 pumps (Waters Associates Milford, MA), a WISP 710B autoinjector, and a dual channel model 441 absorbance detector. For optimum seperation of steroids, Waters Baseline 810 Controller Software was used to create a binary solvent gradient of acetonitrile-water on a

Waters Nova.Pak C_{18} (8 mm x 100 mm, 4μm) column. In total, 21 steroid standards were eluted at room temperature over 35 min at a rate of 2 ml per min. The multistep gradient mobile phase consisted of 29.5, 35, 39, and 75% acetonitrile in water at time 0, 10, 12.5, and 32 min, respectively, in the run. Retention times for each steroid were determined by injecting 20 ng of each standard, with absorbance monitored at 254nm and 280nm. Fractions (0.5 min, progesterone and estrogen; 1 min, testosterone and cortisol) were collected automatically with an LKB RediFrac fraction collector. Aliquots (200 μl) from each fraction were then dried under nitrogen gas, resuspended in an equal amount of EIA buffer and assayed in duplicate as described earlier to evaluate immunoreactivity.

Blood Steroid Hormonal Analyses

Radioimmunoassays

Appropriate radioimmunoassays (RIA) were used to determine steroid concentrations in serum samples. Blood samples collected from females (n=23) during breeding seasons (1991-1997) were quantified for progesterone, estradiol, and luteinizing hormone (LH) concentrations.

Serum progesterone and estradiol were measured using progesterone and estradiol ^{125}I RIA Kits (Coat-a-Count®, Diagnostic Products Corporation, Los Angeles, CA). All samples were analyzed at neat concentration. Methods and cross-reactivities were provided by kit literature (Coat-a-Count®, Diagnostic Products Corporation, Los Angeles, CA). Serum LH concentrations were quantified with a LH RIA developed by Dr. J.L. Brown (Brown et al., 1991; National Zoological Park Conservation and Research Centre, Front Royal, VA). All samples were analyzed at neat concentration with the exception of samples taken during the LH peak. These samples were analyzed at a 1:50 dilution in RIA buffer [In 1 L pink PBS (0.01M phosphate buffer, 0.9% NaCl, 0.01% thimerosal) add 0.05% BSA, 2 mM EDTA, pH 7.4]. Serum LH was measured using a monoclonal mouse LH antibody (518-B7) and an ovine LH standard (NIH-LH-S18) and LH label (LER-1374A).

The cross-reactivities for the antibody were supplied by Dr. J.L. Brown and are listed in appendix II B (Matteri et al., 1987).

A primary antibody stock of 1:10,000 was prepared by dilution of the antibody in RIA buffer and stored at -20°C until required for use in the assay. The LH standard curve ranged 2-fold from 0, 0.08-20ng/ml diluted in RIA buffer. Each standard dilution was stored at -20°C until required.

Just prior to conducting the assay, the primary antibody stock was diluted in RIA buffer to a working concentration of 1:1,000,000 with 1:400 normal mouse serum (NMS). After thawing, samples (in duplicate) and standards (in triplicate) were aliquoted (100 µl) to 12 x 75 mm disposable culture glass tubes followed by the addition of 100 µl of the working antibody to each tube. Duplicate glass tubes also were set aside for total counts and non-specific binding (NSB). The NSB tubes were incubated with 100 µl of 1:400 NMS diluted in RIA buffer. The tubes were agitated (~3 min) and left at room temperature overnight. The tubes then received 100 µl ^{125}I labeled ovine LH which was diluted in RIA buffer (~20,000 c.p.m./100 µl). The tubes were mixed and re-incubated overnight at room temperature. On the final day of the assay, antibody-bound complexes were precipitated by incubation for 1 hr with 1 ml aliquots of goat anti-mouse gamma globulin (GAM; prepared 1:400 in pink PBS with 5% polyethylene glycol). Tubes were centrifuged at 3000 g for 25 min, decanted (except for totals) and each counted in a gamma counter for 1 min.

Gender Determination

Fecal samples collected from males (1998; 5 profiles from 5 animals) and females (1996-1998; 15 profiles from 13 animals) from December 21st (winter solstice) to June 21st (summer solstice) were used in the gender determination study. To test the ability of fecal steroid analysis to distinguish between sexes during the selected time period, samples were divided into 4 periods (Dec. 21- Feb. 3, Feb. 4-March 20, March 21-May 5, May 6-June 21). From each wolf, four fecal samples within each period were randomly selected

and analyzed for P4, E2 and T. Six variables (P4, T, E2, P4/E2, P4/T, E2/T) were compared within each time period among 4 classes (males n=5, pregnant n=3, ovulatory nonpregnant n=9 and nonovulatory females n=3) using factorial ANOVA (Statview version 4.5, Abacus Concepts, Inc., Berkley CA).

Data Evaluation

Fecal Profiles

Females were assigned to 1 of 3 classes according to the occurrence of birth (pregnant; P) and presence (ovulatory nonpregnant; NP) or absence (non-ovulatory or acyclic; AC) of a continued rise in P4, which presumably reflected ovulation. Profiles from individual female red wolves (P, NP) were aligned to a rise in P4 (day 0) from baseline values using the following criteria: a baseline value for each wolf was determined by calculating each individual's mean P4 value from samples collected mid December until mid February. Day 0 was recognized as the value that was elevated above baseline by 2 standard deviations (SD), with subsequent levels remaining elevated for a minimum of 3 consecutive samples. For AC animals, day 0 was assigned based on the location of the individual animal (Southeastern, Northeastern and Northwestern United States) and the mean estimated date of estrus for all wolves in that location (Waddell, 1995). Since fecal samples were not always available on a daily basis, fecal hormone levels were pooled into 3 day means. Reproductive profiles were then divided into 9 cycle stages: pre-ovulatory (days -30 to -13), periovulatory (days -12-8), luteal phases (days 9-17, 18-26, 27-35, 36-44, 45-53 and 54-65) and post (66-71). Comparison of P4 and E2 levels within each cycle stage between the 3 classes (P, NP, and AC) was determined by ANOVA with repeated measures using Statview (version 4.5, Abacus Concepts, Inc., Berkley CA) on a Macintosh computer. Comparison of P4 and E2 levels across all cycle stages within each class (P, NP, and AC) were conducted using a factorial ANOVA analysis (Statview version 4.5, Abacus Concepts, Inc., Berkley CA). All p values reported are derived from

the Fisher's protected least significant difference test. Descriptive data are reported as means ± SEM within a cycle stage.

During the periovulatory period, daily mean P4 and E2 values from NP wolves were compared to basal concentrations using a Student's t-test. Insufficient sampling from P animals did not permit the analysis of daily means. Descriptive data are reported as daily means ± SEM.

Over the 1 year sampling period, male fecal T levels were pooled into weekly means. Comparisons among weekly means and daylength were determined using correlation analysis (Statview version 4.5, Abacus Concepts, Inc., Berkley CA). Descriptive data are reported as weekly means ± SEM. Daylength data was downloaded from the The United States Naval Observatory (www.usno.navy.mil/home.html).

Blood Profiles

As baseline progesterone values in red wolves have never been characterized, alignment of data was determined in a similar manner to that of fecal samples. Individual serum profiles were aligned to a rise in progesterone (day 0). Because of the small number of pre-ovulatory samples, day 0 for each individual was the value that was elevated above a mean baseline (mean progesterone concentration from all wolves February 2nd-22nd) by 2 SD, with subsequent levels remaining elevated for a minimum of 3 consecutive samples. Luteinizing hormone and estradiol profiles were normalized by aligning the serum hormone values of individual animals to the day of progesterone rise. A Student's t test was employed to detect a significant elevation in daily means above basal concentrations of progesterone, estradiol and luteinizing hormone. Values are reported as daily means ± SEM.

RESULTS

Validation of Fecal Assays

Parallelism

The curves generated from the serially diluted pooled fecal extracts from females for P4, E2, T and cortisol were parallel to their respective standard curves (Figures 1a, 2a, 3a, and 4). Serial dilutions of fecal extracts from males gave displacement curves parallel to the standard curves for P4, E2 and T (Figures 1b, 2b, and 3b).

Recovery - Progesterone and Estradiol

The mean percent recoveries of an exogenous source of progesterone from fecal extracts for extraction methods 1, 2 and 3 were 71.2% (range 62.8%-78.5%), 40.9% (range 25.6%-48.8%) and 60.8% (range 58.0%-88.9%), respectively. For estradiol, the mean percent recoveries for the 3 protocols were 78.5% (range 64.1%-92.9%), >100% (range 90.4%-118.3%) and >100% (range 128.0%-173.1%), respectively.

Extraction Efficiency - Progesterone and Estradiol

For progesterone, the mean percentages of exogenous hormone detected from fecal samples for extraction methods 1, 2 and 3 were 77.1% (range 32.0%-89.9%), 64.6% (range 59.8%-71.6%) and 14.9% (range 7.7%-23.1%), respectively. For estradiol the mean extraction efficiencies for the 3 protocols were 76.9% (range 64.1%-92.9%), 74.1% (range 64.0-89.7%) and 31.6% (range 12.2%-51.3%), respectively. Based on the results of the recovery and extraction efficiencies for progesterone and estradiol, extraction method 1 was used to monitor ovarian activity.

Extraction Efficiency - Testosterone and Cortisol

The testosterone assay detected a mean of 94.8% (range 92.2%-95.4%) of the endogenous T added to fecal samples. The mean extraction efficiency of an endogenous source of cortisol from fecal samples was 93.8% (range 85.6%-95.4%).

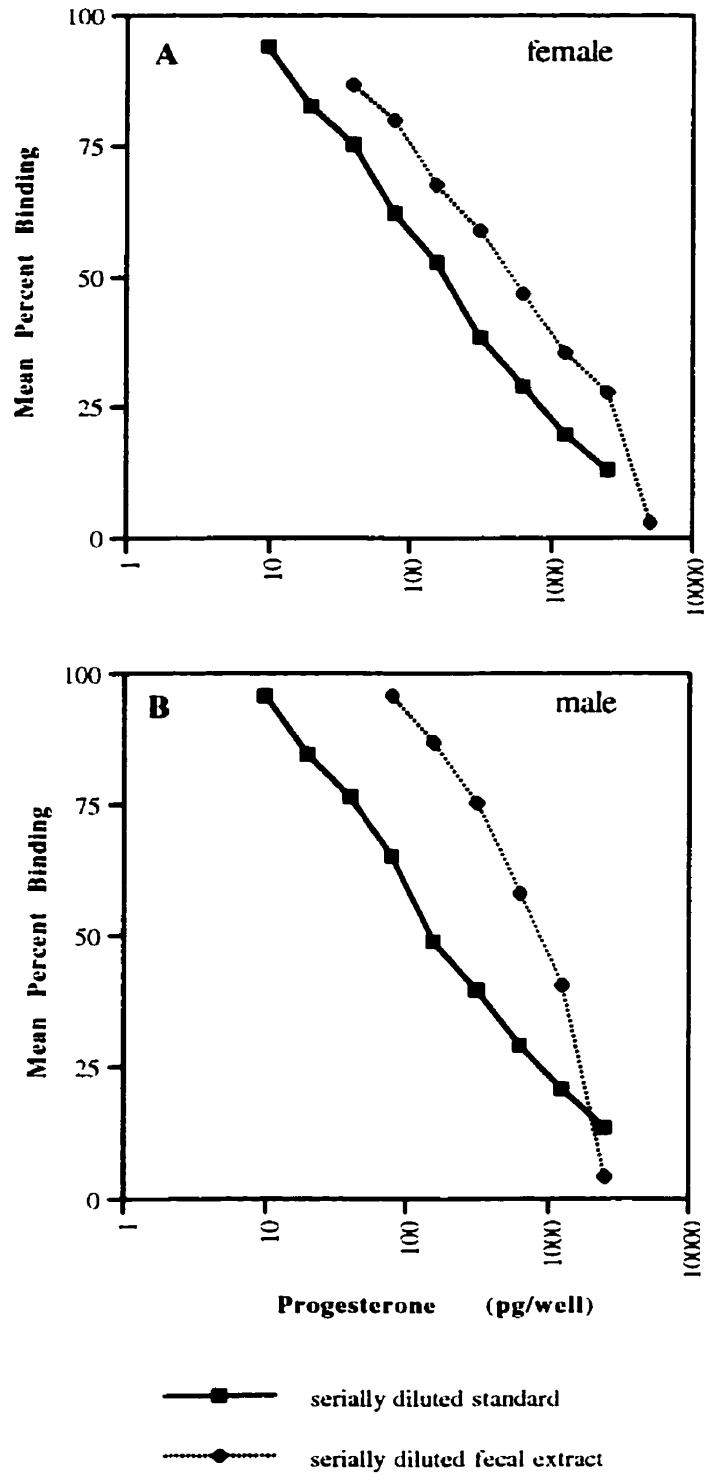


Figure 1. Parallelism curves resulting from anaylsis of serially diluted progesterone standards and pooled fecal extracts from female (A) and male (B) red wolves.

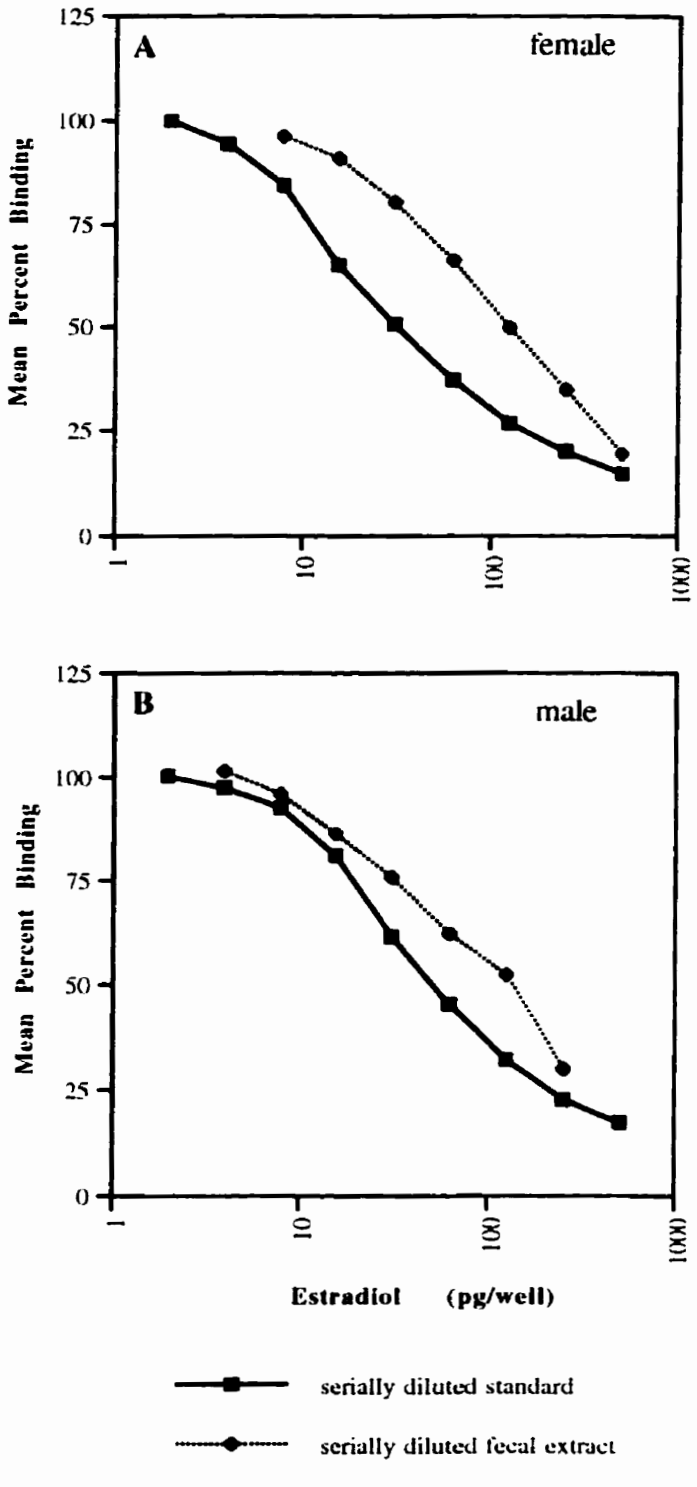


Figure 2. Parallelism curves resulting from anaylsis of serially diluted estradiol standards and pooled fecal extracts from female (A) and male (B) red wolves.

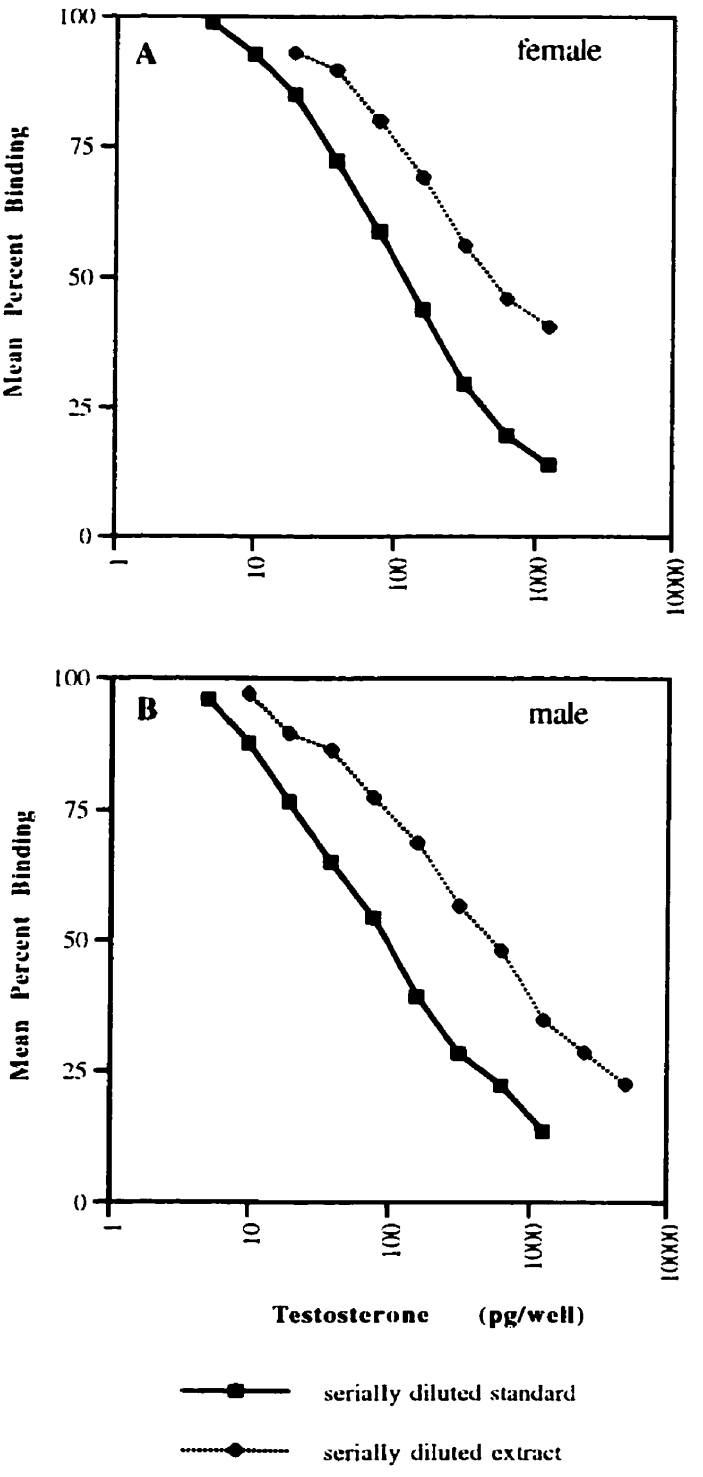


Figure 3. Parallelism curves resulting from anaylsis of serially diluted testosterone standards and pooled fecal extracts from female (A) and male (B) red wolves.

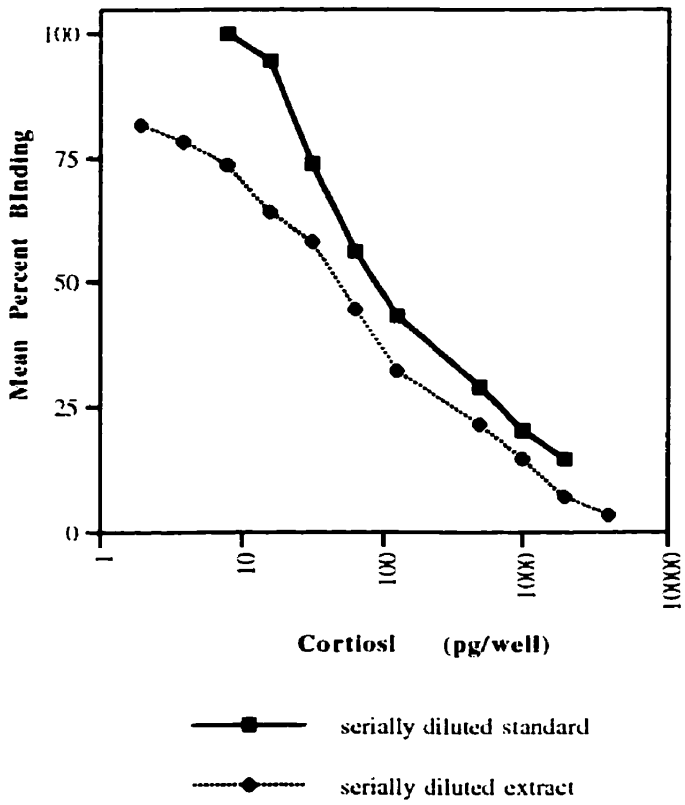


Figure 4. Parallelism curve resulting from anaylsis of serially diluted cortisol standard and pooled fecal extracts from female red wolves.

Assay variation

Assay variation was monitored using 4 individually prepared lots of controls for each EIA type. The inter-assay coefficients of variation (CV) for the progesterone assay were 12.78% and 7.64% (n=86), at mean percent bindings of 32.4% and 67.1%, respectively. The intra-assay CV were 5.2% (n=12) and 7.9% (n=12), at mean percent bindings of 30.1% and 62.1%, respectively.

For the estradiol assay, the inter-assay CV were 13.4% and 16.0% (n=85), at mean percent bindings of 42.3% and 77.1%, respectively. The intra-assay CV were 3.2% (n=12) and 3.6% (n=12), at mean percent bindings of 40.1% and 75.4%, respectively.

For testosterone, the inter-assay CV were 13.7% and 13.7% (n=49), at mean percent bindings of 24.7% and 67.0%, respectively. The intra-assay CV were 9.3% (n=12) and 5.6% (n=12), at mean percent bindings of 23.4% and 60.8%, respectively.

The inter-assay CV of the cortisol assay were 11.1% and 8.9% (n=12), at mean percent bindings of 52.6% and 69.8%, respectively. The intra-assay CV were 2.1% (n=12) and 4.9% (n=12), at mean percent bindings of 50.0% and 72.0%, respectively.

High Performance Liquid Chromatograpy

Progesterone/Estradiol

The resultant P4 immunoreactive profiles of fecal extracts subjected to HPLC from nonpregnant females during the follicular and luteal phases of the estrous cycle are illustrated in Figures 5a and 5b, respectively. Using this HPLC system, progesterone is known to elute within fraction 32 (Figure 8). There were several unconjugated immunoreactive peaks measured by this antiserum; however, the majority of immunoreactive metabolites were found to be less polar progesterone (fractions 33 to 35) in both the follicular and luteal phase. Several minor immunoreactivity peaks were observed in both phases within fractions more polar than progesterone.

The resultant E2 immunoreactive profiles of fecal samples subjected to HPLC from nonpregnant females during the follicular and luteal phases, are illustrated in Figures 6a and

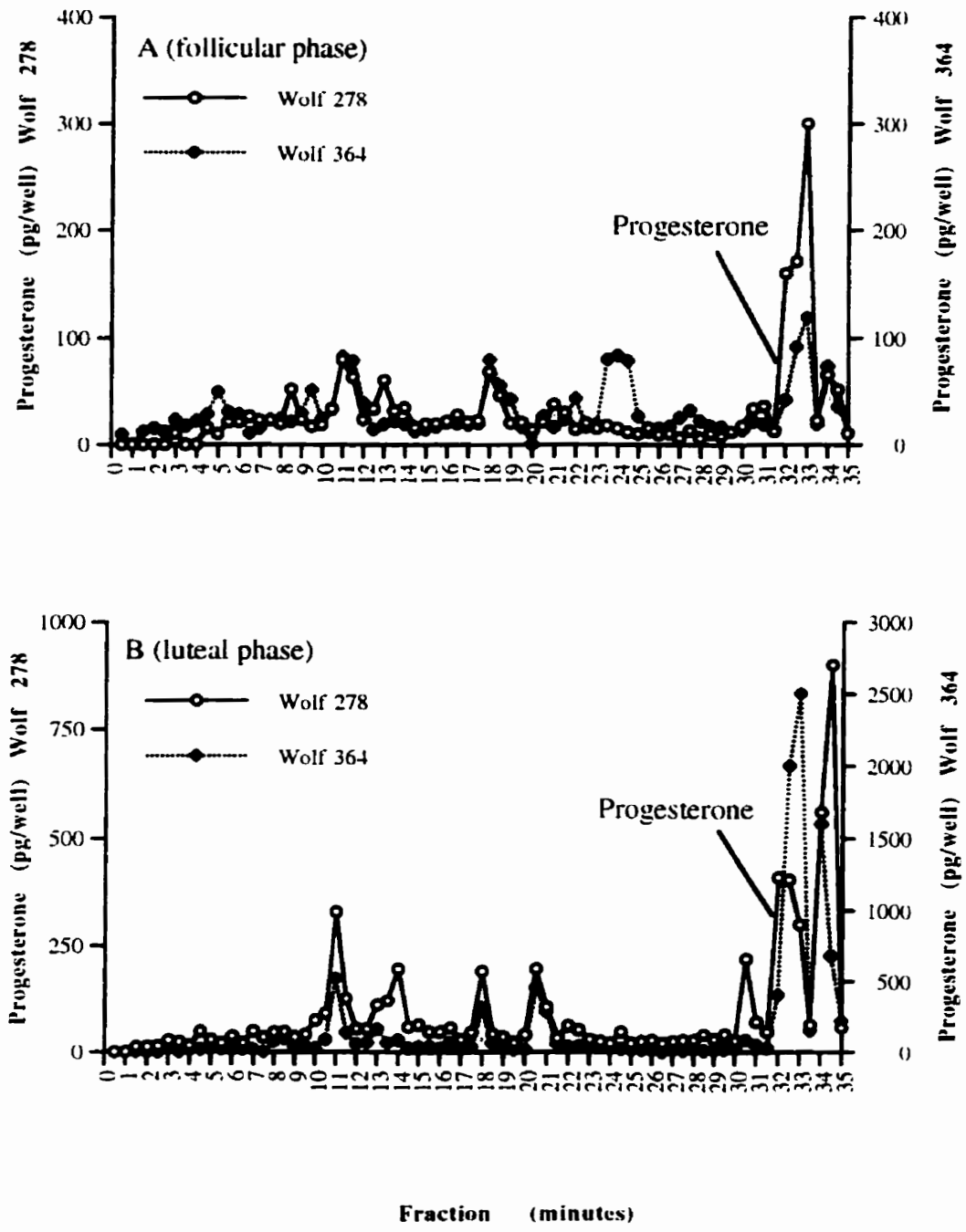


Figure 5. Immunoreactive profiles of fecal extracts from female red wolves during the follicular (A) and luteal (B) phases after fractionation with HPLC and EIA of the fractions obtained with progesterone antiserum (R4861).

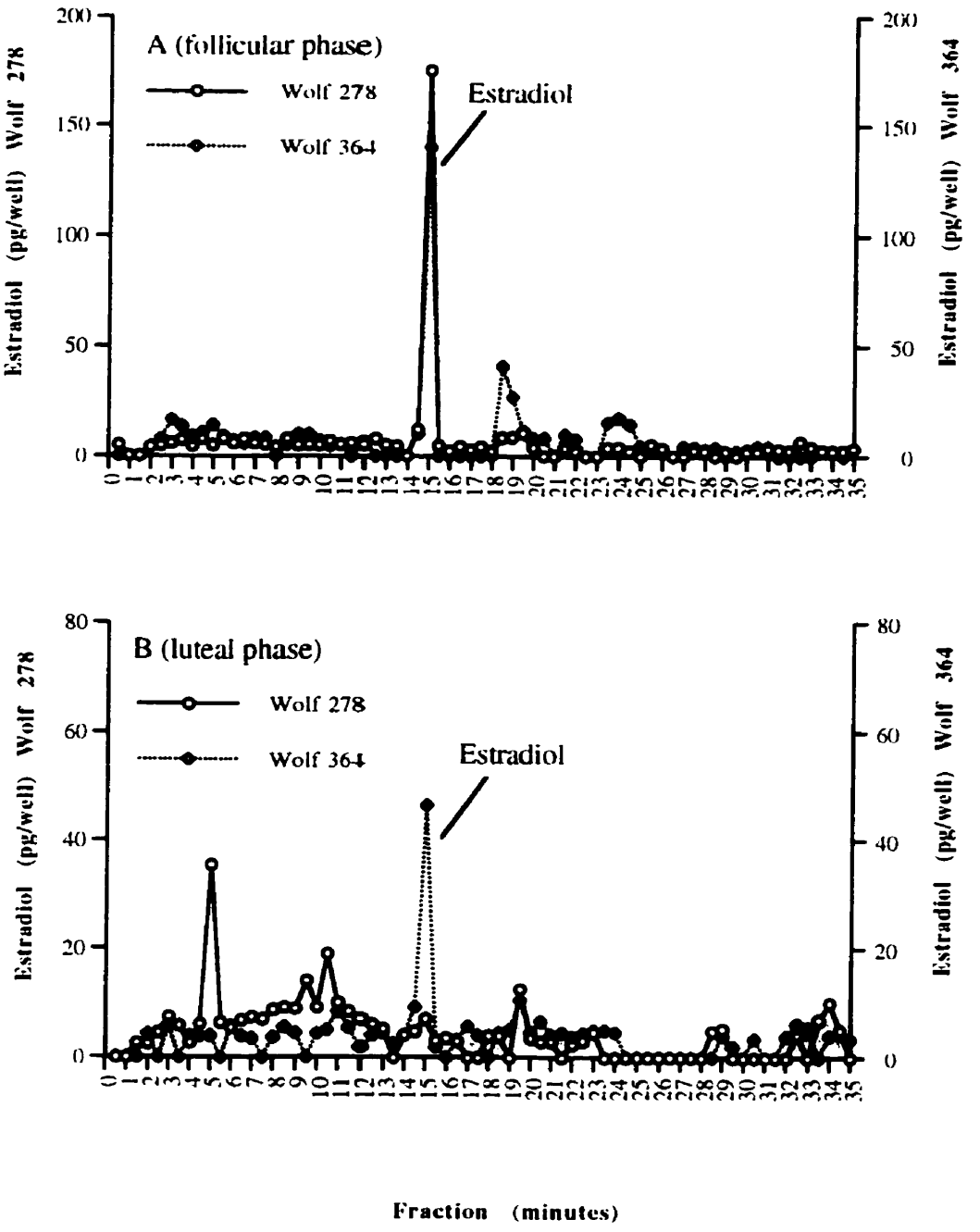


Figure 6. Immunoreactive profiles of fecal extracts from female red wolves during the follicular (A) and luteal (B) phases after fractionation with HPLC and EIA of the fractions obtained with estradiol antiserum (R4972).

6b, respectively. The resultant profiles revealed a large unconjugated immunoreative peak in 3 of the 4 profiles that had a retention time comparable to estradiol in the standard (Fraction 15; Figure 8). In addition, minor immunoreactivity was detected within fractions known to have a similar retention time as estrone in the standard (Fraction 20; Figure 8). The profile from wolf 278 (Figure 6b) suggested that no estradiol was present. This is not unexpected, as the fecal sample was obtained during the luteal phase, however we have no explanation for the large immunoreactive peak that was observed within fraction 5.

In general, with exception of relative amounts of immunoreactivity, the progesterone and estrogen immunoreactive profiles obtained did not dramatically differ between the follicular and luteal phases. Minor individual variation between profiles did exist. However, with the exception of the estrogen profile obtained during the luteal phase from one wolf, the patterns of immunoreactivity detected within profiles were similar between animals.

Cortisol

The resultant cortisol immunoreactive profile of an elelvated cortisol fecal sample subjected to HPLC from a female red wolf is illustrated in Figure 7a. Fraction 6 was comparable to cortisol in the standard (Figure 8). Analysis of the HPLC fractions on the cortisol EIA illustrated a large immunoreactive peak more polar than cortisol (fraction 4-5) and a minor peak less polar than cortisol (fraction 9).

Testosterone

Two elevated T concentration fecal extracts obtained from male wolves during the breeding season were subjected to HPLC. The resulting fractions analyzed with the T EIA revealed several immunoreactive peaks (Figure 7b). Fraction 17 was comparable to testosterone in the standard (Figure 8). The largest amounts of immunoreactivity were detected in peaks less polar than testosterone (between fractions 19 and 27). Two small unidentified immunoreactive peaks also were observed in fractions more polar than testosterone.

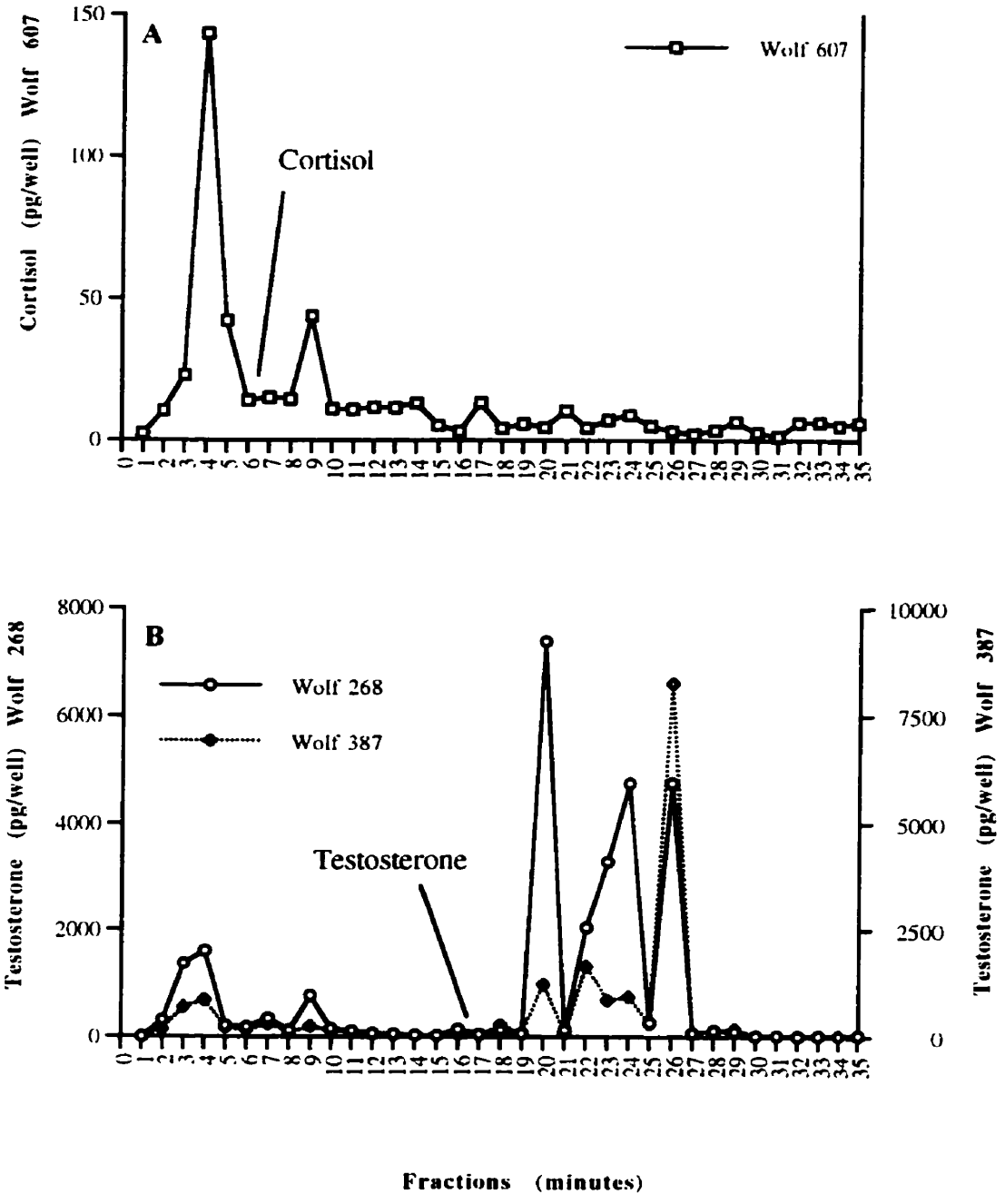


Figure 7. Immunoreactive profiles of fecal extracts from (A) a female red wolf after fractionation with HPLC and EIA of the fractions obtained with cortisol antiserum (R4972) and (B) male red wolves after fractionation with HPLC and EIA of the fractions obtained with testosterone antiserum (R156/7).

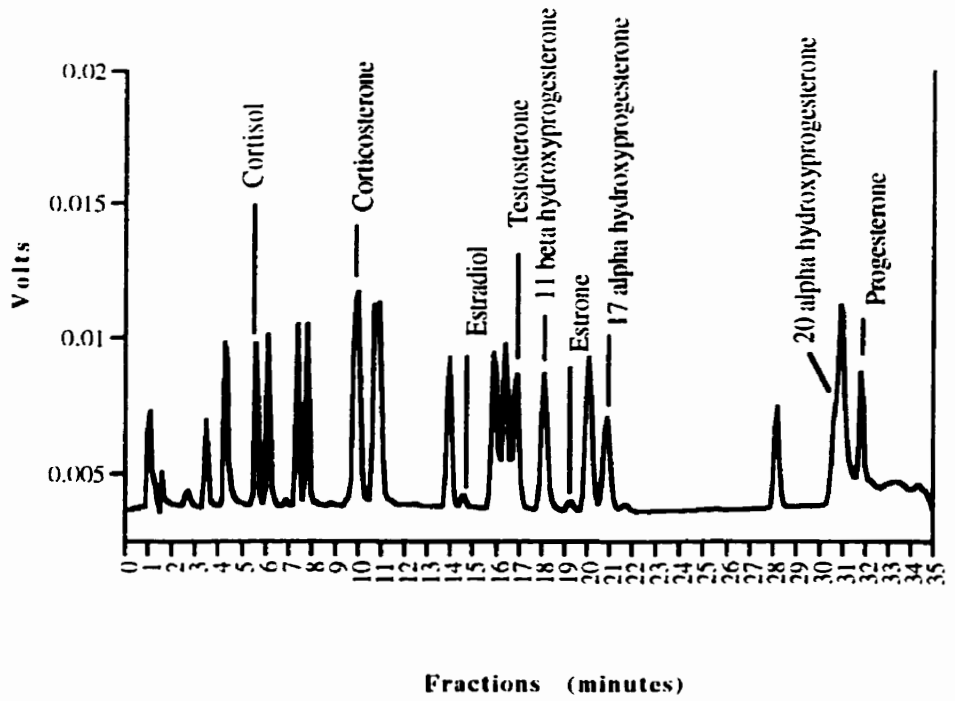


Figure 8. Chromatograph of 21 purified steroid standards using reverse phase high performance liquid chromatography.

Female Profiles

Longitudinal Fecal Profiles

Concentrations of fecal P4 and E2 metabolites were analyzed for 19 cycles (n=17 animals). Of the 19 cycles examined 3 were eliminated due to poor sample collection and therefore could not be classified. Figure 9 illustrates the longitudinal 3 day mean composite fecal P4 and E2 excretory profiles for pregnant (P; n=3), ovulatory nonpregnant (NP; n=9) and nonovulatory or acyclic animals (AC; n=3). Data are aligned by a rise in P4 (day 0). The ovarian steroids and cortisol concentrations during the reproductive cycle of two female wolves, one which endured repeated restraint episodes (wolf 607) and one that did not (wolf 545) appear in Figure 10.

Within Classes of females

Mean P4 and E2 values (Table I) were compared across cycle stages within each class (P, NP, and AC) based on factorial ANOVA analysis. Progestins remained at low levels in all females sampled until the periovulatory period, when a steep increase in P4 levels was observed in the profiles of cycling (P and NP) animals. During the luteal phase, mean P4 levels in P (range, 49.1-2961.0 ng/g feces; $p \leq 0.003$) and NP (range, 52.9-3541.0 ng/g feces; $p \leq 0.006$) animals remained significantly elevated above baseline levels (pre) until the mid- to late luteal phase. Mean P4 concentrations reached maximum values later in P females (days 18-26) than NP females (days 9-17). Following day 54, mean P4 levels were not significantly different from baseline levels in either P ($p=0.347$) or NP ($p=0.190$) animals. Mean P4 AC concentrations during the different cycle stages did not significantly differ from mean AC baseline values ($p \geq 0.060$).

Mean E2 levels in P animals were significantly elevated above mean P baseline levels during the periovulatory period ($p=0.034$), days 27-35 ($p=0.007$) and days 36-44 ($p= 0.041$). Mean E2 NP levels were significantly elevated above mean NP baseline levels during the periovulatory period ($p<0.001$) and days 27-35 ($p=0.014$). Mean AC E2

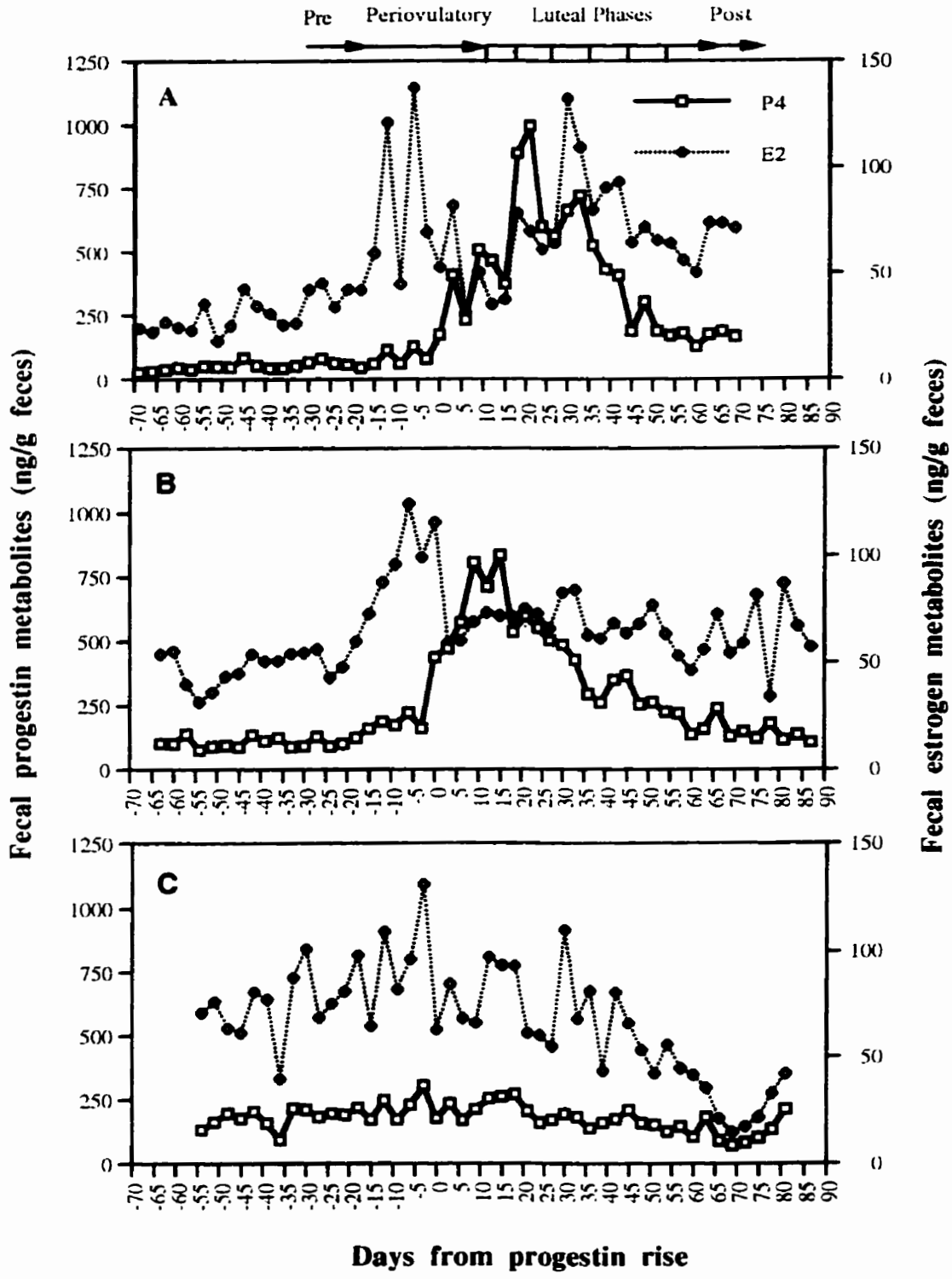


Figure 9. Mean longitudinal profiles of fecal progestin and estrogen metabolite concentrations for the three female reproductive classes: pregnant (A; n=3), ovulatory nonpregant (B; n=9) and acyclic (C; n=3) red wolves.

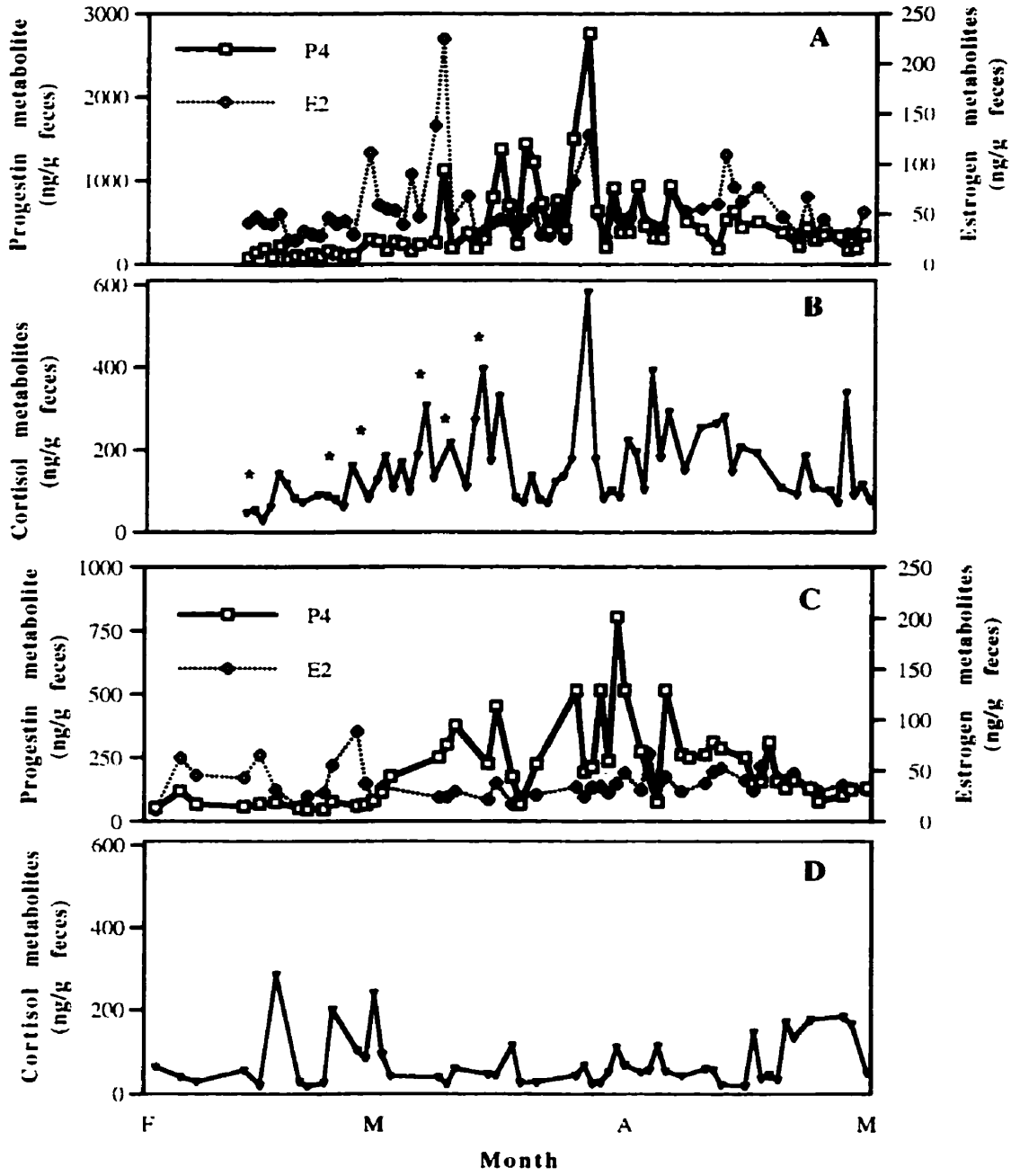


Figure 10. Longitudinal fecal progestin, estrogen (A,C) and cortisol (B,D) metabolite profiles from 2 female red wolves. Wolf 607 (AB) experienced restaint episodes for serum sample collection to time for artifical insemination. Asterisks indicate restraint episodes and blood samples taken. Wolf 545 (CD) was not restrained and became pregnant naturally.

	Day	P4	SEM	E	SEM
Pregnant					
Pre	-30 to -13	65.7	6.6	44.6	5.6
Periovulatory	-12 to 8	163.5	28.0	76.6	12.9
Luteal	9 to 17	409.9	93.8	38.4	6.5
	18 to 26	680.2	181.3	60.1	12.2
	27 to 35	581.5	111.3	91.9	19.0
	36 to 44	435.7	51.7	80.3	13.8
	45 to 53	223.2	42.5	66.4	6.6
	54 to 65	158.5	13.9	61.7	7.1
	66 to 71	178.0	10.6	72.5	1.2
Ovulatory nonpregnant					
Pre	-30 to -13	116.5	8.2	56.5	2.6
Periovulatory	-12 to 8	302.2	29.5	98.9	9.7
Luteal	9 to 17	705.7	97.0	68.6	5.2
	18 to 26	541.8	63.4	73.9	6.6
	27 to 35	492.0	47.2	83.1	7.8
	36 to 44	317.6	38.5	66.5	4.0
	45 to 53	288.2	33.9	70.6	7.5
	55 to 65	197.3	20.6	56.7	4.6
Post	66 to 71	163.3	37.6	61.3	8.7
Nonovulatory					
Pre	-30 to -13	218.2	24.9	94.8	12.5
Periovulatory	-12 to 8	213.6	22.5	92.9	11.8
Luteal	9 to 17	266.7	32.8	107.1	24.8
	18 to 26	251.1	69.5	90.1	22.0
	27 to 35	188.6	19.5	88.4	23.2
	36 to 44	152.6	17.9	72.9	17.5
	45 to 53	170.6	18.7	63.1	13.4
	54 to 65	133.9	20.1	45.7	7.9
Post	66 to 71	81.6	8.2	18.1	3.3

Table I. Mean ± SEM fecal progestin and estrogen metabolite concentrations for all cycle stages, pre-ovulatory (days -30 to -13), periovulatory (days -12-8), luteal (days 9-17, 18-26, 27-35, 36-44, 45-53 and 54-65) and post (66-71) for pregnant, ovulatory nonpregnant and nonovulatory red wolves.

concentrations from all cycle stages did not significantly differ from mean AC baseline values ($p \geq 0.066$).

Between Classes of females

Concentrations of P4 and E2 within each cycle stage were compared between the 3 classes (P, NP and AC) of females by ANOVA with repeated measures. During the pre-ovulatory period (days -30 to -13) P4 concentrations between P and NP wolves were relatively low and did not differ ($p=0.234$, Figure 9). Progesterone concentrations from AC animals during this time period were elevated in comparison to P and NP wolves ($p \geq 0.017$). Fecal progestin concentrations did not significantly differ between P and NP animals during any stage of the luteal phase (days 9-65: $p \geq 0.270$). Progesterone values remained elevated above AC values longer in P animals (days 36-44: $p=0.035$) than in NP animals (days 27-35: $p=0.017$). Insufficient samples precluded comparison of post-samples.

Fecal estrogen metabolite concentrations during the pre-ovulatory phase (days -30 to -13) did not differ between NP and P animals ($p=0.824$). However, during the pre-ovulatory phase, E2 concentrations in AC animals were significantly greater than E2 values in NP animals ($p=0.034$) but not P animals ($p=0.088$). During the periovulatory and luteal phase (days -12-65) E2 concentrations did not significantly differ among the three classes (P, NP and AC) of females ($p \geq 0.130$). Similar to P4, insufficient samples precluded comparison of E2 post-samples.

In addition to the analysis of adult wolves, 1 yearling wolf, housed within a family unit (with mother, father and 2 brothers) was examined for evidence of cyclicity. Appendix III A(a) illustrates that the young wolf did experience a surge in E2 followed by a dramatic rise in P4. Concentrations of P4 and E2 were within ranges observed for cycling adult females.

Periovulatory Profiles

For consistency, days -16 to -12 were used as baseline values for comparison between daily means in both fecal and serum profiles [day -16 corresponds to the earliest samples collected in the baseline time frame for serum samples (February 2nd-22nd)].

Fecal

In addition to the calculation and statistical analysis of 3 day means, daily mean P4 and E2 concentrations were calculated for 9 cycles (n=7 NP animals) to more specifically evaluate endocrine trends occuring in the periovulatory period (Figure 11). Limited data from P females did not allow for the calculation of daily means during the periovulatory period. Progesterone metabolite values rose steadily above mean basal values (days -16 to -12: 142.2 ± 15.6 ng/g feces; range 37.8 - 453.6 ng/g feces) from day 0 (399.7 ± 89.1 ng/g feces; range 222.2 - 1033.2 ng/g feces; p=0.010), reaching maximum daily mean concentrations by day 16 (1157.8 ± 348.0 ng/g feces).

Basal E2 values (day -16 to -12) ranged from 25.4 - 153.0 ng/g feces (mean 72.2 ± 6.1 ng/g feces). Daily mean E2 values demonstrated a gradual increase from day -12, achieving peak levels at day -5 (187.20 ± 51.89 ng/g feces), followed by a gradual decline from days -4 to 3. However, at no point were daily mean E2 values significantly elevated above mean basal E2 values ($p \geq 0.068$). Of the 9 cycles examined, 6 exhibited a single marked elevation in E2 [Day -7 (n=1), Day -6 (n=3), Day -5 (n=1) and Day -4 (n=1)] with peak values ranging from 169.9 - 1032.9 ng/g feces.

Serum

Daily mean concentrations of serum progesterone (P4s), estradiol (E2s) and luteinizing hormone (LH) were calculated and analyzed for 23 cycles from 12 NP animals. Figure 12 illustrates the endocrine relationship of P4s and E2s to LH. Lack of data on specific days (days -7 and -1) was due to infrequent sample collection and small amounts of serum obtained. In all cases, priority was given to P4s analysis.

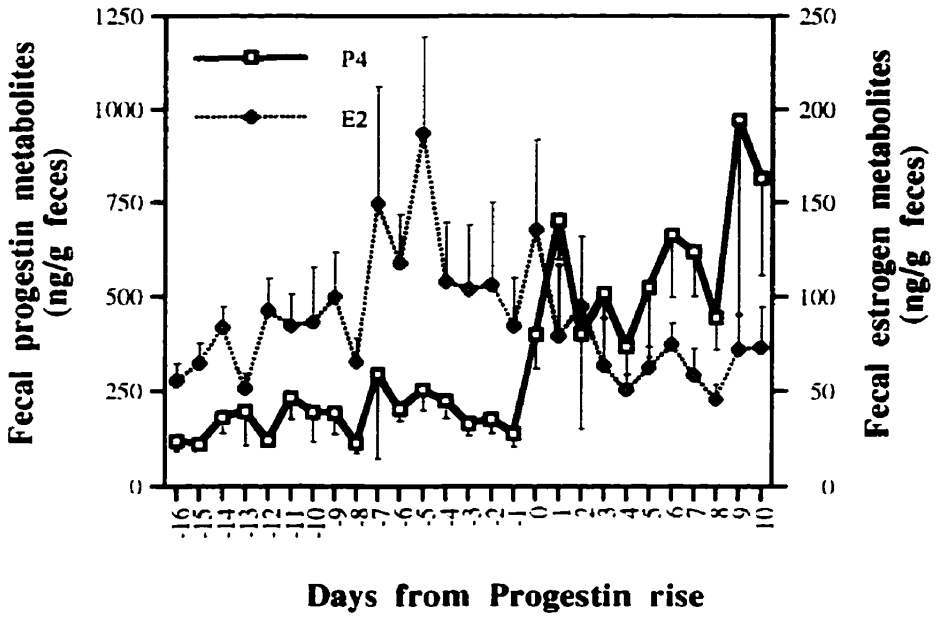


Figure 11. Daily mean +/-SEM (n=9 cycles) fecal progestin and estrogen metabolite concentrations from ovulatory nonpregnant female red wolves (n=7).

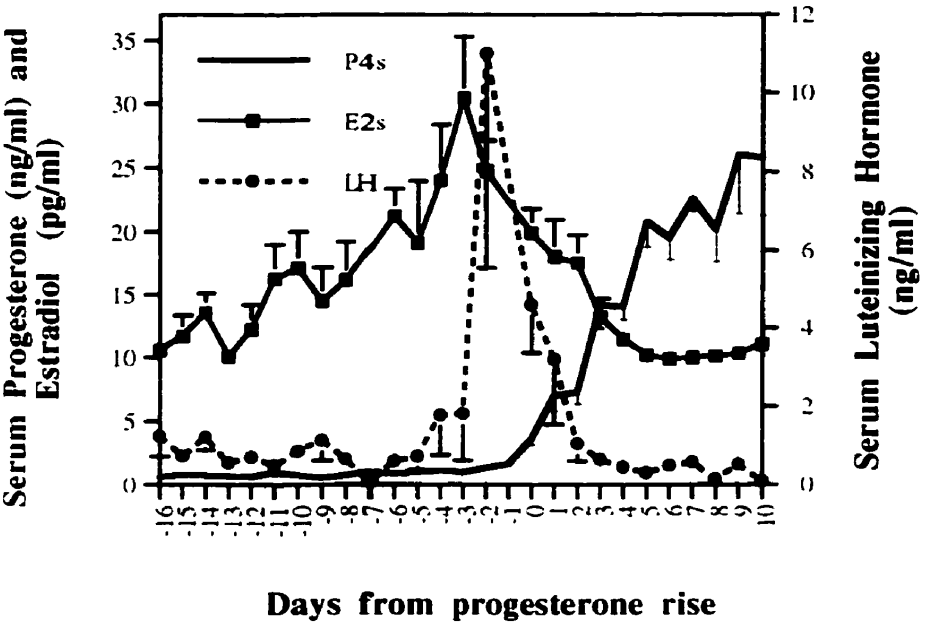


Figure 12. Daily mean +/-SEM (n=23 cycles) serum progesterone, estradiol and luteinizing hormone concentrations for ovulatory nonpregnant female red wolves (n=12).

The mean serum profile demonstrated that there was minimal variation in mean baseline P4s concentrations values (days -16 to -12; 0.7 ng/ml ± 0.1 ng/ml, range 0.2-1.1 ng/ml). Despite the initial alignment of each individual profile to a rise in P4s [day 0 = basal values (P4s values from all wolves February 2nd to 22nd) + 2 SD], statistically, the first significant increase in mean P4s concentration from mean baseline concentrations (days -16 to -12) occurred at day -2 (1.3 ± 0.1 ng/ml; $p<0.05$), which corresponded with the onset of the LH surge. Serum progesterone levels rose sharply until day 5 (20.8 ± 2.0 ng/ml), and ranged from 8.9 - 33.6 ng/ml between days 6-10. The greatest mean value measured was on day 9 (26.0 ± 4.6 ng/ml).

Individual basal E2s values (days -16 to -12) ranged from 10.0-20.1 pg/ml (mean 11.9 ± 0.7 pg/ml). Estradiol began an apparent rise on day -11. Daily mean E2s concentrations continued to gradually increase and were significantly greater by day -4 ($p<0.05$) when compared to basal values. Significant elevations of E2s above basal values were detected over an ~ 7 day interval (day -4 to 2; $p<0.05$) with a peak mean value of 30.4 ± 4.8 pg/ml measured on day -3. Possibly due to infrequent blood sampling, only 13 of the 23 cycles examined exhibited a marked elevation in E2s. Examination of these 13 individual cycles revealed that E2s peaked on day -6 (n=2), day -5 (n=1), day -4 (n=2), day -3 (n=3) and day -2 (n=5) with values ranging from 17.9 - 52.1 pg/ml. Mean estradiol values declined coincidentally with the onset of the LH surge. By day 3, mean E2s concentrations returned to basal values and remained at nadir concentrations through until day 10 ($p>0.05$).

Baseline LH concentrations (days -16 to -12) ranged from 0.3-3.2 ng/ml (mean 0.9 ± 0.1 ng/ml). The first significant rise from baseline was observed on day -2 ($p<0.05$) reaching a peak mean value of 11.0 ± 5.5 ng/ml. Daily mean LH values remained above baseline until day 1 ($P<0.05$). From day 2 through day 10, daily mean LH levels remained at nadir levels ($p>0.05$). Of the 23 cycles examined, 15 exhibited a peak in LH with

values ranging from 4.0 - 70.7 ng/ml. Individual peaks occurred on day -4 (n=3), day -2 (n=6) and day 0 (n=6).

Male Profile

Concentrations of fecal T were analyzed for 5 intact adult male red wolves. Figure 13 illustrates the weekly mean longitudinal fecal T excretory profile over a 1 year period. Between mid-spring and early autumn, T concentrations were at their lowest values (May - late October: mean 103.1 ± 6.3 ng/g feces; range 3.6-507.7 ng/g feces). Levels increased above mean baseline values (p=0.001) as daylengths were declining during late autumn (November-December; weekly mean range 136.3 ± 21.4 to 259.7 ± 48.5 ng/g feces; individual range 31.2 to 840.1 ng/g feces). During Autumn [September 21st (Autumn Equinox) to December 21st (Winter Solstice)] weekly T concentrations and daylength were inversely related (r= -0.749; p=0.006). From the winter solstice until mean peak T concentrations were reached in late February (838.2 ± 243.8 ng/g feces), mean weekly T concentrations and daylength were positively related (r= 0.921; p<0.001). Thereafter, T concentrations declined steadily to basal concentrations and from early March to the summer solstice (June 21st) T concentrations and daylength were inversely related (r= -0.865, p<0.001).

Fecal samples were collected (January - June, 1998) from one intact yearling (February - March was his first breeding season), housed in a family unit (mother, father, sister and brother), and were analyzed for T concentrations. Results for this animal demonstrate the occurrence of seasonality in T secretion in a pattern similar to adult animals [Appendix III. A(b)].

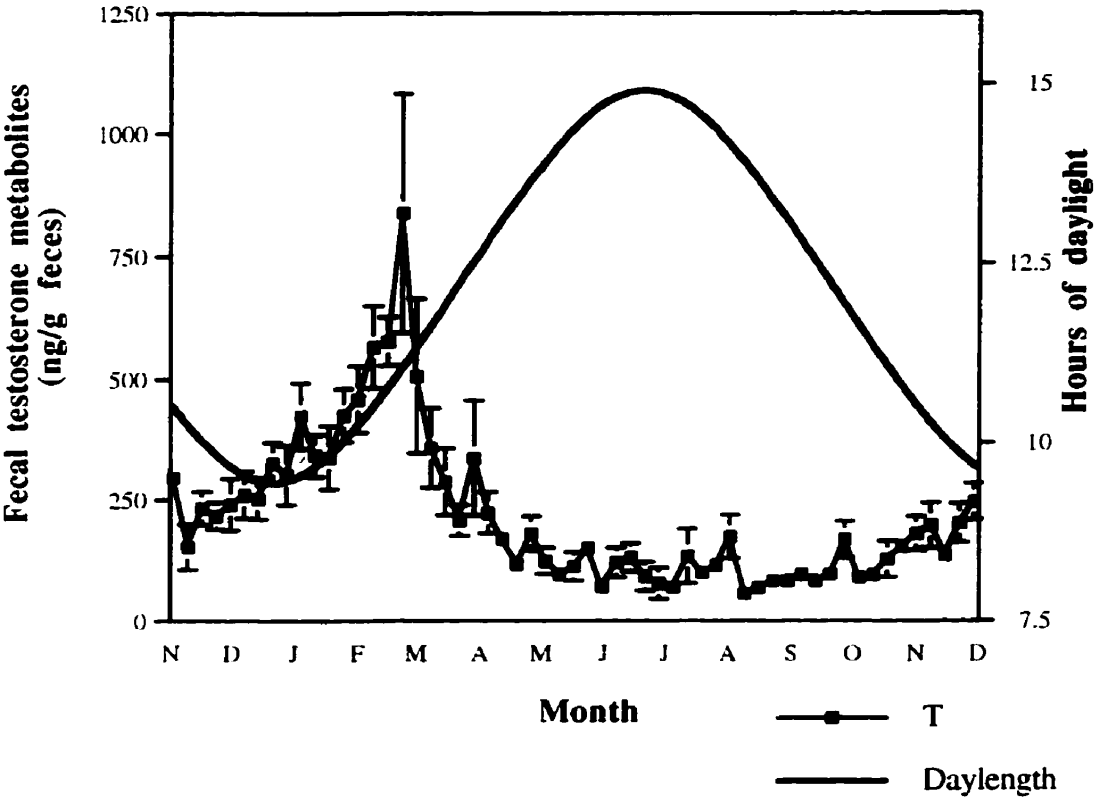


Figure 13. Weekly mean (+/- SEM) changes in fecal testosterone metabolite concentrations for male red wolves (n=5) in relation to changing daylength over a one year peroid.

Gender Determination

ANOVA was performed to determine which of 6 variables (P4, E2, T, P4/T, P4/E2 and T/E2) demonstrated significant differences among the classes (P, NP, AC and males) within the four different time periods (December 21st - February 3rd, February 4th - March 20th, March 21st - May 5th, May 6th - June 21st). To test the ability of fecal steroid analysis to distinguish between the sexes, only significant differences detected between the 3 classes of females and males were of interest. Mean ± SEM for each variable within each period for P, NP, AC and male animals are presented in Table II. Shaded areas represent female variables that were significantly different ($p<0.005$) from the respective male variable. In all four periods, differences among female and male variables were detected. However only 3 of the 4 periods (from December 21st - May 5th) demonstrated a variable that was significantly different from males for all 3 classes of females. The ratios P4/T and T/E2 were the two most informative variables during these 3 time periods (December 21st to May 5th). Testosterone also was an informative variable to characterize between the sexes; however, significant differences from males for all 3 classes of females were only demonstrated from February 4th to May 5th (Table II). Among the 6 variables, P4 and E2 concentrations along with P4/E2 ratios were the least informative to compare male and female hormone levels.

	Acyclic	±SEM	Nonpregnant	±SEM	Pregnant	±SEM	Male	±SEM
December 21st to February 3rd								
P4	**215.7**	37.9	**91.0**	7.1	**76.1**	18.2	**86.1**	13.9
E2	**70.7**	10.3	**45.8**	2.8	**37.2**	7.0	**47.3**	7.3
T	**170.5**	63.3	**151.5**	59.2	**74.1**	26.9	**355.0**	62.6
P4/T	**3.5**	1.4	**1.8**	0.3	**2.5**	1.4	**0.3**	0.6
P4/E2	**3.1**	0.5	**2.1**	0.2	**2.2**	0.5	**2.2**	0.3
T/E2	**2.1**	0.6	**3.4**	1.2	**1.7**	0.4	**7.5**	0.7
February 4th to March 20th								
P4	**296.6**	36.7	**320.1**	49.6	**172.5**	35.2	**96.1**	11.7
E2	**90.5**	16.9	**84.0**	11.2	**73.8**	23.8	**55.4**	6.7
T	**61.9**	17.5	**232.2**	100.2	**48.3**	12.0	**657.9**	78.9
P4/T	**8.1**	1.5	**5.2**	0.9	**4.3**	1.0	**0.2**	0.1
P4/E2	**4.4**	1.0	**5.1**	0.9	**4.0**	1.2	**1.9**	0.2
T/E2	**0.8**	0.2	**2.5**	1.0	**0.9**	0.2	**11.9**	1.0
March 21st to May 5th								
P4	**221.8**	55.8	**386.7**	37.9	**537.5**	72.5	**200.6**	113.7
E2	**80.3**	18.2	**63.6**	4.5	**68.7**	10.9	**54.9**	8.5
T	**94.8**	33.4	**77.3**	13.2	**108.9**	27.9	**197.1**	22.9
P4/T	**4.2**	0.9	**7.7**	0.8	**8.5**	1.9	**1.0**	0.4
P4/E2	**2.8**	0.2	**6.5**	0.6	**9.1**	1.4	**2.6**	0.6
T/E2	**1.4**	0.4	**1.4**	0.3	**1.8**	0.6	**4.1**	0.4
May 6th to June 21st								
P4	**210.1**	80.2	**173.5**	16.3	**175.1**	32.2	**95.3**	21.3
E2	**36.0**	5.5	**57.4**	4.3	**67.4**	11.1	**41.3**	5.0
T	**82.0**	27.7	**106.0**	32.0	**111.2**	34.3	**141.0**	30.5
P4/T	**6.9**	4.0	**4.0**	0.6	**2.5**	0.6	**2.0**	0.8
P4/E2	**5.8**	1.6	**3.3**	0.3	**2.7**	0.5	**2.4**	0.4
T/E2	**3.9**	2.0	**1.9**	0.5	**2.1**	0.9	**3.4**	0.6

Table II. Mean ± SEM concentrations and ratios of fecal progestin (P4), estrogen (E2) and testosterone (T) metabolites for acyclic, nonpregant, pregnant and male red wolves during 4 time periods surrounding and including the breeding season. Within rows, shaded areas represent significant differences ($p<0.005$) between the variables for the female reproductive class(es) and males.

DISCUSSION

Validation

One of the main objectives of this study was to develop a non-invasive method to endocrinologically monitor the reproductive status of both male and female red wolves. In addition, the adaptation of a method to non-invasively monitor adrenocortical activity was investigated. Progestins and estrogens have been successfully measured for a number of carnivores in fecal (felids: Shille et al., 1991; Graham et al., 1993; Brown et al., 1994; Czekala et al., 1994; Brown et al., 1995; Graham et al., 1995; Brown et al., 1996b, canids: Wasser et al., 1995; Hay, 1996; Monfort et al., 1997; Gudermuth et al., 1998; Velloso et al., 1998) and urine samples (canid: Batchelor et al., 1972, Monfort et al., 1997). Testosterone metabolites also have been quantified in urine (felid: Brown et al., 1996a) and feces (felid: Brown et al., 1996a, canid: Monfort et al., 1997; Velloso et al., 1998), as have corticosteroids (urine; felids: Carlstead et al. 1992; Carlstead et al., 1993; canid: Jones et al, 1990, feces; felid: Graham & Brown, 1996, canid: Creel et al. 1996). In this study, fecal samples were chosen as the substrate for analysis based on the following: 1) feces were readily available and easily collected during the daily husbandry routine, while urine was rapidly absorbed into the ground or masked by bushes and trees within the holding area, and 2) in previously studied carnivore species, it has been demonstrated that feces is the primary route of excretion for the steroid metabolites of interest in this study (progestins and estrogens: Shille et al., 1984; Shille et al., 1990; Gross, 1992; Brown et al., 1994; Monfort et al., 1997, cortisol: Graham & Brown, 1996, testosterone: Brown et al., 1996a; Velloso et al., 1998).

Achievement of the first objective required the adaptation and validation of fecal extraction procedures for use with our laboratory's enzyme immunoassays. Results obtained from the dose-response experiments indicated that ovarian hormone (method 1) and corticosteroid extraction protocols were effective at removing exogenous progestin/estrogen and endogenous cortisol metabolites, respectively, from the feces of the

female red wolf. Similarly, the androgen hormone extraction protocol (method 1) was efficient at removing endogenous testosterone metabolites from the feces of the male red wolf. Comparison between different progestin and estrogen extraction protocols demonstrated that exogenous progestin and estrogen removal was improved through the addition of aluminum oxide (Method 1 vs. 2). The addition of aluminum oxide aided in the removal of background pigments, resulting in more consistent percent recoveries and extraction efficiencies (Lucas et al., 1991; Graham et al., 1995). In all but one case, the removal of ovarian fecal metabolites was greater utilizing methods 1 (~90% methanol) than method 3 (~20% methanol). These results are not unexpected as Palme et al. (1996) and Young (1998) demonstrated that the recovery of steroids was improved by utilizing increasing amounts of methanol.

Tests of parallelism suggested that extracted steroids from both males and females behave in an immunologically similar manner to steroids used in the standard curve and are present in quantities which are measurable by the assay systems. A more detailed examination of unconjugated steroids present in fecal samples was facilitated by HPLC analysis. Caution should be exercised when interpreting the data, as only a few samples were subjected to HPLC analysis. However, results from this study suggest that there is little variation between individuals or stage of the reproductive cycle regarding which progestin and estrogen metabolites are excreted into the feces. Examination of the immunoreactive estrogen profile suggested that estradiol and estrone are the major forms of metabolites present in the feces of the red wolf. These results are in agreement with previous HPLC based studies in which it has been demonstrated that estradiol (Brown et al., 1994; Graham et al., 1995; Monfort et al., 1997; Velloso, 1998) and estrone (Brown et al., 1994; Monfort et al., 1997) constitute the major forms of estrogen metabolites excreted in carnivore feces. In contrast to estrogen, the majority of fecal progesterone metabolites in canids do not appear to be in the native form of progesterone (Monfort et al., 1997; Möstl & Brunner, 1997; Velloso et al., 1998). This appears to be true of the red wolf as well.

Although progesterone appears to be present in the feces, the majority of immunoreactivity was detected in fractions that were less polar than progesterone, suggesting the presence of pregnanes. This result is not unexpected, since recent publications in the domestic dog (Möstl & Brunner, 1997) and African wild dog (Monfort et al., 1997) identify 20-oxo-pregnanes as the major form of progestin metabolites. The numerous minor peaks also observed in red wolf feces suggest the likelihood of metabolism of progesterone to several more polar products, possibly including 11β-hydroxyprogesterone, 20α-dehydroxyprogesterone and 17α-hydroxyprogesterone. Collectively, this information suggests that an antibody with a broader range of cross-reactivities and/or higher cross-reactivity with pregnanes would increase the efficiency of monitoring ovarian activity in the red wolf.

It also appears that the majority of cortisol-like immunoreactivity in carnivore feces is not from the native steroid, but rather from other metabolite forms. The physiological relevance of cortisol-like metabolites in the feces of the African wild dog (Creel et al., 1996) and domestic cat (Graham & Brown, 1996) have been demonstrated using a corticosterone RIA; however, neither cortisol nor corticosterone were present in their native form in the feces. Data from the present study provide similar results, indicating that cortisol is not excreted in its native form in the feces of the red wolf. Although the cortisol-like metabolites in the feces of the red wolf act immunologically similar to a cortisol standard, as evidenced from parallelism results, and repeated restraint episodes appeared to increase corticosteroid metabolites in one wolf, further investigation into the validation of this assay system is required before it can be applied to study 'stress' events in this species.

In one canid species, the African wild dog, HPLC analysis suggests that testosterone is present in the feces (Monfort et al., 1997). Separation of radiolabelled metabolized testosterone in the maned wolf revealed that several testosterone metabolites exist, with free testosterone constituting only a small percentage of the total metabolites (Velloso et al., 1998). In contrast, although several radiolabelled fecal testosterone

metabolites were detected in the feces of the male domestic cat, none were associated with free testosterone (Brown et al., 1996a). The results in this present study agree with the observations made by Brown et al. (1996a), as no free testosterone appeared to be excreted in the feces of the red wolf. Although testosterone was not observed, the presence of several large immunoreactive peaks less polar than testosterone suggests the possiblity of 5α androstanediol, 17β-hydroxy-5α-androstan-3one and 3β-hydroxy-5α-androstan-17one (standards not shown) crossreacting with the testosterone antibody used in this study.

Female Reproductive Cycle

Longitudinal Profiles

Another goal of this project was to establish a database of normal endocrine profiles for the female red wolf. The findings of this study indicate that the major hormonal events which occur during the reproductive cycle of the red wolf are reflected in fecal steroid concentrations. Patterns observed are comparable to those reported for several canid species, including serum steroid profiles in the domestic dog (Smith & MacDonald, 1974; Concannon et al., 1975; Concannon et al., 1977a; Wildt et al., 1978; Wildt et al., 1979; Olson et al., 1982; Concannon et al., 1989) coyote (Stellflug et al., 1981) and the gray wolf (Seal et al., 1979; Seal et al., 1987), as well as fecal steroid profiles in the maned wolf (Wasser et al., 1995; Velloso et al., 1998), African wild dog (Monfort et al., 1997) and domestic dog (Gudermuth et al., 1998).

The measurement of reproductive hormones in the feces of the red wolf has a practical application for detecting the presence or absence of ovulation. In contrast to pregnant and nonpregnant animals, progesterone and estrogen concentrations did not fluctuate throughout the breeding season in acyclic red wolves. In fact, concentrations were elevated above cyclic animals prior to the onset of estrus. In domestic dogs the administration of a progestin treatment as a means of contraception, maintains gonadotropin secretion in an anestrual state. This prevents the elevation of gonadotropin secretion which

would normally terminate anestrus and initiate proestrus (Concannon, 1995). Although several potential explanations exist (age, health, genetic variability, stress) it is possible that the elevated progesterone levels present in acyclic red wolves prior to the breeding season acted in a similar manner, thereby maintaining the wolves in an anestrual state.

One objective for the female red wolf longitudinal study was to examine differences that occurred between the endocrine profiles of pregnant and non-pregnant animals. Gestation length in the red wolf, as determined from a rise in progesterone, was 64-65 days in duration. This is comparable to gestation lengths reported in the gray wolf (60-65: Seal et al., 1979), maned wolf (65 days: Velloso et al., 1998) and domestic dog (64-66 days: Concannon et al., 1989). The detection of pregnancy-specific differences post-implantation (~day 20 in the domestic dog: Thatcher et al., 1994) in serum and plasma ovarian hormone concentrations is controversial. Plasma or serum concentrations of progesterone (Smith & MacDonald, 1974) and estrogen (Concannon et al., 1977b) have been reported to be greater in pregnant animals than in the nonpregnant luteal phase of domestic dog. However, most studies have not observed these pregnancy-specific differences in either progesterone (Hadely, 1975; Nett et al., 1975; Austad et al., 1976; Reimers et al., 1978; Concannon et al., 1989; Onclin & Verstegen, 1997) or estrogen (Edqvist et al., 1975; Hadely, 1975; Nett et al., 1975; Austad et al., 1976; Graf, 1978; Reimers et al., 1978). In general, pregnancy-specific increases may not be clearly observed in plasma and serum steroid concentrations because of the increased hemodilution, metabolism and clearance associated with pregnancy (Concannon et al., 1977b). However, it is possible that differences in ovarian steroid hormone production during pregnancy could be evident in fecal samples. In recent reports for the domestic dog (Gudermuth et al., 1998) and maned wolf (Velloso et al., 1998), pregnancy-specific differences in progestin and estrogen metabolites were observed in the feces. In the present study, although fecal progestin and estrogen metabolite levels remained elevated slightly longer in pregnant than non-pregnant animals, a dramatic difference between the

concentrations of fecal ovarian steroids in pregnant and non-pregnant animals was not detected. Perhaps a larger group of pregnant study animals and/or an antibody more efficient at monitoring changes in progestin concentrations would allow for the detection of pregnancy-specific endocrine changes in the red wolf

Although not observed in this study, pregnancy-specific differences in ovarian steroid concentrations following implantation are believed to be of luteal origin as there is no evidence of placental steroid secretion in the domestic dog (Concannon, 1986b). It also has been suggested that, although the stimulus for increased steroid production by the corpus luteum is unknown, prolactin is likely involved (Gudermuth et al., 1998). Prolactin is a required luteotrophin in the dog (Concannon et al., 1987), and it has been demonstrated that following implantation there is a pregnancy-specific elevation in serum prolactin between days 25-30 (Graf et al., 1978; DeCoster et al., 1983; Onclin & Verstegen, 1997). Gudermuth et al. (1998) demonstrated that when prolactin rises in serum, pregnancy-specific elevations in fecal ovarian steroids occur. Perhaps, pregnancy diagnosis could be achieved through the validation of a sensitive immunoassay (RIA or EIA) to non-invasively monitor urinary prolactin, similar to previous success with LH (Czekala et al., 1988; Brannian et al., 1989; Robeck et al., 1993; Jeffcoate & England, 1997) and FSH (Walker et al., 1988).

Periovulatory Profiles

A final objective of the study concerning female red wolves was to describe the endocrine relationships between ovarian steroids during the periovulatory period. Characterization of the periovulatory period through fecal and serum steroid hormone analysis indicated that the red wolf is very similar to the domestic dog (Concannon et al., 1975; Concannon et al., 1977a; Wildt et al., 1979) and gray wolf (Seal et al., 1979).

The mean serum profile demonstrated that hormone values in the red wolf were within similar ranges reported for the gray wolf (Seal et al., 1979) and domestic dog (Edqvist et al., 1975; Austad et al., 1976; Concannon et al., 1975; Concannon et al.,

1977a; Wildt et al., 1979; Olson et al., 1982). The proestrous surge in estradiol began prior to the surge in LH and occurred over a ~7 day period attaining peak values 3 days prior to a rise in progesterone. Mean estradiol values declined coincidentally with the onset of the LH surge and then slowly returned to basal values. Similar trends have been reported for the gray wolf (Seal et al., 1979) and domestic dog (Concannon et al., 1975; Nett et al., 1975; Austad et al., 1976; Wildt et al., 1979; Olson et al., 1982). In addition, the preovulatory LH surge was similar in magnitude and duration (~4 days) to other canid species (Seal et al., 1979; Concannon et al., 1989) and occurred on the same day as the first significant increase in mean progesterone concentrations. As blood samples could not be drawn on a daily basis, no conclusive evidence could be drawn regarding the range in which LH and estradiol surges occurred in relation to a rise in serum progesterone. However, based on the data obtained, LH peaks generally occurred within 0-2 days prior to a rise in progesterone and peak estradiol levels were observed 2-6 days before a rise in progesterone.

Examination of the mean fecal profile for red wolves during the periovulatory period of the nonpregnant animal revealed similar endocrine trends to that of the serum profile. Peak estrogen concentrations were observed 5 days (range 4-7) before a rise in progesterone and although values were not significantly elevated above basal concentrations, the estrogen surge appeared to last for ~8 days. Compared to serum samples, fecal progesterone and estrogen concentrations were ~140 to 10 fold higher during basal concentrations, respectively, and ~40 to 10 fold higher at peak levels of steroid concentrations, respectively. This is not unexpected, since fecal concentrations tend to be much more concentrated than steroid levels in the blood (Hay, 1996; Möstl & Brunner, 1997). Behavioural observations, although not reported, indicated that overt estrous behaviours, including tail deflection, presentation of ano-genital area and mounting, were generally observed during falling estrogen levels and rising progesterone

values, similar to that of the domestic dog (Concannon et al., 1979b; Beach et al., 1982; Feldman & Nelson, 1987).

Results from this study suggest that because serum and fecal ovarian steroid profiles in the red wolf exhibit similar trends and are similar to reproductive patterns observed in the domestic dog, the measurement of ovarian hormones in the feces of the red wolf has a practical application for detecting the approximate timing of ovulation. Although mean fecal concentrations increased ~2-10 fold between basal and peak levels of steroid concentrations, elevations in fecal steroids concentrations during the estrogen surge and the rise in progesterone were only clearly observed in comparison to concentrations during the early stages of the breeding season in a given animal. These results are similar to a recent report in the domestic dog (Gudermuth et al., 1998). Therefore, the application of fecal steroid monitoring to detect ovulation must be applied on an individual basis, requiring the daily collection of fecal samples several weeks prior to the anticipated time of estrus to accurately estimate ovulation.

When using feces as an alternative to serum for monitoring steroid concentrations, a time lag between circulating steroid levels and concentrations measured in the feces is expected. In the domestic dog, fecal steroid concentrations have been demonstrated to be positively correlated within a ~24 hour period with serum concentrations (Hay, 1996; Gudermuth et al., 1998). Although serum and fecal concentrations were not collected simultaneously in this study, we would expect to see similar trends in the feces of the red wolf. Although one must be careful in assuming that the time it takes for food items to pass through the gut closely reflects the lag between steroid secretion in blood and its excretion rate in the feces, the transit time of small pieces of surveyors tape or food colouring within a meatball occurred within an ~12-16 hour period in the red wolf (S. Berhns, personal communication). Based on observations in the red wolf and those described for the domestic dog, it is suggested that steroid concentrations within red wolf

feces most likely represented a pool of circulating steroid concentrations from the previous 12-24 hours.

Studies in the domestic dog have suggested that the peak fertility period is estimated to be 4-5 days following the LH surge (Concannon et al., 1989) based on ovulation occurring within 1-3 days following the LH surge (Phemister et al., 1973; Concannon et al., 1977a; Wildt et al., 1978) and 2-3 days for oocyte maturation (Holst & Phemister, 1971; Anderson & Simpson, 1973; Phemister et al., 1973; Concannon et al., 1989). Studies have demonstrated that declining estrogen concentrations concomitant with increasing progesterone concentrations are associated with the LH surge in the domestic bitch (Smith & MacDonald, 1974; Concannon et al., 1975, Concannon et al., 1977a; Concannon et al., 1979b; Wildt et al., 1979; Olson et al., 1982) and that the initial sharp elevation in progesterone concentrations occurs coincidentally with the LH surge (Concannon et al., 1977a; Penton et al., 1991). Therefore, timing for AI in the domestic dog is routinely based on circulating progesterone concentrations (Linde-Forsberg & Forsberg, 1993; Wilson, 1993; Fontbonne & Badinand; 1993). Previous attempts at AI for the red wolf required manual restraint of females for blood sample collection and vaginal cytology. Results from the present study indicate that non-invasive fecal steroid monitoring could be used as an alternative to blood sampling to estimate ovulation and time AI. Blood sampling from a stress-susceptible non-domestic animal such as the red wolf has the potential to negatively impact on AI success. Stress may interrupt normal endocrine patterns, and possibly disrupt fertilization, implantation or embryonic development (Moberg, 1985; Liptrap, 1993; deCanranzaro & MacNiven, 1995). The application of monitoring ovarian steroid concentrations through fecal samples has the potential to improve AI success rate and possibly aid in the genetic and conservation management of this endangered species.

Male Reproductive Cycle

For the male red wolf, one objective of this study was to investigate the relationship between changes in fecal testosterone levels and photoperiod. Previous work in male non-domestic canids has demonstrated events consistent with photoperiod synchronization (Green et al., 1984; Smith et al., 1985; Asa et al., 1987; Mitsuzuka, 1987; Seal et al. 1987; Monfort et al., 1997; Velloso et al., 1998). Similar to other non-domestic canids, the results of this study demonstrated changes in fecal testosterone metabolite concentrations in relation to photoperoid and further support seasonal regulation of androgenesis and spermatogenesis in the male red wolf.

It has been suggested that the gray wolf cannot be clearly classified into either a spring (long-day) or fall (short-day) breeder (Asa et al., 1987). This appears to be true of the red wolf as well. As observed in this study, the peak breeding periods in red wolves occurs during mid- to late winter as days are lengthening. However, fecal testosterone levels began to rise in late autumn, reaching peak levels coincident with estrus in late winter. Thus, although peak breeding occurs during lengthening days, the early phases of sexual recrudescence occur when days are becoming shorter. As with the gray wolf, it is not known which of these photoperoidic phases is more dominant or important, or if both have equally significant roles in the annual cycle (Asa et al., 1987).

In addition to the measurement of fecal steroid concentrations from adult wolves, fecal samples also were collected from two wolves entering their first breeding season (1 male and 1 female). The wolves were housed in a family unit, consisting of the mother, father and 2 young males and 1 young female. Their steroid profiles indicated that the young animals did in fact exhibit seasonal changes in reproductive hormones and the concentrations observed were within ranges reported for adult wolves. These endocrine data support the observation that young wolves can produce pups in their first breeding season (Medjo & Mech, 1976; Zimen, 1976; Waddell, 1995).

Management and Conservation Applications

Overall, this project demonstrates the ability to non-invasively monitor ovarian and testicular endocrine function in the female and male red wolf, respectively. The knowledge gained from this study greatly improves our understanding of the basic endocrinology of the endangered red wolf and has direct application towards the management of both captive and wild populations.

The fact that fecal samples are readily available, easily collected and reflect changes which occur during the reproductive cycle in the female has important implications for the captive breeding program. Fecal samples can provide crucial information on peak fertility periods and could therefore be used to time AI. The establishment of endocrine norms for male and female red wolves over a long-term period allows for recognition of any disturbances in these patterns in future analyses of individual reproductive function. Therefore, fecal steroid analysis may aid in the diagnosis of possible cases of infertility or test the effectiveness of different contraceptive treatments. This kind of information is important with regard to management decisions such as future relocations and/or pairing of animals. In addition, the non-invasive nature of this technique eliminates any undue 'stress' that animals would endure if blood samples were required to achieve the same objectives and therefore may more accurately reflect ovarian function.

For free-ranging red wolves, information gained from this study could serve as an indicator of normal reproductive patterns. Additionally, results from the present study suggest that gender determination through fecal steroid analysis can be achieved during the breeding season from late December until the beginning of May and could possibly be used in certain aspects of wild population studies such as sex ratio and sample identification within a territory. However, due to individual variation a larger database is needed before this technique could be used in practical studies.

Further validation of the cortisol assay is required before adrenal endocrine function can be non-invasively monitored in the feces of the red wolf. However, the potential

application of this technique could be used to determine if potential 'stressors', such as daily husbandry routines or other environmental factors, negatively impact on the well-being of the wolves and possibly interfere with reproductive success.

SUMMARY AND CONCLUSION

In light of recent concerns of hybridization of wild red wolves and coyotes, the importance of and reliance on a genetically healthy and viable captive population is crucial for the future management of this endangered carnivore. This investigation was undertaken primarily as a means to improve our basic knowledge of red wolf reproductive biology. The fundamental biological data accumulated from this study will prove to be an effective aid in refining and improving the captive breeding management of this species. Although red wolves have been maintained in captive populations for almost 20 years this is the first report to examine female and male endocrinology. While it is generally assumed that reproductive aspects of the red wolf would mimic those of the domestic dog, it is necessary to document all aspects of reproductive biology prior to undertaking assisted reproduction. Studies have demonstrated that the reproductive biology of a non-domestic species can differ from its domestic model [sperm characteristics differ between red wolves and domestic dogs (Goodrowe et al., 1998), clouded leopards (Neofelis nebulosa), although 3-8 times the body weight, require the same dosage of gonadotropin as the domestic cat (Felis domesticus) to elicit comparable follicular growth (Howard et al., 1992)].

To facilitate the non-invasive monitoring of ovarian, testicular and adrenocorticoid steroids within the feces of the red wolf, validation of enzyme immunoassay systems used in our laboratory, through dose response and parallelism experiments, was required. Results from these experiments demonstrated that extraction protocols selected for use in this study were effective at removing progestin, estrogen, testosterone and cortisol metabolites from fecal samples in this species. Facilitated by HPLC, a more detailed examination of unconjugated steroids present in fecal samples revealed that it is best to use antiserum specific for estradiol to detect follicular activity, but for monitoring products of luteal and testicular steroidogenesis, antiserum with a broader spectrum of cross-reactivities would be more favourable. Although cortisol-like metabolites in the feces of the red wolf

behaved immunologically similar to a cortisol standard and repeated restraint episodes appeared to increase cortisol metabolites in one wolf. HPLC results indicated cortisol is not present in its native form but rather as other cortisol-like metabolites. Further investigation into the validation of this assay system is required before it can be applied to study 'stress' events in this species.

Longitudinal monitoring of ovarian steroids was conducted on female red wolf fecal samples collected during the breeding season. In general, the major hormonal events which occurred during the adult reproductive cycle were reflected in steroid concentrations in the feces. Similar reproductive patterns also were observed in a single female red wolf experiencing her first breeding season. Mean estrogen metabolite values in pregnant and ovulatory non-pregnant female red wolves peaked prior to a rise in progestins and were significantly elevated above mean baseline concentrations during the mid-luteal phase. Mean progesterone metabolite values were significantly higher than baseline concentrations until the mid- to late luteal phase in pregnant and ovulatory non-pregnant wolves. Although mean fecal progestin and estrogen metabolite concentrations in pregnant females remained elevated longer during the luteal phase than those measured in ovulatory non-pregnant females no significant difference between the two profiles, for either progesterone or estrogen, was demonstrated. Therefore, pregnancy detection using the techniques outlined in this study is not possible. However, measurement of reproductive hormones in the feces is useful for the detection of normal reproductive patterns, by evaluating the presence or absence of ovulation through a significant rise in progesterone. In some females, fecal progestin and estrogen concentrations remained at baseline concentrations throughout the breeding season and were therefore considered to be acyclic.

The traditional method of timing AI in the female red wolf involved physical restraint for vaginal cytology and blood sampling for progesterone analysis. Females were generally subjected to AI procedures 2-3 times (beginning 5-6 days after progesterone levels became elevated above 1.0 ng/ml) and were restrained on average 11 times before the

suspected period of ovulation could be determined. Although serum and fecal values were not simultaneously monitored in this study, the serum and fecal profiles obtained during the periovulatory period exhibited similar trends and were similar to those observed in the domestic dog. This suggests that the non-invasive measurement of ovarian steroids in the feces could serve as a practical application for the detection of the approximate time of ovulation. Unfortunately, due to individual variation, there was no defined concentration that coincided with ovulation. Therefore, the use of non-invasive hormonal monitoring to detect a rise in progestin concentrations from baseline values must be applied on an individual basis. Perhaps the development of a more appropriate antiserum to progestin metabolites excreted in the feces of the female red wolf would minimize individual variation allowing for a broader application and possibly pregnancy detection.

Longitudinal monitoring of testicular steroids was conducted on male red wolf fecal samples collected over a 1 year period. The changes in hormonal patterns demonstrated events consistent with photoperiod synchronization, further supporting seasonal regulation of androgenesis and spermatogenesis in the red wolf. Testicular steroid measurements in a single male red wolf experiencing its first breeding season exhibited similar patterns to those observed adult males. Adult fecal testosterone concentrations remained at baseline levels throughout the summer months until late autumn (November-December) when levels significantly rose above baseline values and were negatively correlated with shortening daylengths. From the winter solstice (December 21st) until peak concentrations were attained during a period coincident with estrus (late February) testosterone metabolites were positively correlated with increasing daylength. Thereafter, testosterone metabolite concentrations declined back to nadir levels.

Experiments from this study also demonstrate the ability to sex red wolves through fecal steroid analysis. Although practical application of this technology is yet to be implemented, results from this study indicate that gender determination from unknown fecal samples is possible because of the differences that exist between female and male fecal

progestin, estrogen and testosterone metabolite concentrations. Unfortunately, this technology can only be utilized during the breeding season (late December - early May) as concentrations of steroids beyond this period do not contrast sufficiently to detect differences between the sexes.

In summary, this study demonstrates the capability to non-invasively monitor ovarian and testicular function in female and male red wolves, respectively, through fecal steroid analysis. It has greatly enhanced our basic knowledge of the hormonal events which occur during the reproductive cycle of this species. Although the techniques described in this study are adequate for practical application, future investigations should focus on the development of assays more specific to metabolites excreted in the feces of this species. In addition, non-invasive fecal steroid analysis should be used to time future AI attempts, thereby eliminating stressful restraint episodes and possibly improving AI success rate. Improving the management of the captive breeding program by utilizing techniques developed in this study has the potential to greatly improve the reproduction of genetically valuable individuals for reintroduction and ensure the continued existence of red wolves in the wild.

LITERATURE CITED

Abbott DH, McNeilly AS, Lunn SF, Hulme MJ, Burden FJ. Inhibition of ovarian function in subordinate female marmoset monkeys (Callithri jacchus). J Reprod Fertil 1963; 63: 335-345.

Adam CL, Atkinson T. Effect of feeding melatonin to red deer (Cervus elaphus) on the onset of the breeding season. J Repro Fertil 1984; 72: 463-466.

Alexander G, Signoret JP, Hafez ESE. Sexual and maternal behaviour. In: Hafez (ed.) Reproduction in Farm Animals. Philadelphia, Lea & Febiger, 1974: 222-254.

Aman RP. Reproductive physiology and endocrinology of the dog. In: Morrow DA (ed.) Current Therapy in Theriogenology 2. Philadelphia: WB Saunders Co., 1986: 532-538.

Anderson AC, Simpson ME. The ovary and reproductive cycle of the dog (Beagle). Los Altos, California, Geron-X Press, 1973.

Anderson AC. Reproduction. In: Anderson AC (ed.) The Beagle as an Experimental Dog. Ames, IA, Iowa State University Press, 1970: 31-39.

Anderson D. The rate of passage of the mammalian ovum through various portions of the fallopian tube. Amer J Physiol 1927; 82(3): 557-569.

Anderson K. Fertility of frozen dog semen. Acta Vet Scand 1972; 13: 128-130.

Anderson K. Intrauterine insemination with frozen semen in dogs. Proc 12th Nordic Vet Cong, Iceland 1974: 153-154.

Arbeiter K, Brass W, Ballabio R, Jöchle W. Estrus induction and fertility with cabergoline, a prolactin inhibitor, in the bitch. J Sm Anim Prac 1988; 29: 781-788.

Asa CS, Seal US, Letellier M, Plotka ED, Peterson EK. Pinealectomy or superior cervical ganglionectomy do not alter repoduction in the wolf (Canis lupus). Biol Reprod 1987; 37: 14-21.

Atkins DL, Dillon LS. Evolution of the cerebellum in the genus Canis. J Mammol 1971; 52: 96-107.

Atkinson S, Gilmartin WG. Seasonal testosterone pattern in Hawaiian monk seals (Monachus schauinslandi). J Reprod Fertil 1992; 96: 35-39.

Audubon JJ, Bachman J. The quadrupeds of North America. New York, 1851, Vol 2 334p.

Audy MC, Bonnin M, Souloumiac J, Ribes C, Kerdelhue B, Mondain-Monval M, Scholler R, Canivenc R. Seasonal variations in plasma luteinizing hormone and testosterone levels in the European badger Meles meles L. Gen Comp Endrocrinol 1985; 57: 445-453.

Austad R, Lunde A, Sjaastad OV. Peripheral plasma levels of oestradiol-17β and progesterone in the bitch during the oestrous cycle, in normal pregnancy and after dexamethasone treatment. J Reprod Fertil 1976; 46: 129-136.

Austin CR, Short RV. Germ cells and Fertilization. In: Reproduction in Mammals. London: Cambridge University Press, 1972: 82.

Bailey V. Biological survey of Texas. N American Fauna No 25, 1905, 222p.

Bangs O. The land mammals of peninsular Florida and the coast region of Georgia. Proc Boston Soc Nat Hist 1898; 28; 157-235.

Barb CR, Kraeling RR, Rampacek GB, Fonda ES, Kiser TE. Inhibition of ovulation and LH secretion in the gilt after treatment with ACTH or hydrocortisone. J Reprod Fertil 1982; 64: 85-92.

Bartness TJ, Goldman BD. Mammalian pineal melatonin. Experienta 1989; 45: 939-945.

Bartram W. Travels. Philadelphia, 1791; 522p.

Batchelor A, Bell ET, Christie W. Uninary oestrogen excretion in the beagle bitch. Br Vet J 1972; 128: 560-565

Battista M, Parks J, Concannon PW. Canine sperm post-thaw survival following freezing in straws or pellets using PIPES, lactose tris, or TEST extenders. Proc 11th Int Congr Anim Reprod & AI, Dublin 1988; 3: 229-231.

Beach FA, Dunbar IF, Buehlar MG. Sexual characteristics of female dogs during successive phases of the ovarian cycle. Horm Behav 1982; 16: 414-442.

Bekoff M. Social behaviour and ecology of the African Canidae: a review. In: Fox MW (ed.) The Wild Canids. New York: Van Nostrand Reinhold, 1975: 120-142.

Ben Sadd M, Bayle JD. Seasonal changes in plasma testosterone, thyroxine, and cortisol levels in wild rabbits (Oryctolagus cuniculus algirus). Gen Comp Endocrinol 1985; 57: 383-388.

Bondestam S, Alitalo I, Karkkainen M. Realtime ultrasound pregnancy diagnosis in the bitch. J Sm Anim Pract 1983; 24: 145-151.

Bouchard GF, Gross S, Ganjan VK, Youngquist RS, Concannon PW, Krause GF, Reddy CS. Oestrus induction in the bitch with the synthetic oestrogen diethylstibestrol. J Reprod Fertil Suppl 1993; 47: 515-516.

Bowman LA, Dilley SR, Keverne EB. Suppression of oestrogen-induced LH surges by social subordination in talapoin monkeys. Nature, London 1978; 275: 56-58.

Braden AWH, Moule GR. Effects of stress on ovarian morphology and oestrus cycles in ewes. Aust J Agric Res 1964; 15: 937-949.

Brady CA, Ditton MK. Management and breeding of maned wolves at the National Zoological Park, Washington. Int Zoo Yearbk 1979; 19: 171-176.

Brannian JD, Griffin F, Terranova PT. Urinary androstenedione and luteinizing hormone concentrations during musth in a mature African elephant. Zoo Biol 1989; 8: 165-170.

Brown JL, Terio KA, Graham LH. Fecal androgen metabolite analysis for non invasive monitoring of testicular steroidogenic activity in felids. Zoo Biol 1996a; 15: 425-434.

Brown JL, Wasser SK, Wildt DE, Graham LH. Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured non-invasively in feces. Biol Reprod 1994; 51: 776-786.

Brown JL, Citino SB, Bush M, Lehnhardt J, Phillips LG. Cyclic patterns of luteinizing hormone, follicle stimulating hormone, insulin, and progesterone secretion in the asian elephant (Elephas maximus). J Zoo Wild Med 1991; 22: 49-57.

Brown JL, Wildt DE, Graham LH, Byers AP, Collin SL, Barrett S, Howard JG. Natural versus chorionic gonadotrophin-induced ovarian responses in the clouded leopard (Neofelis nebulosa) assessed by fecal steroid analysis. Biol Reprod 1995; 53: 93-102.

Brown JL, Wildt DE, Wielebnowski N, Goodrowe KL, Graham LH, Wells S, Howard JG. Reproductive activity in captive female cheetahs (Acinonyx jubatus) assessed by faecal steroids. J Reprod Fertil 1996b; 106: 337-346.

Bubenik GA, Schams D, Leatherland JF. Seasonal rhythms of prolactin and its role in the antler cycle of the white-tailed deer. In: Fennessy PF, Drew KR (eds.) Biology of deer production. R Soc NZ Bull 1985; 22: 257-262.

Burdick HO, Whitney R. Acceleration of the rate of passage of fertilized ova through the fallopian tubes of mice by massive injections of an estrogenic substance. Endocrinology 1937; 21: 637-634.

Busch RH. The Wolf Almanic. New York: Lyons & Burford, 1995.

Carley CJ. Activites and findings of the Red Wolf Field Recovery Program from late 1973 to July 1, 1975. USFWS Report, Albuquerque, NM, 1975, 215p.

Carley CJ. Status summary: The red wolf Canis rufus. USFWS Endangered Species Report No 7, Albuquerque, NM, 1979, 36p.

Carlstead K, Brown JL, Strawn W. Behavioural and physiological correlates of stress in laboratory cats. Appl Anim Behav Sci 1993; 38: 143-158.

Carlstead K, Brown JL, Monfort SL, Killens RK, Wildt DE. Urinary monitoring of adrenal responses to phychological stressors in domestic and nondomestic felids. Zoo Biol 1992; 11: 165-176.

Chakraborty P. Reproductive hormone concentrations during estrus, pregnancy, and pseudopregnancy in the labrador bitch. Theriogenology 1987; 27: 827-840.

Chang MC, Yanagimachi R. Effect of estrogens and other compounds as oral antifertility agents on the development of rabbit ova and hamster embryo. Fertil Steril 1965; 16: 281-291.

Christian JJ, LeMunyan CD. Adverse effects of crowding on laction and reproduction of mice and two generations of their progeny. Endocrinology 1958; 63: 517-529.

Christiansen IJ. Reproduction in the Dog and Cat. London, Bailliere Tindall, 1984.

Christie DW, Bell ET. Some observations on the seasonal incidence and frequency of estrus in breeding bitches in Britain. J Sm Anim Pract 1971; 12: 159-167.

Christie DW, Bell ET. Studies on canine reproductive behavior during the normal estrus cycle. Amin Behav 1972; 20: 621-631.

Collu R, Gibb W, Ducharme JR. Effects of stress on the gonadal function. J Endocrinol Invest 1984; 7: 529-537.

Colon J, Kimball M, Hansen B, Concannon PW. Effects of contraceptive doses of the progestagen megestrol acetate on luteinizing hormone and follicle-stimulating hormone in female dogs. J Reprod Fertil Suppl 1993; 47: 519-521.

Concannon PW. Effects of hypophysectomy and of LH administration on luteal phase plasma progesterone levels in the beagle bitch. J Reprod Fertil 1980; 58: 407-410.

Concannon PW. Reproductive physiology and endocrine patterns of the bitch. In: Kirk RW (ed.) Current Veterinary Therapy, Small Animal Practice, Vol. VIII. Philadelphia, WB Saunders Co., 1983: 886-900.

Concannon PW. Canine physiology of reproduction. In: Burke T (ed.) Small Animal Reproduction and Infertility. A Clinical Approach to Diagnosis and Treatment. Philadelphia, Lea and Febiger, 1986a: 23-77.

Concannon PW. Canine pregnancy and partuition. Veterinary Clinics of North America: Small Animal Practice 1986b; 16: 453-475.

Concannon PW. Induction of fertile oestrous in anoestrous dogs by constant infusion of GnRH agonist. J Reprod Fertil Suppl 1989; 39: 149-160.

Concannon PW. Reproduction in the dog and cat. In: Reproduction in Domestic Animals (4th Edn). San Diego, Academic Press Inc., 1991: 517-554.

Concannon PW. Biology of gonadotrophin secretion in adult and prepubertal female dogs. J Reprod Fertil Suppl 1993; 47: 3-27.

Concannon PW. Reproductive endocrinology, contraception, and pregnancy termination in dogs. In Ettinger SJ, Feldman EC (eds.) Texbook of Veterinary Internal

Medicine. Diseases of the dog and cat. Philadelphia. WB Saunders Co.. 1995:1625-1636.

Concannon PW, Battista M. Canine semen freezing and artificial insemination. In: Kirk RW (ed.) Current Veterinary Therapy. Small Animal Practice. Vol.X. Philadelphia. WB Saunders CO.. 1989: 1247-1259.

Concannon PW, Castracane VD. Serum androstenedione and testosterone concentrations during pregnancy and nonpregnant cycles in dogs. Biol Reprod 1985: 33: 1078-1083.

Concannon PW, Hansel W. Effects of estrogens and progesterone on plasma LH, sexual behaviour, and pregnancy in beagle bitches. Fed Proc 1975: 34: 323 (Abstract).

Concannon PW, Hansel W. Prostaglandin F2α induced luteolysis, hypothermia and abortions in beagle bitches. Prostaglandins 1977: 13: 533-542.

Concannon PW, Lein DH. Hormal and clinical correlates of ovarian cycles, ovulation, pseudopregnancy, and pregnancy in dogs. In: Kirk RW (ed.) Current Veterinary Therapy X. Small Animal Practice. Philadelphia. WB Saunders CO.. 1989: 1269-1282.

Concannon PW, Temple M. Periovulatory decline and late anestrus increase in naloxone-inducible LH release in adult domestic bitches. Biol Reprod 1988: 38(suppl): 101.

Concannon PW, Cowan RG, Hansel W. LH release in ovariectomized dogs in response to estrogen withdrawl and its facilitation by progesterone. Biol Reprod 1979a: 20: 523-531.

Concannon PW, Hansel W, McEntree K. Changes in LH, progesterone and sexual behaviour associated with preovulatory lutenization in the bitch. Biol Reprod 1977a: 17: 604-613.

Concannon PW, Hansel W, Visek W. The ovarian cycle of the bitch: plasma estrogen, LH and progesterone. Biol Reprod 1975: 13: 112-121.

Concannon PW, McCann JP, Temple M. Biology and endocrinology of ovulation, pregnancy and parturition in the dog. J Reprod Fertil Suppl 1989: 39: 3-25.

Concannon PW, Whaley S, Anderson SP. Increased LH pulse frequency associated with termination of anestrus during the ovarian cycle of the dog. Biol Reprod 1986: 34 (Suppl.): 119 (Abstract).

Concannon PW, Powers ME, Holder W, Hansel W. Pregnancy and parturition in the bitch. Biol Reprod 1977b: 16: 517-526.

Concannon PW, Weigand N, Wilson S, Hansel W. Sexual behaviour in ovariectomized bitches in response to estrogen and progesterone administration. Biol Reprod 1979b: 20: 799-809.

Concannon PW, Weintein R, Whaley S, Frank D. Suppression of luteal function in dogs by luteinizing hormone anti-serum and bromocriptine. J Reprod Fertil 1987: 81: 175-180.

Concannon PW, Whaley S, Lein D, Wissler R. Canine gestation length: varation related to time of mating and fertile life of sperm. Am J Vet Res 1983; 44: 1819-1821.

Concannon PW, Bulter WR, Hansel W, Knight PJ, Hamilton JM. Parturition and lactation in the bitch: serum progesterone, cortisol, and prolactin. Biol Reprod 1978; 19: 1113-1118.

Concannon PW, Isaman L, Frank DA, Michel FJ, Currie WB. Elevated concentrations of 13, 14-dihydro-15-keto-prostaglandin F-2α in maternal plasma during prepartum luteolysis and partuition in dogs (Canis familiaris). J Reprod Fertil 1988; 84: 71-77.

Creel S, Creel NM, Monfort SL. Stress and social dominance. Nature, London 1996; 379: 212.

Cunningham DJ. Cape hunting dogs (Lycaon pictus) in the gardens of the Royal Zoological Society of Ireland. Proc. R. Soc. Edinburgh 1905; 25: 843-848.

Czekala NM, Durrant BS, Callison L, Williams M, Millard S. Fecal steroid hormone analysis as an indicator of reproductive function in the cheetah. Zoo Biol 1994; 13: 119-128.

Czekala NM, Roser JF, Mortensen RB, Reichard T, Lasely BL. Urinary hormone analysis as a diagnostic tool to evaluate the ovarian function of female gorillas (Gorilla gorilla). J Reprod Fertil 1988; 82: 255-261.

Deanesly R. Further observations on the effects of oestradiol on tubal eggs and implantation in the guinea pig. J Reprod Fertil 1963; 5: 49-57.

deCatanzaro D. Effect of predator exposure upon early pregnancy in mice. Physiol Behav 1988; 43: 691-696.

deCatanzaro D, MacNiven E. Psychogenic pregnancy disruptions in mammals. Neurosci Biobehav Rev 1992; 16: 43-53.

deCatanzaro D, MacNiven E, Ricciuti F. Comparison of the adverse effects of adrenal and ovarian steroids on early pregnancy. Psychoneuroendocrinology 1991; 16: 525-536.

De Coster T, Beackers JF, Beerens D, De May J. A homologous radioimmunoassay for canine prolactin: plasma levels during the reproductive cycle. Acta Endocrinol Copenh 1983; 103: 473-478.

DePalatis L, Moore J, Falvo RE. Plasma concentrations of testosterone and LH in the male dog. J Reprod Fertil 1978; 52: 201-207.

Dickson AD. Delay of implantation in super-ovulated mice subjected to crowded conditions. Nature, London 1964; 201: 829-840.

Doak RI, Hall A, Dale HE. Longevity of spermatozoa in the reproductive tract of the bitch. J Reprod Fertil 1967; 13: 51-58.

Doney JM, Gunn RG, Griffiths JG. The effect of premating stress on the onset of oestrus and on ovulating rates in Scottish blackface ewes. J Reprod Fertil 1973; 35: 381-384.

Doney JM, Gunn RG, Smith WF, Carr WR. Effects of pre-mating environmental stress, ACTH, cortisone acetate or metyrapone on oestrus and ovulation in sheep. J Agric Sci 1976; 87: 127-132.

Dowling TE, Minckley WL, Marsh PC, Demaris BD. Response to Wayne, Nowak, and Phillips and Henry: Use of molecular characters in conservation biology. Cons Biol 1992; 6(4): 600-603.

Durrant BS, Pratt NC, Russ KD, Bolamba D. Isolation and characterization of canine advanced preantral and early antral follicles. Theriogenology 1998; 49: 917-932.

Dutt RH. Critical period for early embryo mortality: ewes exposed to high ambient temperature. J Anim Sci 1963; 22: 713-725.

Edquist LE, Johansson EDB, Kasstrom H, Olsson SE, Richkind M. Blood plasma levels of progesterone and oestradiol in the dog during the oestrus cycle and pregnancy. Acta Endocinol 1975; 75: 554-564.

Eik-Nes KB. Secretion of testosterone in anesthetized dogs. Endocrinology 1962; 71: 101-106.

Elder WH, Hayden CM. Use of discriminant function in taxonomic determination of canids from Missouri. J Mammol 1977; 58:17-24.

Elvidge H, Challis JRG, Robinson JS, Roper C, Thorburn, GD. Influence of handling and sedation on plasma cortisol in Rhesus monkeys (Macaca mulatta). J Endocrinol 1976; 70: 325-326.

England GCW. Effect of progestens and androgens upon spermatogenesis and steroidgenesis in dogs. J Reprod Fertil Suppl 1997; 51: 123-138.

Euker JS, Riegle GD. Effects of stress on pregnancy in the rat. J Reprod Fertil 1973; 34: 343-346.

Evans HM, Cole HH. An introduction to the study of the estrous cycle of the dog. Mem Univ Calif 1931; 9: 65-103.

Ewer RF. The Carnivores. Ithaca, NY: Cornell University Press. 1973.

Falvo RE, DePalatis LR, Moore J, Kepic TA, Miller J. Annual variations in plasma levels of testosterone and luteinizing hormone in the laboratory male mongrel dog. J Endocrinol 1980; 86: 425-430.

Farstad W, Anderson-Berg K. Factors influencing the success rate of artifical insemination with frozen semen in the dog. J Reprod Fertil Suppl 1989; 39: 289-292.

Farstad W. Bitch fertility after natural mating and after artifical insemination with fresh or frozen semen. J Sm Anim Pract 1984; 25: 561-565.

Feldman EC, Nelson RW. Canine Female Reproduction In: Feldman EC, Nelson RW (eds.) Canine and Feline Endocrinology and Reproduction. Philadelphia, WBSaunders Co., 1987: 399-524.

Fenske M, Grospietsch G, Konig A. Plasma glucocorticosteriod and aldosterone levels during physiological and stress conditions in rabbits. II. Mature animals. Endokrinologie 1981; 77: 59-64.

Fenske M. Adrenal function in the Mongolian gerbil (Meriones unguiculatus): Influence of confinement stress upon glucocorticosteroid, progesterone, dehydroepiandrosterone, testosterone and androstenedione plasma levels, adrenal content and in-vitro secretion. Exp Clin Endocrinol 1986; 87: 15-25.

Fernandes PA, Bowen RA, Kostas AC, Sawyer HR, Nett TM, Olson PN. Luteal function in the bitch: changes during diestrus in pituitary concentrations and the number of luteal receptors for luteinizing hormone and prolactin. Biol Reprod 1987; 37: 804-811.

Ferrell RE, Morizot DC, Horn J, Carley CJ. Biochemical markers in species endangered by introgression: the red wolf. Biochem Genet 1978; 18:39-49.

Flesness NR. Mammalian extinction rates: Background to the black-footed ferret drama. In: Seal US, Thorne ET, Bogan MA, Anderson SH (eds.) Conservation Biology and the Black-footed ferret. New Haven: Yale University Press, 1989: 3-9.

Fontbonne A, Badinand F. Canine artificial insemination with frozen semen: comparison of intravaginal and intrauterine deposition of semen. J Reprod Fertil Suppl 1993; 47: 325-327.

Foote RH, Swierstra EE, Hunt WL. Spermatogenesis in the dog. Anat Rec 1972; 173: 341-352.

Fowler ME. Zoo & Wild Animal Medicine. Philadelphia: WB Saunders Co., 1986.

Franzmann AW, Flynn A, Arneson PD. Serum corticoid levels relative to handling stress in Alaskan moose. Can J Zool 1975; 53: 1424-1426.

Fuller GB, Hobson WC, Reyes FI, Winter JSD, Faiman C. Influence of ketamine anesthesia on adrenal steroids, progesterone, and gonadotropins in rhesus monkeys. Proc Soc Exp Biol Med 1984; 175: 487-490.

Fuller TK. Denning behaviour of wolves in northcentral Minnesota. Am Midl Nat 1989b: 121: 184-188.

Fuller TK. Population dymanics of wolves in northcentral Minnesota. Wildl Monogr 1989a: 105: 1-41.

Gensch W. Notes on breeding Timber Wolves Canis lupus occidentalis at Dresden Zoo. Int. Zoo Yearb 1968: 8: 15-16.

Ghosal SK, LaMarche PH, Bhanja P, Joardar S, Segupta, A, Midya T. Duration of meiosis and spermatogenesis in the dog. Can J Genet Cytol 1983; 25: 678-681.

Gidley-Baird AA, O'Neill C, Sinosich MJ, Porter RN, Pike IL, Saunders DM. Failure of implantation in human in vitro fertilization and embryo transfer patients: The effects of altered progesterone estrogen ratios in humans and mice. Fertil Steril 1986; 45: 69-74.

Gidley-Baird AA. Endocrine control of implantation and delayed implantation in rats and mice. J Reprod Fertil Suppl 1981; 29: 97-109.

Gier HT. Ecology and behaviour of the coyote (Canis latrans). In: Fox MW (ed.) The Wild Canids. New York: Van Nostrand Reinhold, 1975: 247-262.

Gipson PS, Gipson IK, Sealander JA. Reproductive biology of wild Canis (Canidae) in Arkansas. J Mammol 1975: 56(3): 605-612.

Gipson PS, Sealander JA, Dunn JE. The taxonomic status of wild Canis in Arkansas. Syst. Zool 1974; 23: 1-11.

Goldman EA. Classification of Wolves. In:Young SP, Goldman EA (eds) The Wolves of North America. Washington: Amer Wild Inst, 1944: 389-636.

Goodrowe KL. Applications of reproductive biotechnology to wildlife conservation programs. Proceedings for Annual Meeting; September 17-20; Montreal, Canada. Hastings, Nebraska: Society for Theriogenology: 1997: 30-37.

Goodrowe KL, Hay MA, Platz CC, Behrns SK, Jones MH, Waddell WT. Characteristics of fresh and frozen-thawed red wolf (Canis rufus) spermatozoa. An Reprod Sci 1998: 53: 299-308.

Graf KJ. Serum oestrogen, progesterone and prolactin concentrations in cyclic, pregnant and lactating beagle dogs. J Reprod Fertil 1978; 52: 9-14.

Graham LH, Brown JL. Cortisol metabolism in the domestic cat and implications for non-invasive monitoring of adrenocortical function in endangered felids. Zoo Biol 1996; 15: 71-82.

Graham LH, Goodrowe KL, Raeside JI, Liptrap RM. Non-invasive monitoring of ovarian function in several felid species by measurement of fecal estradiol-17β and progestins. Zoo Biol 1995; 14: 223-237.

Graham LH, Raeside JI, Goodrowe KL, Liptrap RM. Measurements of faecal oestradiol and progesterone in non-pregnant and pregnant domestic exotic cats. J Reprod Fertil Suppl 1993; 47: 119-120.

Green JS, Adam RA, Woodruff RA, Stellflug JN. Seasonal variation in semen production by captive coyotes. J Mammol 1984; 65: 506-509.

Greenwald GS. Luteolytic effect of estrogen on the corpora lutea of the hamster. Endocrinology 1965; 76: 1213-1219.

Griffiths WFB, Amoroso EC. Proestrus, oestrus, ovulation and mating in the grayhound bitch. Vet Rec 1939; 51: 1279-1284.

Gross TS. Development and use of faecal steroid analyses in several carnivore species. In: Proc Int Symp Faecal Steroid Monit Zoo Anim. Zool and Bot Gardens, Rotterdam, Germany, 1992: 55-61.

Gudermuth DF, Concannon PW, Daels PF, Lasely BL. Pregnancy-specific elevations in fecal concentrations of estradiol, testosterone and progesterone in the domestic dog (Canis familiaris). Theriogenology 1998; 50: 237-248.

Hadley JC. Total unconjugated oestrogen and progesterone concentrations in cyclic, pregnant and lactating beagle dogs. J Reprod Fertil 1975; 44: 453-460.

Hagion N, Watanabe M, Golzieher JW. Inhibition by adrenocorticotrophin-induced ovulation in immature female rats. Endocrinology 1969; 84: 308-314.

Hamilton GD, Weeks HP. Cortisol and aldosterone comparisons of cottontail rabbits collected by shooting, trapping and falconry. J Wildl Dis 1985; 21: 40-42.

Hamlett GWD. The reproductive cycle of the coyote. US Dept Agric Tech Bull 1938: 1-12.

Harper MJK. Effects of androstenedione on pre-implantation stages of pregnancy in rats. Endocrinology 1967; 81: 1091-1099.

Harper MJK. Estrogenic effects of dehydroepiandrosterone and its sulfate in rats. Endocrinology 1969; 84: 229-235.

Hartley FGL, Follet BK, Harris S, Hirst D, McNeilly AS. The endocrinology of gestation failure in foxes (Vulpes vulpes). J Reprod Fertil 1994; 100:341-346.

Hay MA. Canine gametes - Evaluation of ooctye maturation and penetrating potential of spermatozoa pre-freeze and post thaw. Guelph, Ontario, Canada: University of Guelph; 1996. 182p. PhD Thesis.

Heape W. The sexual season of mammals and the relationship of "pro-estrus" to menstruation. Part I Q J Micribiol Sci 1900; 44: 1-70.

Hegstad RL, Johnston SD, Pasternak DM. Concentrations and pulse analyses of adrenocorticotrophin and luteinizing hormone in plasma from dogs in pro-oestrus, oestrus, dioestrus or anoestrus. J Reprod Fertil Suppl 1993; 47: 85-91.

Hensleigh PA, Johson DC. Heat stress effects during pregnancy. II. Pituitary gonadotropins in intact, adrenalectomized, and ovariectomized rats. Fertil Steril 1971; 22: 528-535.

Hoffmann B, Schneider S. Secretion and relese of luteinizing hormone during the luteal phase of the oestrous cycle in the dog. J Reprod Fertil Suppl 1993; 47: 85-91.

Holst PA, Phemister RD. Onset of diestrus in the Beagle bitch: Definition and Significance. Am J Vet Res 1974; 35: 401-406.

Holst PA, Phemister RD. Temporal sequence of events in the estrous cycle of the Bitch. Am J Vet Res 1975; 36: 705-706.

Holst PA, Phemister RD. The prenatal development of the dog; pre-implantation events. Biol Reprod 1971; 5: 194-206.

Howard JG, Roth TL, Byers AP, Swanson WF, Wildt DE. Sensitivity to exogenous gonadotrophins for ovulation induction and laparoscopic artifical insemination in the cheetah and clouded leopard. Biol Reprod 1997; 56: 1059-1068.

Howarth B, Hawk HW. Effect of hydrocortisone on embryonic survival in sheep. J Anim Sci 1968; 27: 117-121.

Hutchins M, Wiese RJ. Beyond genetic and demographic management: The future of the Species Survival Plans and related AAZPA conservation efforts. Zoo Biol 1991; 10: 285-292.

Jackson P, Furr B, Hutchinson F. A preliminary study of pregnancy termination in the bitch with slow-release formulations of prostaglandin analogues. J Sm Anim Pract 1982; 23: 287-294.

Jeffcoate IA. Endocrinology of anoestrous bitches. J Reprod Fertil Suppl 1993; 47: 69-76.

Jeffcoate IA, England GCW. Urinary LH, plasma LH and progesterone and their clinical correlates in the periovulatory period of domestic bitches. J Reprod Fertil Suppl 1997; 51: 267-275.

Jöchle W, Arbeiter K, Post K, Ballabio R, D'Ver AS. Effects of pseudopregnancy, pregnancy and interoestrous intervals of pharmacological suppression of prolactin secretion in female dogs and cats. J Reprod Fertil Suppl 1989; 39: 199-207.

Johnston LA, Armstrong DL, Brown JL. Seasonal effects on seminal and endocrine traits in the captive snow leopard (Panthera uncia). J Reprod Fertil 1994; 102: 229-236.

Johnston SD, Mather EC. Canine plasma cortisol (hydrocortisone) measured by radioimmunoassay: Clinical absence of diurnal variation and results of ACTH stimulation and dexamethasone suppression tests. Am J Vet Res 1978; 39: 1766-1770.

Johnston SD. Breeding management of the bitch. In: Ettinger SJ, Feldman EC (eds.) Textbook of Veterinary Internal Medicine. Diseases of the dog and cat. Philadelphia, WB Saunders Co., 1995: 1604-1606.

Jones CA, Refsal KR, Lippert AC, Nachreiner RF, Schwacha MM. Changes in the adrenal cortisol secretion as reflected in the urinary cortisol/creatinine ratio in dogs. Dom Anim Endocrinol 1990; 7: 559-572.

Jones GE, Baker K, Fahmy DR, Boyns AR. Effect of luteinizing hormone releasing hormone on plasma levels of luteinizing hormone, oestradiol and testosterone in the male dog. J Endocrinol 1976; 68: 469-474.

Jones GE, Boyns AR, Cameron EHD, Bell ET, Christie DW, Parkes MF. Plasma oestradiol, lutenizing hormone and progesterone during pregnancy in the Beagle bitch. J Reprod Fertil 1973; 35: 187-189.

Jones GE, Boyns AR. Inhibition by oestradiol of the pituitary response to luteinizing hormone releasing hormone in the dog. J Endocrinol 1976; 68: 475-479.

Jurke MH, Czekala NM, Linburg DG, Millard SE. Fecal corticoid metabolite measurement in the cheetah (Acinonyx jubatus). Zoo Biol 1997; 16: 133-147.

Kaplan JB, Mead RA. Seasonal changes in testicular function and seminal characteristics of the male eastern spotted skunk (Spilogale putorius ambarvilus). J Mammol 1994; 75(4): 1013-1020.

Kawakami E, Hori T, Tsutsui T. Changes in plasma luteinizing hormone, testosterone and estradiol-17β levels and semen quality after injections of gonadotropin releasing hormone agonist and human chorionic gonadotropin in three dogs with oligozoospermia and two dogs with azoospermia. Anim Reprod Sci 1997; 47: 157-167.

Kennelly JJ, Johns BE. The estrous cycle of the coyotes. J Wildl Manage 1976; 40: 272-277.

Kennelly JJ. Coyote reproduction.I. The duration of the spermatogenic cycle and epididymal sperm transport. J Reprod Fertil 1972: 31: 163-170.

Kiso Y, Yamauchi S. Hisotchemical study on hydroxysteriod dehydrogenases in the trophoblast of the dog placenta. Jap J Vet Sci 1984: 46: 219-233.

Knight PJ, Hamilton JM, Hiddleston WA. Serum prolactin during pregnancy and lactation in the beagle bitch. Vet Rec 1977: 101: 202.

Knol BW, Dieleman SJ, Bevers MM, van der Brom WE. GnRH in the male dog: dose-response relationship with LH and testosterone. J Reprod Fertil 1993: 98: 159-161.

Koehler JK, Platz CC Jr., Waddell W, Jones MH, Smith R, Behrns S. Spermophagy in semen in the red wolf (Canis rufus). Mol Reprod Dev 1994: 37: 457-461.

Koehler JK, Platz CC Jr., Waddell W, Jones MH, Behrns S. Semen parameters and electron microscope observations of spermatozoa of the red wolf, Canis rufus. J Reprod Fertil 1998: 114: 95-101.

Kreeger TJ, Seal US, Cohen Y, Plotka ED, Asa CS. Characterization of prolactin secretion in gray wolves (Canis lupus). Can J Zool 1991: 69: 1366-1374.

Kuroda H, Hiroe K. Studies on the metabolism of dog sperm. I. Seasonal variation on the semen quality and the of the aerobic metabolism of spermatozoa Jpn J Anim Reprod 1972: 17: 89-98.

Kurten B, Anderson E. Pleistocene Mammals of North America. New York: Columbia University Press, 1980.

Lacoste D, Arnaud R, Bleanger A, Labrie F. A pure antiandrogen does not interfere with the LHRH agonist-induced blockage of testicular androgen secretion in the dog. Mol Cell Endocrinol 1988: 56: 141-147.

Lammond DR. Synchronization of oestrus and ovulation in beef heifers. Proc Aust Soc Anim Prod 1962; 4: 72-78.

Lasely BL, Kirkpatrick JF. Monitoring ovarian function in captive and free-ranging wildlife by means of urinary and fecal steroids. J Zoo Wildl Med 1991; 22: 23-31.

Lawrence B, Bossert WH. Multiple character analysis of Canis lupus, latrans, and familiaris, with a discussion of the relationships of Canis niger. Amer Zool 1967; 7:223-232.

Lentfer JW, Englund J. Notes on the captive wolf (Canis lupus) colony, Barrow, Alaska. Can J Zool 1973: 51: 623-627.

Lincoln GA. Seasonal Aspects of Testicular Function. In: Burger H, de Kretser D (eds.) The Testis. New York: Raven Press Ltd, 1989: 329-385.

Linde-Forsberg C, Forsberg M. Fertility in dogs in relation to semen quality and the time and site of insemination with fresh and frozen semen. J Reprod Fertil Suppl 1989; 39: 299-310.

Linde-Forsberg C, Forsberg M. Results of 527 controlled artificial inseminations in dogs. J Repord Fertil Suppl 1993; 47: 313-323.

Lindeberg H, Jalkanen L, Savolainen R. In vitro culture of silver fox embryos. Theriogenology 1993; 40: 779-788.

Liptrap RM. Stress and reproduction in domestic animals. In: Corticotropin-Releasing Factor and Cytokines: Role in the Stress Response. Ann NY Acad Sci 1993; 697: 275-283.

Lloyd GH, Englund J. The reproductive cycle of the red fox in Europe. J Reprod Fertil Suppl 1973; 19: 119-130.

Lucas Z, Raeside JI, Betteridge K. Non-invasive assessment of the incidences of pregnancy and pregnancy loss in the feral horses of Sable Island. J Reprod Fertil 1991; 44: 479-488.

MacKenzie AH, Thwaites CJ, Edey TN. Oestrous, ovarian and adrenal response of the ewe to fasting and cold stress. Aust J Agric Res 1975; 26: 545-552.

MacMillan KL, Watson JD. Short estrous cycles in New Zealand dairy cattle. J Dairy Sci 1971; 54: 1526-1529.

Mason JW. "Over-all" hormonal balance as a key to endocrine orginization. Psychosom Med 1968; 30: 791-808.

Matsuda DM, Bellem AC, Gartley CJ, Madison V, King WA, Liptrap RM, Goodrowe KL. Endocrine and behavioral events of estrous cyclicity and synchronization in wood bison (Bison bison athabascae). Theriogenology 1996; 45: 1429-1441.

Matteri, R.L., Roser, J.F., Baldwin, D.M., Lipovetsky, V.,and H. Papkoff. Characterization of a monoclonal antibody which detects luteinizing hormone from diverse mammalian species. Dom Anim Endocrinol 1987; 4:157-165.

McCann JP, Altszuler N, Hampshire J, Concannon PW. Growth hormone, insulin, glucose, luteinizing hormone, and diabetes in beagle bitches treated with medroxyprogesterone acetate. Acta Endocrinol Copenh 1987; 116: 73-80.

McCann JP, Temple M, Concannon PW. Pregnancy-specific alterations in the metabolic endocrinology of domestic dogs including insulin resistance and modified regulation of growth hormone. Proc 11th Int Congr Anim Reprod & AI, Dublin 1988; 2: 103-105.

McCarley H, Carley CL. Recent changes in distribution of wild red wolves (Canis rufus). USFWS Endangered Species Report No 4, Albuquerque, NM, 1979, 38p.

McCarley H. The taxonomic status of wild Canis (Canidae) in the South-central United States. S W Nat 1962; 7: 227-235.

McGuckin MA, Blackshaw AW. Mating-associated peak in plasma testosterone concentration in wild gray-headed flying foxes (Pteropus poliocephalus). J Reprod Fertil 1991a; 92: 347-352.

McGuckin MA, Blackshaw AW. Seasonal changes in testicular size, plasma testosterone concentration and body weight in captive flying foxes (Pteropus poliocephalus and P. scapulatus). J Reprod Fertil 1991; 92: 339-346.

McLeod PJ, Moger WH, Ryon J, Gadbois S, Fentress JC. The relation between urinary cortisol and social behaviour in captive timber wolves. Can J Zool 1996; 74: 209-216.

McRae GI, Roberts BB, Worden AC, Bajka A, Vickery BH. Long-term reversible suppression of oestrus in bitches with nafarelin acetate, a potent LHRH agonist. J Reprod Fertil 1985; 74: 389-397.

Mech DL. Longevity in wild wolves. J Mammal 1988; 69: 197-198.

Mech DL. Premature reproductive activity in wild wolves. J Mammal 1987; 68: 871-873.

Mech DL. The Way of the Wolf. Stilwater, MN: Voyageur Press Inc., 1991.

Mech DL. The Wolf. New York, NY: Doubleday, 1970.

Medjo D, Mech DL. Reproductive activity in nine and ten month old wolves. J Mammol 1976; 57: 406-408.

Mialot JP, Thibier M, Toublanc JE, Castanier M, Scholler R. Plasma concentrations of luteinizing hormone, testosterone, dehydroepiandrosterone, androstenedione between birth and one year in the male dog: Longitudinal study and hCG stimulation. Andrologia 1988; 20: 145-154.

Miller MW, Hobbs NT, Sousa MC. Detecting stress responses in Rocky Mountain bighorn sheep (Ovis canadensis canadensis): Reliability of cortisol concentrations in urine and feces. Can J Zool 1991; 69: 15-24.

Mirarchi FE, Howland BE, Scanlon PF, Kirkpatrick RL, Sanford LM. Seasonal variation in plasma LH, FSH, prolactin, and testosterone concentrations in adult male white-tailed deer. Can J Zool 1978; 56: 121-127.

Mitsuzuka M. Collection, evaluation and freezing of wolf sperm. In: Frank H (ed.) Man and Wolf: Advances, Issues, and Problems in Captive Wolf Research. Dordrecht, Netherlands: Dr W Junk Publishers, 1987: 127-141.

Moberg GP. Influence of stress on reproduction: Measure of well-being. In: Moberg GP (ed.) Animal Stress. Bethesda: American Physiological Society, 1985a: 245-267.

Moberg, GP, Watson JG, Hayashi K. Effects of adrenocorticotrophin treatment on estrogen, lutenizing hormone, and progesterone secretion in the female rhesus monkey. J Med Primatol 1982; 11: 235-241.

Moehlman PD. Introduction. In: Sheldon JW (Ed) Wild Dogs. The natural history of the nondomestic candae. San Diego: Academic Press Inc., 1992: 1-3.

Möller OM. Progesterone concentration in the perphieral plasma of the blue fox (Alopex lagopus) during pregnancy and the oestrous cycle. J Endocrinol 1973; 59: 429-438.

Mondain-Monval M, Smith AJ, Simon P, Möller OM, Scholler R, McNeilly AS. Effect of melatonin implantation on the seasonal variation of FSH secretion in the male blue fox (Alopex lagopus). J Reprod Fertil 1988; 83: 345-354.

Monfort SL, Wasser SK, Machburn KL, Burke M, Brewer BA, Creel SR. Steroid metabolism and validation of noninvasive endocrine monitoring in the African wild dog (Lycacon pictus). Zoo Biol 1997; 16: 533-548.

Morgan PJ, Mercer JG. Symposium on 'Seasonality'. Proc Nutr Soc 1994; 53: 483-493.

Morton DJ, Anderson E, Foggin CM, Kock MD, Tiran EP. Plasma cortisol as an indicator of stress due to capture and translocation in wildlife species. Vet Rec 1995; 136: 60-63.

Möstl E, Brunner I. Comparison of different progestagen assays for measuring progesterone metabolites in faeces of the bitch. J Vet Med 1997; 44: 573-578.

Möstl E, Lehmann H, Wenzel U. Gestagens in the faeces of mink and cats for monitoring corpus luteum activity. J Reprod Fertil (Suppl) 1993; 47: 540-541.

Munro CJ, Stabenfeldt F. Development of a cortisol enzyme immunoassay in plasma. Clin Chem 1985; 31: 956 (Abstract).

Munro CJ, Stabenfeldt GH, Cragun JR, Addiego LA, Overstree JW, Lasley BL. The relationship of serum estradiol and progesterone concentrations to the excretion profiles of their major urinary metabolites as measured by enzymeimmunoassay and radioimmunoassay. Clin Chem 1991; 37: 838-844.

Nalbandov AN. Reproductive Physiology. San Francisco, CA; Freeman. 1964, p 135.

Nequin LG, Schwartz NB. Adrenal participation in the timing of mating and LH release in the cyclic rat. Endocrinology 1971; 88: 325-331.

Nett TM, Akbar AM, Phemister RD, Holst PA, Reichert LE, Jr., Niswender GD. Levels of lutenizing hormone, estradiol and progesterone in the serum during the estrous cycle and pregnancy in the beagle bitch. Proc Soc Exp Biol Med 1975; 148: 134-139.

Nowak RM. North Americam Quaternary Canis. Museum of Natural History, University of Kansas Monograph 6. 1979, 154p.

Nowak RM. The mysterious wolf of the south. Nat Hist 1972; 81: 51-53.

Nowak RM. The red wolf is not a hybrid. Cons Biol 1992; 6(4): 593-595.

Nowak RM, Federoff NE. Systematics of Wolves in Eastern North America. Proc Defenders of Wildlife's Wolves of North America: Albany, NY 1996; 187-194.

Okkens AC, Bevers M, Dieleman S, Willemse AH. Shortening of the interestrous interval and lifespan of the corpus luteum of the cyclic dog by bromocriptine treatment. Vet Q 1985a; 7: 173-176.

Okkens AC, Bevers MM, Dieleman SJ, Willemse AH. Evidence for prolactin as the main luteotrophic factor in the cyclic dog. Vet Q 1990; 12: 193-201.

Okkens AC, Dieleman SJ, Bevers MM, Lubberink AAME, Willemse AH. Influence of hypophysectomy on the lifespan of the corpus luteum in the cyclic dog. J Reprod Fertil 1986; 77: 187-192.

Okkens AC, Dieleman SJ, Bevers MM, Willemse AH. Evidence for the noninvolvement of the uterus in the lifespan of the corpus luteum in the cyclic dog. Vet Q 1985b; 7: 169-173.

Olar TT. Using frozen canine semen: a guide for practitioners. Vet Med 1985; March: 22-30.

Olson PN, Bowen RA, Behrendt M, Olson JD, Nett TM. Concentrations of reproductive hormones in canine serum throughout late anestrus, proestrus and estrus. Biol Reprod 1982; 27: 1196-1206.

Olson PN, Bowen RA, Behrendt MD, Olson JD, Nett TM. Concentrations of testosterone in canine serum during late anestrus, proestrus, estrus and early diestrus. Am J Vet Res 1984b; 45: 145-148.

Olson PN, Bowen RA, Behrendt MD, Olson JD, Nett TM. Concentrations of progesterone and luteinizing hormone in the serum of diestrous bitches before and after hysterectomy. Am J Vet Res 1984c; 45: 149-153.

Olson PN, Husted PW, Allen TA, Nett TM. Reproductive endocrinology and physiology of the bitch and queen. Veterinary Clinics of North America: Small Animal Practice 1984a; 14: 927-946.

Olson PN, Nett TM, Bowen RA, Sawyer HR, Niswender GD. Endocrine regulation of the corpus luteum of the bitch as a potential target for altering fertility. J Reprod Fertil Suppl 1989; 39: 27-40.

Onclin K, Verstegen JP. In vivo investigation of luteal function in dogs: effects of cabergoline, a dopamine agonist, and prolactin on progesterone secretion during mid-pregnancy and -dioestrus. Dom Anim Endocrinol 1996; 14: 25-38.

Onclin K, Verstegen JP. Secretion patterns of plasma prolactin and progesterone in pregnant compared with nonpregnant dioestrous bitches. J Reprod Fertil Suppl 1997; 51: 203-208.

Othen LS, Bellem AC, Gartley CJ, Aukland K, King WA, Liptrap RM, Goodrowe KL. Hormonal control of estrous cyclicity and attempted superovulation in wood bison (Bison bison athabascae). Theriogenology 1999; 54: 313-323.

Paradis M, Post K, Mapletoft RJ. Effects of prostaglandin F2α on corpora lutea formation and function in mated bitches. Can Vet J 1983; 24: 239-242.

Paradiso JM, Nowak RM. A report on the taxonomic status and distribution of the red wolf. USFWS Spec Sci Rept Wildl 1971; No 145: 1-36.

Paramo RM, Renton JP, Ferguson JM, Concannon PW. Effects of medroxyprogesterone acetate or gonadotrophin-releasing hormone on suppression of spermatogenesis in the dog (Cains familiaris). J Reprod Fertil Suppl 1993; 47: 387-397.

Parker WT. The red wolf. In: Chandler WJ, Labate L. (Eds.) Audubon Wildlife Report 1988/1989. San Diego: Academic Press, Inc., 1988: 597-607.

Parkes AS, Bellerby CA. Studies on the internal secretions of the ovary. 2. The effects of injections of the oestrus producing hormone during pregnancy. J Physiol 1926; 62: 145-151.

Parkes AS, Bruce HM. Pregnancy-block in female mice placed in boxes soiled by males. J Reprod Fertil 1962; 4: 303-308.

Pauiraj S, Sundararajan N, Manimozhi A, Walker S. Reproduction of the Indian wild dog (Cuon alpinus) in captivity. Zoo Biol 1992; 11: 235-241.

Penton JP, Boyd JS, Eckersall PD, Ferguson JM, Harvey MJA, Mullaney J, Perry B. Ovulation, fertilization and early embryonic development in the bitch (Canis familiaris). J Reprod Fertil 1991; 93: 221-231.

Phemister RD, Holst PA, Spano JS, Hopwood ML. Time of ovulation in the beagle bitch. Biol Reprod 1973; 8: 74-82.

Phillips MK, Henry VG. Comments on red wolf taxonomy. Cons Biol 1992; 6: 596-599.

Pimlott DH, Joslin PWD. The status and distribution of the red wolf. Trans N Am Wildl Nat Res 1968; 33:373-389.

Porton IJ, Kleiman DG, Rodden M. Aseasonality of bush dog reproduction and the influence of social factors on the estrous cycle. J Mammal 1987; 68: 867-871.

Puswell BJ, Wilcke JR. Response to gonadotrophin-releasing hormone by the intact male dog: serum testosterone, luteinizing hormone and follicle-stimulating hormone. J Reprod Fertil Suppl 1993; 47: 335-341.

Putney DJ, Drost M, Thatcher WW. Embryonic development in superovulated dairy cattle exposed to elevated ambient temperatures between days 1 to 7 post insemination. Theriogenology 1988; 30: 195-209.

Ramaley JA. Stress and Fertility. In: Gilmore D, Cook B (eds.) Enviormental factors in mammal reproduction. London:MacMillian Publishers Ltd, 1981: 127-141.

Reimers T, Phemister R, Niswender G. Radioimmunological measurement of follicle stimulating hormone and prolactin in the dog. Biol Reprod 1978: 19: 673-679.

Rich TD, Turman EJ, Hillier JC. A comparison of the ovulation rate, fertilization rate and embryo survival of hand-mated and lot-mated gilts. J Anim Sci 1968; 27: 443-446.

Richkind M. Possible use of early moring urine for dectection of pregnancy in dogs. Vet Med/Small Animal Clinic 1983; 78: 1067-1068.

Riley GA, McBride RT. A survey of the red wolf (Canis rufus). USFWS Spec Sci Rep Wildl 1972; No 162: 1-15.

Robeck TR, Schneyer AL, McBain JF, Dalton LM, Walsh MT, Czekala NM, Kraemer DC. Analysis of urinary immunoreactive steroid metabolites and gonadotropins for characterization of the estrous cycle, breeding period, and seasonal estrous activity of the captive killer whales (Orcinis orca). Zoo Biol 1993; 12: 173-187.

Roblero LS, Garavagno AC. Effect of oestradiol-17β and progesterone on oviductal transport and early development of mouse embryos. J Reprod Fertil 1979; 57: 91-95.

Rodden MD, Sorenson LG, Sherr A, Kleiman DG. Use of behavioural measures to assess reproductive status in maned wolves (Chrysocyon brachyurus). Zoo Biol 1995: 15: 565-585.

Rowell TE. Baboon menstrual cycles affected by social environments. J Reprod Fertil 1970; 21: 133-141.

Roy MS, Geffen E, Smith D, Ostrander EA, Wayne RK. Patterns of differentiation and hybridization in North American wolflike canids, revealed by analysis of micosatellite loci. Mol Biol Evol 1994a; 11: 553-570.

Roy MS, Girman DJ, Taylor AC, Wayne RK. The use of museum specimens to reconstruct the genetic variability and relationships of extinct populations. Experientia 1994b; 50: 551-557.

Runner ML. Embryocidal effects of handling pregnant mice and its prevention with progesterone. Anat Rec 1959; 133: 330-331.

Schulte BA, Seal US, Plotka ED, Letellier MA, Verme LH, Ozaga JJ, Parsons JA. The effect of pinealectomy on seasonal changes in prolactin secretion in the white-tailed deer (Odocoileus virginianus borealis). Endocrinol 1981; 108: 173-178.

Schwartz NB. Acute effects of ovariectomy on pituitary LH, uterine weight and vaginal cornification. Am J Physiol 1964; 207: 1251-1259.

Scott GH, Williams RJ. Causes of low breeding efficiency in dairy cattle associated with seasonal high temperatures. J Dairy Sci 1962; 45: 1369-1375.

Seager SWJ, Platz CC, Hodge W. Successful pregnancy using frozen semen in the wolf. Int Zoo Yearbk 1975; 15: 140-143.

Seal US, and Mech LD. Blood indicators of seasonal metabolic patterns in captive adult gray wolves. J Wildl Mange 1983; 47: 704-715.

Seal US, Plotka ED, Mech DL, Packard JM. Seasonal metabolic and reproductive cycles in wolves. In: Frank H (ed.) Man and Wolf: Advances, Issues, and Problems in Captive Wolf Research. Dordrecht, Netherlands: Dr W Junk Publishers, 1987: 109-125.

Seal US, Plotka ED, Packard JM, Mech DL. Endocrine correlates of reproduction in the wolf.I. Serum progesterone, estradiol and LH during the estrous cycle. Biol Reprod 1979; 21: 1057-1066.

Sheldon JW. Wild Dogs, The natural history of the nondomestic canidae. San Diego: Academic Press Inc., 1992.

Shideler SE, Ortuno AM, Moran FM, Moorman EA, Lasley BL. Simple extraction and enzyme immunoassays for estrogen and progesterone metabolites in the feces of Macaca fascicularis during non-conceptive and conceptive ovarian cycles. Biol Reprod 1993; 48: 1290-1298.

ShilleVM, Stabenfeldt GH. Clinical reproductive physiology in dogs. In: Morrow DA (ed.) Current Therapy in Theriogenology, Philadelphia: WB Saunders Co., 1986: 571-574.

Shille VM, Haggerty MA, Shackleton C, Lasley BL. Metabolites of estradiol in serum, bile, intestine and feces of the domestic cat (Felis catus). Theriogenology 1990; 34: 779-794.

Shille VM, Kollias GV, Thatcher MJ, Waterman L. Determination of reproductive status in the serval and bobcat using a validated, direct radioimmunoassay of fecal estradiol. Biol Reprod 1991; (Suppl 1) 44: 121.

Shille VM, Wing AE, Lasley BL, Banks JA. Excretion of radiolabeled estradiol in the cat (Felis catus, L.): A preliminary report. Zoo Biol 1984; 3: 201-209.

Smith AJ, Clausen OPF, Kirkhus B, Jahnsen T, Möller OM, Hannson V. Seasonal changes in spermatogenesis in the blue fox (Alopex lagopus), quantified by DNA flow cytometry and measurement of soluble Mn^{2+} -dependant adenylate cyclase activity. J Reprod Fertil 1984; 72: 453-461.

Smith AJ, Mondain-Monval M, Berg KA, Gordeladze JO, Clausen OPF, Simon P, Scholler R. Sexual development in the immature male blue fox (Alopex lagopus), investigated by testiscular histology, DNA flow cytometry and measurements of plasma FSH, LH, testosterone and soluble testicular Mn^{2+} -dependant adenylate cyclase activity. J Reprod Fertil 1987; 81: 505-515.

Smith AJ, Mondian-Monval M, Möller OM, Scholler R, Hansson V. Seasonal variations of LH, prolactin, androstenedione, testosterone, and testicular FSH binding in the male blue fox (Alopex lagopus). J Reprod Fertil 1985; 74: 449-458.

Smith DM, Biggers JD. The oestrogen requirement for implantation and the effect of its dose on the implantation response in the mouse. J Endocrinol 1968; 41: 1-9.

Smith FO, Graham EF. Cryopreservation of canine semen: Technique and Performance. Proc 10th Int Congr Anim Reprod & AI, Urbana, Champaign, 1984: 216.

Smith MS, McDonald LE. Serum levels of luteinizing hormone and progesterone during the estrous cycle, pseudopregnancy and pregnancy in the dog. Endocrinology 1974; 94: 404-412.

Soulé M, Gilpin M, Conway W, Foose T. The millenium ark: How long a voyage, how many staterooms, how many passengers? Zoo Biol 1986; 5: 101-113.

Stabenfeldt GH, Shille VM. Reproduction in the dog and cat. In: Cole HH, Cupps PT (eds.) Reproduciton in Domestic Animals. New York, Academic Press, 1977: 499-527.

Steinetz B, Goldsmith L, Lust G. Plasma relaxin levels in pregnant and lactating dogs. Biol Reprod 1987; 37:719-725.

Steinetz BG, Goldsmith LT, Harvey HJ, Lust G. Serum relaxin and progesterone concentrations in pregnant, pseudopregnant, and ovariectomized, progestin-treated, pregnant bitches: detection of relaxin as a marker of pregnancy. Am J Vet Res 1989; 50: 68-71.

Stellflug JN, Muse PD, Everson DO, Louis TM. Changes in serum progesterone and estrogen of the nonpregnant coyote during the breeding season. Proc Soc Exp Bio Med 1981; 167: 220-223.

Stoebel DP, Moberg GP. Effects of adreoncorticotropin and cortisol on lutenizing hormone surge and estrous behaviour of cows. J Dairy Sci 1982; 65: 1016-1024.

Stone GM, Emmens CW. The action of oestradiol and dimethylstilboestrol on early pregnancy and deciduoma formation in the mouse. J Endocrinol 1964; 29: 137-145.

Strott CA, Sundel H, Stahlman MT. Maternal and fetal plasma progesterone, cortisol, testosterone and 17β-estradiol in preparturient sheep: Response to fetal ACTH infusion. Endocrinology 1975; 95: 1327-1332.

Taha MB. Studies on semen composition and testicular function in the dog, with particular reference to the effect of season, age and exogenous hormones. London: University of London; 1980. PhD Thesis.

Thatcher MJD, Shille VM, Buhi WC, Alvarez IM, Concannon PW, Thibeault D. Canine conceptus appearance and de novo protein synthesis in relation to the time of implantation. Theriogenology 1994; 41: 1679-1692.

Tsutsui T. Gamete physiology and timing of ovualtion and fertilization in dogs. J Reprod Fertil Suppl 1989; 39: 269-275.

Tsutsui T. Studies on reproduction in the dog. V. On cleavage and transpost of fertilized ova in the oviduct. Jpn J Anim Reprod 1975; 21; 70-75.

Tsutsiu T, Shimizu T. Studies on the reproduction in the dog. IV. On the fertile period of ovum afer ovualtion. Jpn J Anim Reprod 1975; 21: 65-69.

Tudge C. Last Animals at the Zoo. Washington DC: Island Press, 1992. 266p.

Ulberg LC. Effect of macro- and mircoenvironment on the biology of mammalian reproduction. In: Shaw ROH (ed) Ground Level Climatology. Washinton DC: Am Assoc Adv Sci. 1967: 265-276.

[USFWS]. Red Wolf recovery plan. U.S. Fish and Wildlife Service, Atlanta, GA. 1984. 37p.

[USFWS]. Red Wolf recovery plan. U.S. Fish and Wildlife Service, Atlanta, GA. 1990. 110p.

Valtonen MH, Rajakoski EJ, Makela JI. Reproductive features in the female raccoon dog (Nyctereutes procyonoides). J Reprod Fertil 1977; 51: 517-518.

van Haaften B, Bevers MM, van der Brom WE, Dieleman SJ, Okkens AC, van Sluijs FJ, Willemse AH. Increasing sensitivity of the pituitary for GnRH from early to advanced anoestrus in the beagle bitch. Proc 12th Inter Cong Anim Reprod & AI 1992; 4: 1829-1831.

van Haaften B, Dieleman SJ, Okkens AC, Bevers MM, Willemse AH. Induction of oestrus and ovulation in dogs by treatment with PMSG and/or bromocriptine. J Reprod Fertil Suppl 1989; 39: 330-331.

Vanderlip SL, Wing AE, Felt P, Linke D, River J, Concannon PW, Lasley BL. Ovulation induction in anestrous bitches by pulsaltile administration of gonadotrophin-releasing hormone. Lab Anim Sci 1987; 37: 459-464.

Velerado JT. Action of adrenocorticotropin on pregnancy and litter size in rats. Am J Physiol 1957; 191: 319-322.

Velloso AI, Wasser SK, Monfort SL, Dietz JM. Longitudinal fecal steroid excretion in maned wolves. Gen Comp Endocrinol 1998; 112: 96-107.

Vickery B, McRae G. Effects of synthetic PG analogue on pregnancy in beagle bitches. Biol Reprod 1980; 22: 438-442.

Vickery BH, Bergstrom K, Miller M, Goodpasture JC. Effect of Detirelix, a long acting LHRH antagonist, on luteal function and pregnancy in beagle bitches. Biol Reprod 1987; 36 (Supppl): 145 (Abstract)

Vincent IC, Michell AR. Comparison of cortisol concentrations in saliva and plasma of dogs. Res Vet Sci 1992; 53: 342-345.

Waddell, WT. Red Wolf (Canis rufus) International Studbook, 1995.

Waddell WT. AZA Species Survival Plan Profile: Red Wolves. Endangered Species Update 1996; 13 (10 & 11): 10-11.

Waddell, WT. Red Wolf (Canis rufus) International Studbook, 1998 (in preparation, 1999).

Waddell, WT, Henry GV. Red wolf: recovery of a native species. In: Wagner RO (ed.) AZA Regional Conference Proceedings. Wheeling, WV, 1996: 388-392.

Wagon KA, Rollins WC, Cupps PT, Carroll FD. Effects of stress factors on the estrous cycles of beef heifers. J Anim Sci 1972; 34: 1003-1010.

Walker LA, Cornell L, Dahl KD, Czekala NM, Dargan CM, Joseph B, Hsueh AJW, Lasley BL. Urinary concentrations of ovarian steroid hormone metabolites and bioactive follicle-stimulating hormone in killer whales (Orcinus orca) during ovarian cycles and pregnancy. Biol Reprod 1988; 39: 1013-1020.

Wasser SK, Velloso AL, Rodden MD. Using fecal steroids to evaluate reproductive function in female maned wolves. J Wildl Manage 1995; 59: 889-894.

Wasserman GF, Baldi A, del Castillo E, Charreau E. Metabolism of [^{3}H]-testosterone by the adrenal of the dog: the action of ACTH. J Steroid Biochem 1973; 4: 217-226.

Wayne RK, Gittleman JL. The problematic red wolf. Scientific America, July 1995: 36-39.

Wayne RK, Jenks SM. Mitochrondrial DNA analysis implying extensive hybridization of the endangered red wolf Canis rufus. Nature, London 1991; 351: 565-568.

Wayne RK. On the use of morphologic and molecular genetic characters to investigate species status. Cons Biol 1992; 6: 591-592.

Wiese RJ, Hutchins M (eds). Species Survival Plans: Strategies for Wildlife Conservation. Bethesda, MD: American Zoo and Aquarium Association, 1994, 64p.

Wesson JA, Scanlon PF, Kirkpatrick RL, Mosby HS, Butcher RL. Influence of chemical immobilization and physical restraint on steroid hormone levels in blood of white-tailed deer. Can J Zool 1979; 57: 768-776.

Whitney R, Burdick HO. Tube-locking of ova by oestrogenic substances. Endocrinology 1936; 20: 643-647.

Wiebold JL, Stanfield PH, Becker WC, Hillers JK. The effects of restraint stress in early pregnancy in mice. J Reprod Fertil 1986; 78: 185-192.

Wildt DE, Chakraborty PK, Panko, Seager WB. Relationship of reproductive behaviour, serum luteinizing hormone and time of ovulation in the bitch. Biol Reprod 1978; 18: 561-570.

Wildt DE, Howard JG, Chakraborty PK, Bush M. Reproductive physiology of the clouded leopard. II. A circannual analysis of adrenal-pituitary-testicular relationships during electroejaculation or after an adrenocorticotropin hormone challenge. Biol Reprod 1986; 34: 949-959.

Wildt DE, Levinson CJ, Seager SWJ. Laparoscopic exposure and sequential observation of the ovary of the cycling bitch. Anat Biol 1977; 189: 443-450.

Wildt DE, Meltzer D, Chakraborty PK, Bush M. Adrenal-testicular-pituitary relationships in the cheetah subjected to anesthesia/electroejaculation. Biol Reprod 1984; 30: 665-672.

Wildt DE, Panko WB, Chakraborty P, Seager SW. Relationship of serum estrone, estradiol-17β and progesterone to LH, sexual behaviour and time of ovulation in the bitch. Biol Reprod 1979; 20: 648-658.

Wildt DE, Seager SWJ, Chakraborty PK. Behavioral, ovarian and endocrine relationships in the pubertal bitch. J Anim Sci 1981; 53: 182-191.

Wilson EO. Conservation: The next hundred years. In: Western D, Pearl M (eds.) Conservation for the twenty-first century. New York: Oxford University Press. 1989: 3-7.

Wilson MS. Non-surgical intrauterine artifical insemination in bitches using frozen semen. J Reprod Fertil Suppl 1993; 47: 307-311.

Winter M, Pirmann J, Falvo RE, Schanbacher BD, Miller J. Steroid control of gonadotrophin secretion in the orchidectomized dog. J Reprod Fertil 1982; 64: 449-455.

Worgul TJ, Santen RJ, Samojlik E. Evidence that brain aromatization regulation LH secretion in the male dog. Am J Physiol 1981; 241: E241-E250.

Wright PJ, Stelmasiak T, Black D, Sykes D. Medroxyprogesterone acetate and reproductive processes in male dogs. Aust Vet J 1979; 55: 437-438.

Yonjun X, Shuxian J, Yuren T, Jianhua S, and Guosen W. Seasonal changes in testicular size and serum testosterone levels and the relationship between testosterone concentrations and reproductive performance in male raccoon dogs. Scientifur 1994; 18: 39-43.

Young KM. Reproductive biology of the black-footed ferret (Mustela nigripes). Guelph, Ontario, Canada: University of Guelph; 1998, 119 p. MSc Thesis.

Zimen E. On the regualtion of pack size in wolves. Z Tierpsychol 1976; 40: 300-341.

Zondek B, Tamari I. Effects of audigenic stimulation on genital function and reproduction. Am J Obstet Gynecol 1960; 80: 1041-1048.

APPENDIX I.A

CHEMICALS

Acetonitrile	Caledon Laboratories Ltd., Georgetown, Ont., Canada
ABTS 2,2' - Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)	Fisher Scientific, Unionville Ont., Canada
Aluminum oxide powder	BDH Ltd., Burlington, Ont., Canada
Bovine serum albumin (Fraction V)	Sigma Chemical Co., St. Louis, MO, USA
Citric acid	Fisher Scientific, Unionville Ont., Canada
Cortisol antiserum (R4866)	C.J. Munro, University of California, Davis, USA
Cortisol/horseradish peroxidase	C.J. Munro, University of California, Davis, USA
Diethyl ether	Caledon Laboratories Ltd., Georgetown, Ont. Canada
EDTA	Sigma Chemical Co., St. Louis, MO, USA
Estradiol-17β antiserum (R4972)	C.J. Munro, University of California, Davis, USA
Estradiol-17β standard (1,3,5 [10]-Estratriene-3,17β-diol)	C.J. Munro, University of California, Davis, USA
Estradiol-17β /horseradish peroxidase	C.J. Munro, University of California, Davis, USA
Hydrocortisone standard (11β, 17α, 21-Trihydroxypregn-4-ene-3, 20-dione)	Sigma Chemical Co., St. Louis, MO, USA
Hydrogen peroxide	Fisher Scientific, Unionville, Ont., Canada
Lutenizing hormone antiserum (518-B7)	J.Brown, National Zoological Park Conservation Research Center, Front Royal VA, USA
Lutenizing hormone ovine standard	J.Brown, National Zoological Park Conservation Research Center, Front Royal, VA, USA

Lutenizing hormone ovine tracer	J.Brown. National Zoological Park Conservation Research Center. Front Royal. VA. USA
Methanol	BDH Inc.. Toronto. Ont.. Canada
Normal Mouse Serum	Sigma Chemical Co.. St. Louis. MO. USA
Phenol red	Sigma Chemical Co.. St. Louis. MO. USA
Polyethylglycol (PEG)	Fisher Scientific. Nepean. Ont.. Canada
Progesterone antiserum (R4861)	C.J. Munro. University of California. Davis. USA
Progesterone/horseradish peroxidase	C.J. Munro. University of California. Davis. USA
Progesterone standard (4-pregnen-3,20-dione)	Sigma Chemical Co.. St. Louis. MO. USA
Sodium carbonate	Fisher Scientific. Nepean. Ont.. Canada
Sodium chloride	Fisher Scientific. Nepean. Ont.. Canada
Sodium hydrogen carbonate	Fisher Scientific. Nepean. Ont.. Canada
Sodium phosphate monobasic	BDH Ltd.. Burlington. Ont.. Canada
Sodium phosphate dibasic	BDH Ltd.. Burlington. Ont.. Canada
Testosterone antiserum (R156/7)	C.J. Munro. University of California. Davis. USA
Testosterone/horseradish peroxidase	C.J. Munro. University of California. Davis. USA
Testosterone standard (17β-hydroxy-4-androsten-3-one)	Steraloids. New Hampshire. USA
Thimerosal	Sigma Chemical Co.. St. Louis. MO. USA
Tween 20	Sigma Chemical Co.. St. Louis. MO. USA

APPENDIX I.B

EQUIPMENT

Centrifuge, Labofuge 400	VWR/Canlab, Mississauga, Ont., Canada
Dynatech Ultrawash II Microplate Washer	VWR/Canlab, Mississauga, Ont., Canada
Dynatech 700 Plate Reader	VWR/Canlab, Mississauga, Ont., Canada
Dynex MRX Plate Reader	Dynex Tecnologies, Chantilly, VA, USA
Fisher Platelet Mixer Model 348	Fisher Scientific Co., Ltd., Guelph, Ont., Canada
LKB RediFrac fraction collector	LKB Wallac, Turku, Finland
Nunc-Immuno 96F Maxisorp plates	Mandel Scientific Co., Ltd., Guelph, Ont., Canada
Gamma Counter	Isodata ICN Pharmaceuticals, Costa Mesta, CA, USA.
Gilson P200 Diamond tips	Mandel Scientific Co., Ltd., Guelph, Ont., Canada
Radial compression column	Waters Scientific, Mississauga, Ont., Canada
Rotator American Instruments R4140	VWR/Canlab, Mississauga, Ont., Canada
Sep-Pak C_{18} cartridges	Waters Scientific, Mississauga, Ont., Canada
Waterbath 180 Series	Lab-Equip, Markham, Ont., Canada

APPENDIX I.C

ENZYME IMMUNOASSAY SOLUTIONS

Coating Buffer

Na_2CO_3	1.52 g	
$NaHCO_3$	2.93 g	
distilled H_2O (dH_2O)	1000 ml	pH to 9.6

EIA Buffer

Stock A: 0.2M NaH_2PO_4 — 27.8 g/ 1000 ml dH_2O
Stock B: 0.2M Na_2HPO_4 — 28.4 g/ 1000 ml dH_2O

Stock A	195 ml	
Stock B	305 ml	
dH_2O	500 ml	
NaCl	8.7 g	
BSA (Fraction V)	1.0 g	pH to 7.0

Wash Solution Concentrate

NaCl	87.66 g	
Tween 20	5.0 ml	
dH_2O	1000 ml	

Substrate Buffer

Citrate buffer	Citric Acid	9.61 g	
	dH_2O	1000 ml	pH to 4.0
40 mM ABTS	ABTS	0.55 g	
	dH_2O	25 ml	pH to 6.0
0.5M H_2O_2	H_2O_2 (30%)	500 µl	
	dH_2O	8 ml	

Fecal Extraction Solution

Wash conc 10 X	NaCl	87.66 g	
	Tween 20	5.0 ml	in 1000 ml dH_2O
20% Methanol wash	wash conc 10 X	100 ml	
	methanol	200 ml	in 700 ml dH_2O
Stock A:	NaH_2PO_4	27.8 g/1000 ml 20% methanol wash	
Stock B:	Na_2HPO_4	28.4 g/1000 ml 20% methanol wash	
Extraction buffer	Stock A	195 ml	
	Stock B	305 ml	
	NaCl	8.7 g	
	BSA	1.0 g	
	20% Methanol wash	500 ml	pH to 7.0

APPENDIX I.D

RADIOIMMUNOASSAY SOLUTIONS

Pink PBS

0.5 M monobasic NaPO4	19.54 ml	
0.5 M dibasic NaPO4	83.31 ml	
NaCl	40.85 g	
Thimerosal	0.5 g	
Phenol red	a few drops	
distilled H_2O (dH_2O)	5 litres	pH to 7.4

RIA Buffer

Pink PBS	1 litre	
EDTA (non free acid)	0.672 g	
BSA (fraction V)	0.5 g	pH to 7.4

APPENDIX II.A

EIA ANTIBODY CROSS-REACTIVITIES

PROGESTERONE (R4861)

progesterone	100 %	11α-hydroxyprogesterone	40 %
5α-pregnane-3,20-dione	12.19 %	17α-hydroxyprogesterone	0.38 %
20α-hydroxyprogesterone	0.13 %	20β-hydroxyprogesterone	0.13 %
pregnanediol	< 0.01 %	pregnenolone	0.12 %
estradiol-17β	< 0.01 %	estrone	<0.01 %
testosterone	< 0.01 %	cortisol	0.04 %

ESTRADIOL (R4972)

estradiol-17β	100 %	estrone	3.3 %
progesterone	0.8 %	testosterone	1.0 %
androstenedione	1.0 %	cortisol	< 1.0 %
dihydrotestosterone	< 1.0 %		

TESTOSTERONE (R156/7)

testosterone	100 %	5α-dihydrotestosterone	57.37 %
androstenedione	0.27 %	androsterone	0.04 %
dehydroepiandrosterone	0.04 %	cholesterol	0.03 %
β-estradiol	0.02 %	progesterone	< 0.02 %
pregnenolone	< 0.02 %	hydrocortisone	< 0.02 %
cholic acid	< 0.02 %	chenodeoxycholic acid	< 0.02 %
cholic acid methyl ester	< 0.02 %	dehydrocholic acid	< 0.02 %
deoxycholic acid	< 0.02 %	lithocholic acid	< 0.02 %
glycholic acid	< 0.02 %	taurodeoxycholic acid	< 0.02 %
taurochenodeoxycholic acid	< 0.02 %	glycochenodeoxycholic acid	< 0.02%
taurocholic acid	< 0.02 %		

CORTISOL (R4972)

cortisol	100.0 %	prednisolone	9.9 %
prednisone	6.3 %	cortisone	5.0 %
corticosterone	0.7 %	deoxycorticosterone	0.3 %
21-deoxycortisone	0.5 %	11-deoxycortisol	0.2 %
progesterone	0.2 %	17α-hydroxyprogesterone	0.2 %
pregnenolone	0.1%	17α-hydroxypregnenolone	0.1 %
androstenedione	0.1 %	testosterone	0.1 %
androsterone	0.1 %	dehydroepiandrosterone	0.1 %
aldosterone	0.1 %	estradiol-17β	0.1 %
estrone	0.1%	estriol	0.1 %
dehydroisoandrosterone-3-sulfate	0.1 %	spironolactone	0.1%
cholesterol	0.1 %		

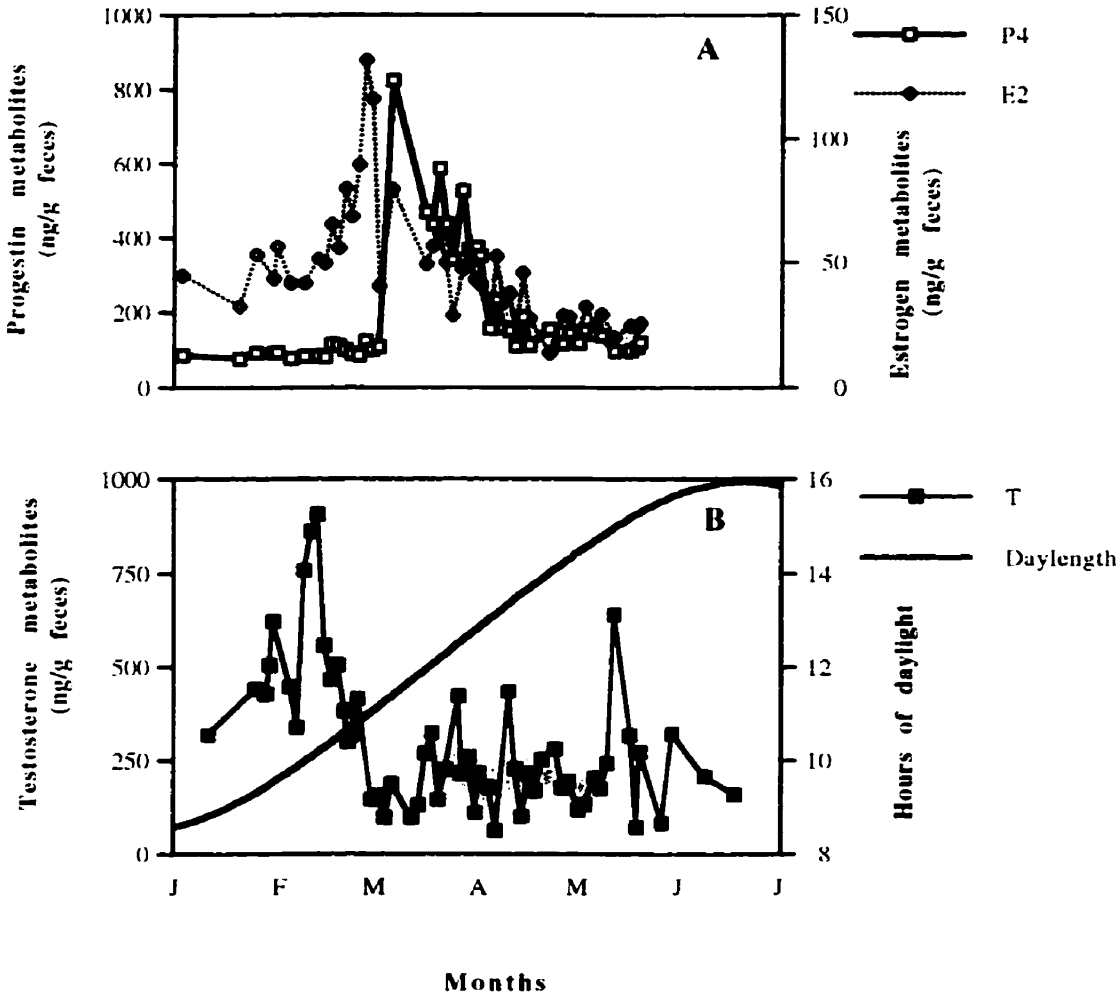
APPENDIX II.B

RIA ANTIBODY CROSS-REACTIVITIES

LUTEINIZING HORMONE ANTIBODY (518-B7)

equine LH	100 %
equine CG	39 %
ovine LH	195 %
bovine LH	264 %
porcine LH	200 %
feline LH	45 %
canine LH	138 %
rabbit LH	83 %
rat LH	191 %
equine FSH	1.4 %
equine TSH	3.8 %
ovine FSH	2.1 %
ovine prolactin	0.9 %
bovine FSH	0.09 %
bovine TSH	0.23 %
bovine growth hormone	0.04 %

APPENDIX III.A



Fecal progestin and estrogen metabolite concentrations from a yearling female red wolf (A) and fecal testosterone metabolite concentrations in relation to daylength from a yearling male red wolf (B).