REPRODUCTIVE BIOLOGY OF THE BLACK-FOOTED FERRET (MUSTELA NIGRIPES)

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ABSTRACT

REPRODUCTIVE BIOLOGY OF THE BLACK-FOOTED FERRET (MUSTELA NIGRIPES)

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Reproductive cycles of the black-footed ferret were studied in captive colonies by fecal enzyme immunoassay. Estradiol, vaginal cornification and vulval area increased through proestrus, to reach maximal values during estrus and declined following intromission. Progesterone remained low until mating, increasing to day 10, plateaued and then declined to the day of expected parturition. Progesterone concentrations were significantly elevated in pregnant females beyond 14 days post-mating. Gestation length and litter size were 41.5 ± 0.5 days and 4.0 ± 0.3 kits, respectively. Animals > 3 years and those experiencing more restraint episodes had poor reproductive success.

A physical restraint/ACTH challenge consisting of four treatments was conducted on each ferret to evaluate adrenocortical function: cage restraint, saline, liquid ACTH, and gelatin ACTH. Only ACTH regimens significantly elevated fecal cortisol levels above pre-treatment baselines. Post-study basal cortisol values were significantly greater than pre-treatment counterparts, suggesting a stress effect caused by repeated restraint.

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DECLARATION OF WORK PERFORMED

I declare that with the exception of the items indicated below, all work reported in this thesis was performed by myself.

Fecal samples, breeding records and vaginal cytology data were collected by the black-footed ferret animal care staff of both the Metro Toronto Zoo and Omaha's Henry Doorly Zoo. Enzyme immunoassay buffers and extraction solutions were prepared by A.C. Bellem, L. Carnio, M.A. Hay, L.S. Othen, S.L. Walker or myself. Assistance with fecal extraction in the late summer and early fall of 1996 was provided by S.L. Walker. Antibody and HRP conjugate stocks were prepared by Coralie J. Munro, University of California, Davis. The layout for the vaginal cytology figure was conducted by T. Cook.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
AZA	American Zoo and Aquarium Association
BFF	Black-footed ferret
CITES	Convention on the International Trade in Endangered Species of Wild Flora and Fauna
CL	Corpora lutea
COSEWIC	Committee on the Status of Endangered Wildlife in Canada
DF	Domestic ferret
EIA	Enzyme immunoassay
EP	European polecat
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
hCG	Human chorionic gonadotropin
HDZ	Henry Doorly Zoo
HPG	Hypothalamic-pituitary-gonadal axis
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IUCN	International Union for Conservation of Nature and Natural Resources

LH	Luteinizing hormone
MTZ	Metro Toronto Zoo
p.c.	post coitus
PMSG	Pregnant mares' serum gonadotropin
SP	Siberian polecat
SSP©	Species Survival Plan©
USFWS	United States Fish and Wildlife Service
WGFD	Wyoming Game and Fish Department
v/v	volume per volume
w/v	weight per volume

INTRODUCTION

A significant portion of worldwide biological diversity has been eroded within the last century, largely in response to the technological advancement and spread of mankind. The 'apocalyptic forces' which have contributed to this decline include: 1) habitat destruction and fragmentation, 2) introduction of non-native species, 3) secondary effects of civilization such as pollution, and 4) resource exploitation including over-hunting and poaching (Tudge, 1992). Although extinction is a natural phenomenon, the current loss of 1 species every 4 years is 13 - 135 times greater than the rate normally observed in fossil records (Flesness, 1989). In the last 400 years alone, 611 extinctions have been recorded for fauna species (IUCN, 1996). The World Conservation Union (International Union for Conservation of Nature and Natural Resources; IUCN) has identified 5,025 taxa threatened with extinction globally, 25% of which are mammalian (IUCN, 1996). These figures are indeed generous, since < 10 % of the 1.7 million documented species were evaluated, which in turn represents a relatively small proportion of all species believed to exist on this planet (IUCN, 1996).

To combat biotic loss and ensure the recovery of endangered species, captive breeding programs have become a major focus of conservation programs. At the forefront of captive propagation and conservation efforts are programs such as the Species Survival Plan© (SSP©) of the American Zoo and Aquarium Association (AZA), which act to coordinate the preservation of both the species and its habitat (Wiese et al., 1994). To accomplish this task, programs intensively manage the demographics and genetics of the endangered species' population, which is largely maintained at various zoological institutions. These activities have essentially forced the progression of zoos and aquaria from menageries of recreation and entertainment to facilities devoted to public education and scientific research (Wiese et al., 1994; Rabb, 1994). Used effectively, captive breeding programs act as genetic reservoirs to facilitate reintroduction and maintain

healthy, stable captive and wild populations through the infusion of new genes. They should not be misconstrued as a substitution for wild populations.

More recently, assisted reproductive technologies such as artificial insemination, embryo transfer, cryopreservation of germ plasm, and non-invasive endocrine monitoring have been applied to the conservation of endangered species (Wildt, 1992; Wildt et al., 1992; Lasley et al., 1994; Goodrowe, 1997). These techniques allow captive breeding programs to escape spacial holding limitations, thereby ensuring long-term survival and preservation of genetic diversity. But, for biotechnologies to be used effectively and efficiently, basic research must be conducted to improve our fundamental knowledge of a species' and its reproductive norms (Wildt, 1992; Goodrowe, 1997).

Within the family Mustelidae, 13 and 5 of 64 species and subspecies, respectively, are considered to be threatened with extinction (IUCN, 1996). Additionally, 1 species (black-footed ferret; Mustela nigripes) and 1 subspecies (big thicket hog-nosed skunk; Conepatus mesoleucus telmalestes) of North America are known to be extinct in the wild (IUCN, 1996). These figures are alarming when one considers that the reproductive cycles of 19 mustelid species have been 'reasonably well described' and the cycles of another 19 have only been fragmentarily documented (Mead, 1989), <39% of which are included in the IUCN's database of threatened taxa. Future attempts to save mustelids from extinction will only be further confounded by the diverse reproductive strategies within this family. Some species have relatively short gestation lengths while in others it is of variable length and features embryonic dipause (Mead, 1989).

The implementation of in situ and ex situ conservation efforts for one of the world's most endangered mustelids, the black-footed ferret, has led to non-invasive endocrine studies in our laboratory. The goal is that the acquisition of basic knowledge concerning this small carnivore's reproductive biology will greatly improve its breeding success and provide innovative strategies for more effective captive and wild management.

REVIEW OF LITERATURE

Ferrets

Taxonomy

Within the order Carnivora, the family Mustelidae constitutes a diverse group of 64 extant species. They have retained many of the primitive characteristics found in ancestral mammals including relatively small size, short stocky limbs, five toes per foot and a long braincase (Anderson, 1989). Of the six Mustelidae subfamilies, Mustelinae contains the martens, weasels, mink and ferrets. These taxa, like the majority of Mustelidae subfamilies do not represent true relationships, but rather parallel adaptations to similar habitats (Anderson, 1989).

Ferret or polecat taxonomic classification falls under the genus Mustela and subgenus Putorius. The terms ferret and polecat are interchangeable, with polecat being predominately used when referring to wild species of the Old World (Anderson, 1989; Porter and Brown, 1993). Putorius comes from the Latin term putor, denoting stench and refers to the musk scent produced by the anal glands of the ferret (Lewington, 1988). This scent is frequently used to mark territory and may be discharged when alarmed (Porter and Brown, 1993).

Three existing species of ferret are recognized: M. putorius, the European polecat (EP); M. eversmanni, the Siberian or steppe polecat (SP); and M. nigripes, the black-footed ferret (BFF). The domestic ferret (M. putorius furo; DF) is thought to have been derived from the EP through approximately 2,000 years of captive breeding and handling (Hammond and Chesterman, 1972; Lewington, 1988; Porter and Brown, 1993). Although kept as a pet, the DF was originally employed to hunt rabbits and rats (Porter and Brown, 1993).

A common ancestor found in central Europe approximately 3 million years ago is believed to have given rise to both the EP and SP (Anderson, 1989). Similarities in size, coloration, dentition and cranial measurements have suggested that the BFF and SP are

closely related, perhaps even conspecifics (Anderson, 1977; Anderson et al., 1986). Recent evidence from electrophoretic studies substantiates the SP as the closest living mustelid relative to the BFF (O'Brien et al., 1989). However, it also has established them as two distinct species with a separation time of at least 500,000 years.

Black-footed Ferret

Historical Perspective

Having been classified as endangered in the United States (USFWS, 1988) and extirpated from Canada (COSEWIC, 1996), the black-footed ferret is one of the rarest mammals in North America and is the only extant ferret species indigenous to this continent (Anderson, 1989). Uncertainty over the continued existence of the BFF led the IUCN's Species Survival Commission, Mustelid and Viverrid Specialist Group to designate the BFF as its species of greatest concern (Schreiber et al., 1989). The BFF also has been classified as Appendix I by CITES since 1978, thereby prohibiting the trade of BFFs or their products for primarily commercial purposes (CITES, 1996).

The BFF once inhabited the grasslands of the Great Plains, a region extending from Alberta and Saskatchewan southward to New Mexico, Arizona and Texas (Henderson et al., 1974; Hall, 1981). Although its secretive lifestyle as well as subterranean and nocturnal habitat made it difficult to observe, it was estimated that there were more than 500,000 ferrets in 1920 (Clark, 1987). Fossil records demonstrate a wide distribution of the BFF from the Pleistocene until the present (Anderson, et al., 1986), suggesting that the reduction of its numbers occurred fairly recently (Miller et al., 1988).

In the wild, BFFs were regarded as habitat specialists, occurring only with prairie dog (Cynomys spp.) communities, and being virtually dependent upon the prairie dog ecosystem for both food and shelter (Forrest et al., 1985). This close association led E. Coues in 1877 to refer to the BFF as Cynomyonax or 'king of the prairie dogs' (Coues, 1970). Widespread eradication of the prairie dog by poisoning campaigns, large-scale conversion of land for agricultural use and sylvatic plague (Yersinia pestis) greatly

decreased prairie dog numbers (Miller et al., 1988; USFWS, 1988; Thorne and Oakleaf, 1991; Reading et al., 1996). In some U.S. states, land occupied by prairie dog complexes has been reduced by 90-98% of its former size (Nowak, 1991; Miller et al., 1994; Roemer and Forrest, 1996). Although depletion of its prey base and fragmentation of its habitat led to the BFF's primary decline, stochastic events such as disease and genetic constraint contributed to its final demise in many areas (Brussard and Gilpin, 1989; Flesness, 1989; Harris et al., 1989; Thorne and Williams, 1988; USFWS, 1988; Williams et al., 1988).

Prior to the discovery of 2 colonies within the last half of this century, the scarcity of BFF sightings led many to believe this species was extinct. The first colony was found in South Dakota during 1964 and was studied until 1975 when it inexplicably disappeared (Thorne and Oakleaf, 1991). During this time, approximately 90 ferrets and 11 litters were located over a highly dispersed 20,000 km^2 range (USFWS, 1988). Nine ferrets were taken into captivity to initiate a breeding program at Patuxent Wildlife Research Center, Maryland; but 4 ferrets later died from vaccine-induced canine distemper (Carpenter and Hillman, 1978). Only 2 litters of 5 kits each were produced, but none survived more than a few days. The last BFF from this colony died in 1979 (Carpenter, 1985). A second colony occupying a 200 km^2 prairie dog complex was discovered outside of Meteetse, Wyoming in 1981 (USFWS, 1988; Cole, 1989). At its peak in the fall of 1984, the population was estimated to be between 120 - 129 ferrets by mark-recapture studies and night spotlighting (Cole, 1989; Thorne and Belitsky, 1989). Following these estimates, the number of ferrets declined dramatically due to the spread of canine distemper across the colony's center. By the summer and fall of 1985, spotlighting revealed only 58 and 31 ferrets, respectively (Thorne and Belitsky, 1989; Thorne and Oakleaf, 1991). In hopes of launching a captive breeding program and saving the species from extinction, the last 18 wild BFFs were captured between 1985-1987 by collaborative efforts of the Wyoming Game and Fish Department (WGFD) and US Fish and Wildlife Service (USFWS, 1988; Thorne and Belitsky, 1989). The first site of captive

propagation was established at the WGFD's Sybille Wildlife Research and Conservation Education Unit in Wheatland, Wyoming (Thorne and Belitsky, 1989; Thorne and Oakleaf, 1991).

Recovery Plan and Reintroduction

The BFF Recovery Plan was first approved in 1978, but has undergone several revisions, including final alterations in 1988. The current objective of this plan is to reestablish at least 10 free-ranging populations, composed of 1,500 breeding animals total, in the species' former range by the year 2010 (USFWS, 1988). To facilitate this, an extensive captive breeding program has been conducted and managed through the cooperation of the USFWS and the AZA's Black-footed Ferret SSP© (Hutchins et al., 1996). The primary goal of the BFF SSP© is to provide sufficient animals to support the reintroduction and recovery of the ferret in its natural habitat (Thorne and Oakleaf, 1991; Thorne, 1995). There currently are 7 institutions participating in the captive breeding program, with the Metro Toronto Zoo being the only Canadian facility (Thorne, 1996). The institutions within the United States which maintain black-footed ferret colonies include: WGFD's Sybille Wildlife Research and Conservation Education Unit, Omaha's Henry Doorly Zoo, National Zoological Park's Conservation and Research Center, Louisville Zoological Garden, Cheyenne Mountain Zoological Park and the Phoenix Zoo.

As of 1995, after 10 yrs of captive propagation, over 1,500 BFFs have been produced from the lines of 7 founders (Hutchins et al., 1996; Russell, 1996). Due to the limited number of founders, the genetic diversity of the population is severly limited. O'Brien et al. (1989) determined the percentage of polymorphism and average heterozygosity to be 2% and 0.008, respectively, for 12 animals surveyed from the Meeteetse population. These values were much lower than other carnivore orders tested, but were still greater than the South African Cheetah (O'Brien et al., 1989). The current captive population includes 159 males and 200 females, with 46.9% of the population judged to be of prime breeding age (i.e. <3 yrs of age; Thorne, 1996). By 1996, a total of

433 adults and juveniles had been released to 4 sites in the wild: Wyoming, South Dakota, Montana, and Arizona (Hutchins et al., 1996; Thorne, 1996). Two additional future release sites are proposed for Colorado/Utah and Mexico (Hutchins et al., 1996). The survial rate of BFFs returned to the wild from the time of release to the next summer is low at 20%, but is comparable with rates of reintroduction for other species (Hutchins et al., 1996). Attempts are being made to maximize survivorship by: 1) short-term management of predators within release sites, and 2) preconditioning ferrets with exposure to both prairie dogs and their tunnel networks prior to release (Hutchins et al., 1996; A. Vargas, personal communication).

Ferret Reproductive Biology

The vast majority of ferret reproductive studies over the last century have focused on the domestic ferret. Since there is a paucity of information in regard to the wild species, captive management programs have utilized knowledge gained from the domestic ferret in maintaining and breeding their exotic cousins. Only within the last 10 years have there been in-depth studies to understand the basic reproductive biology of the Siberian polecat and black-footed ferret. Most of the following text sections relate to our knowledge of the domestic ferret, but attempts have been made to incorporate information about the wild species where possible. The term 'ferret' will be used to denote the domestic species unless specified otherwise.

Photoperiod

A large number of environmental factors or cues including light, temperature, olfaction, and nutrition are known to have an impact upon the reproductive cycle of mammals (Herbert, 1977). Daylength is one of most important cues, since it provides a reliable indicator of climatic conditions that will be favorable for birth and raising offspring (Sadleir, 1969). Similar to other genera, light is of particular importance to

mustelids for controlling reproductive activities and ensuring their synchronization with the seasons (Herbert, 1989).

In 1932, Bissonnette demonstrated that the exposure of domestic ferrets to long daylength (i.e. greater than 12h) accelerates the onset of estrus in females and testicular development in males. A rhythmic change between light and dark is more effective at controlling the annual cycle of the ferret than constant illumination. Ferrets maintained under 16 hr light:8 hr dark enter estrus in half the exposure time required by ferrets under a continuous lighting regimen (Hart, 1951). Additionally, estrual ferrets kept in long photoperiods will eventually become anestrus, requiring exposure to short days before long days will again stimulate estrous activity (Donovan, 1976). Thorpe and Herbert (1976a) explained this phenomena by proposing that the ferret becomes refractory to the long daylength stimulus. Resensitization by exposure to short photoperiods for approximately 7 weeks removes this photorefractivity and allows long photoperiods to again stimulate estrus.

The offset of the breeding season or end of estrual activity for domestic ferrets exposed to natural light occurs in late summer or early autumn, although there is much more variation in the individual offset compared to estrus onset (Herbert and Vincent, 1972). Two mechanisms have been proposed to account for the offset of the breeding season: 1) decreased daylength associated with the time, since artificially shortened photoperiods prematurely end estrus (Bissonnette, 1935; Thorpe and Herbert, 1976a) and alternatively, 2) photorefractoriness may be responsible for the end of estrual activity (Herbert, 1989).

The receptors of the neuroendocrine system responsible for changes in seasonal reproductive activities of the ferret reside within the retina (Herbert and Klinowska, 1978; Herbert 1989). Neural pathways transmit photoperiod information directly from the eye to the suprachiasmatic nucleus of the hypothalamus (Thorpe, 1975; Thorpe and Herbert, 1976b), where additional signals are propagated to the pineal gland (Moore, 1983; Baum,

1988a). Although the retinal receptors and pineal gland are responsible for perceiving changes in daylength, they are not required to ensure the occurrence of estrus. Ferrets blinded by optic nerve severance or pinealectomized still experience recurrent estrous seasons (Herbert 1969; Herbert et al. 1978). Initially, animals cycle in concert with one another and the proper breeding season (i.e. long photoperiods of spring), but in successive years become asynchronous both with each other and with the season (Herbert and Klinowska, 1978; Herbert et al. 1978). Likewise, these surgeries performed on anestrous ferrets will inhibit estrous activity that is normally associated with lengthening photoperiods (Thomson, 1954; Herbert, 1969).

Melatonin secretion into either the blood or cerebrospinal fluid has been implicated as the method by which the pineal gland controls seasonal reproduction (Guyton, 1991). Melatonin likely acts upon neural mechanisms of the ferret to time or re-time breeding in response to light rather than having an 'anti-gonadotropic' effect (Herbert, 1971; Carter et al., 1982). Plasma and pineal melatonin levels of ferrets are nocturnally elevated, with the duration of elevation being greater with increasing numbers of dark hours (Baum et al., 1986). Evidence suggests that exposure of the hypothalamic-pituitary-gonadal axis (HPG) to different durations of nightly melatonin levels as the primary reason for photoperiod stimulating or inhibiting gonadal activity (Carter et al., 1982; Baum et al., 1986).

Evidence from hamsters suggests a specific region of the hypothalamus is responsible for interpretation of the melatonin signal (Hastings et al. 1985). However, exactly where and how it is translated in the ferret, as well as how the signal specifically modulates the seasonal activity of the HPG axis remains to be determined.

Estrous Cycle

Behavior

In general, all three species of ferret reach sexual maturity by the age of 1 year when maintained under a natural lighting regimen (Stroganov, 1962; Fox, 1988; Miller et

al., 1988; Carvalho et al., 1991; Williams et al., 1991; Williams et al., 1992). Housing 15 week old domestic ferrets under long-day photoperiods (16 hr light : 8 hr dark) stimulates precocious puberty and sexual maturity by 21 - 23 weeks of age (Thorpe, 1967; Ryan, 1984). The limited breeding season of the ferret extends from March until August in the Northern Hemisphere, with most breeding activity occurring in March and April (Asdell, 1964; Stroganov, 1962; Hammond and Chesterman, 1972; Mead et al., 1990; Williams et al., 1991; Williams et al., 1992). It is well documented that SP, EP and DF are seasonally polyestrus since they may enter 'heat' more than once in the same breeding season (Asdell, 1964; Stroganov, 1962; Fox, 1988; Mead et al., 1990). An original report had suggested that the BFF was only seasonally monestrus (Hillman and Carpenter, 1983). Recently, it has been accepted that the BFF may have 2 'heats' within a season and breed successfully at either (Brown, 1997; Young and Goodrowe, unpublished observation).

Although there is knowledge as to the life expectancy of several ferret species, there is no literature concerning their reproductive life or age of senescence. Domestic ferrets usually live until the age of 5 - 6 yrs, but may survive until 8 - 11 yrs with good care (Fox, 1988). European polecats are thought to live 5 - 6 yrs in the wild and a maximum of 14 yrs in captivity (Nowak, 1991). Hillman and Carpenter (1981) estimated the oldest captive black-footed ferret to be 12 yrs of age.

The stages of the ferret reproductive cycle can be described as anestrus, proestrus, estrus and diestrus. Anestrus is the period of reproductive inactivity extending from September to February. Free-ranging ferrets are solitary creatures (Nowak, 1991), so when not in estrus the female is intolerant of the male. The female will refuse to allow neck gripping by a sexually active male and will visciously bite him to avoid further advances (Baum, 1988b). Proestrus is characterized by the visible appearance of vulval swelling and increasing percentages of superficial epithelial cells within vaginal lavages (Williams et al., 1992). The duration of proestrus in all three ferret species is approximately 2 - 3 weeks (Carpenter, 1985; Williams et al., 1991; Williams et al., 1992),

although 1 week has been reported in some Siberian polecats (Mead et al., 1990; Williams et al., 1991).

Estrus in the ferret is defined as the period of maximal vulval swelling (Thorpe, 1967), during which vaginal lavages demonstrate ≥ 90 % cornified superficial epithelial cells (Williams et al., 1992). Estradiol is the only ovarian steroid necessary to stimulate reproductive behaviors in the domestic ferret (Baum, 1979). In response to elevated plasma estrogens, the female domestic ferret will show evidence of proceptive behavior by approaching sexually active males (Baum et al., 1985; Baum, 1988b). Administration of estradiol by subcutaneous injection increases a female domestic ferret's acceptance and tolerance of neck gripping (Baum and Schretlen, 1978; Baum et al., 1982). Copulation-induced ovulation ends estrus in female domestic ferrets (Robinson, 1918; Hammond and Marshall, 1930; Hammond and Walton, 1934). In the absence of a male, estrus may last for up to 5 months in domestic ferrets (Hammond and Marshall, 1930). The consequences of a prolonged estrus in the domestic ferret include leukopenia, thrombocytopenia and hypocellular bone marrow, all of which can prove fatal (Kociba and Caputo, 1981; Ryland, 1982; Hart, 1985; Sherrill and Gorham, 1985). The duration of estrus in Siberian polecats and black-footed ferrets is 30 - 42 days (Mead et al., 1990; Williams et al., 1991).

Courtship and pre-copulatory behaviours are similar in all ferret species (Poole 1966; Miller et al., 1988; Williams et al., 1991). These episodes tend to be brief, including low crouching approaches, male or female stand over, genitalia sniffing, and both neck gripping and licking by the male. In contrast, the process of mating for the ferret is fairly prolonged, often taking 30 min (Baum and Schretlen, 1975) to more than an hour (Hammond and Marshall, 1930) before penile intromission is achieved. Several reports have described similar stages of copulation for domestic and black-footed ferrets (Hillman and Carpenter, 1984; Carroll et al., 1985; Baum, 1988b). Mating is initiated by the male gripping the dorsal surface of the female's neck. The male then mounts the female and

displays a series of intermittent pelvic thrusts which cease when intromission is finally achieved. If the female attempts to free herself, the male simply presses down upon her. Records for the duration of intromission have varied, including 5 - 175 min (Hammond and Marshal, 1930), 15 - 180 min (Hammond and Walton, 1934), 2 - 151 min (Baum and Schretlen, 1975), and 1- 94 min (Carroll et al., 1985).

Following ovulation, the ferret enters diestrus, which is characterized by an elevation of circulating progesterone (Carlson and Rust, 1969; Blatchley and Donovan, 1972; Heap and Hammond, 1974; Blatchley and Donovan, 1976; Daniel, 1976, and Rose et al., 1993) and decreasing vaginal cell cornification (Hamilton and Gould, 1940; Carvalho et al., 1991; Williams et al., 1992). The female's sexual behaviors subside 3 days after coitus while ovarian steroids are still in transition (Villars et al., 1990). Although exogenous progesterone administered to ovariectomized domestic ferrets antagonizes the stimulatory effect of estradiol on sexual receptivity (Baum, 1979; Baum et al., 1986), this is not the case for gonadally intact females. Declines in proceptivity and receptivity (Villars et al. 1990) are primarily attributed to the rise of plasma progesterone and decline of estradiol concentrations, respectively.

Follicular and Hormonal Changes

Throughout much of anestrus, the ovaries of the domestic ferret contain relatively small follicles with a diameter < 500 μm (Hammond and Marshall, 1930). The ovary is not completely quiescent, since waves of pre-antral follicles have been observed to develop and degenerate during this period (Robinson, 1918). Ovarian activity associated with the breeding season commences in late anestrus, just prior to the visible external sign of proestrus; vulval edema (Hammond and Marshall, 1930). Follicles may become as large as 720 μm in diameter during this period. The recruitment of primordial follicles and their progression to the primary stage will even occur in hypophysectomized domestic ferrets, suggesting that the initiation of follicular development is independent of pituitary influence (Murphy, 1979; Murphy, 1989). In contrast, further maturation to the antral

stage requires pituitary gonadotropins, since hypophysectomy precludes the appearance of antral follicles (Murphy, 1979; Murphy, 1989).

During proestrus, antral follicles increase in size to form larger, preovulatory or Graafian follicles (Robinson, 1918). It is suggested that the development of primordial follicles to the antral/early preovulatory stages takes approximately 21 days (Robinson, 1918), while progression from early antral to preovulatory occurs in 6 days (Murphy, 1989). Preovulatory follicles are visible to the naked eye at the ovarian surface and have an approximate diameter of 1224 - 1440 μm (Hammond and Marshall, 1930). Throughout estrus, follicles of the preovulatory stage remain in the same apparent condition, without either ovulating or undergoing atresia (Hammond and Marshall, 1930).

Changes in the temporal patterns of circulating steroid hormones have been documented for the period spanning from anestrus to estrus in the female domestic ferret. Following exposure to long days (16 hr light:8 hr dark), plasma concentrations of androstenedione and progesterone decrease dramatically within 2 weeks, while those of testosterone fall progressively over a 7 week period (Donovan, et al., 1983). Progesterone values during proestrus and estrus are < 2.5 ng/ml and often undetectable by radioimmunoassy (Ryan, 1984; Rose et al., 1993). Plasma levels of estradiol during anestrus and for 5 weeks following exposure to long days are uniformly low, averaging 6 - 10 pg/ml (Ryan 1984; Ryan and Robinson, 1985). Concentrations of estradiol steadily rise after week 5 in concert with vulval edema (Donovan et al., 1983; Ryan, 1984). Mean peak values recorded during estrus range from 34.4 - 45 pg/ml (Ryan, 1984; Ryan and Robinson, 1985; Rose et al., 1993).

Although there have been numerous studies investigating gonadotropin secretion and activity of the hypothalamic-pituitary-gonadal axis in the domestic ferret, a paucity of information still exists in regards to the onset of ovarian activity. Much of our present knowledge extends from examining the endocrine processes of sexual maturation at puberty. Ryan et al. (1985) speculated that the adult anestrous domestic ferret may be

analogous to the prepubertal female, suggesting that hormonal changes and follicular development associated with the onset of estrous activity may be similar in both.

Early investigators found no significant differences between plasma luteinizing hormone (LH) concentrations of anestrous and estrous domestic ferrets (Donovan and ter Haar, 1977a; Gledhill and Donovan, 1981). However, detailed analyses by Ryan et al. (1985) were able to determine variances in secretion patterns with different levels of ovarian activity. Luteinizing hormone secretion during anestrus was episodic, with an average of 0.4 pulses/hr and a tonic secretion below the minimum detectable radioimmunoassay limit (i.e. $<0.36 \pm 0.02$ ng/ml LH). In contrast, LH plasma concentrations in estrous ferrets were undetectable and failed to demonstrate episodic secretion patterns.

Ryan and Robinson (1985) have demonstrated a marked increase in plasma LH pulse frequency prior to female domestic ferrets exhibiting vulval edema or a significant rise in plasma estradiol levels. Further, administration of human chorionic gonadotropin (hCG) to immature ferrets housed in long day photoperiods (16 hr light: 8 hr dark) prior to signs of vulva swelling stimulates a rise in plasma estradiol concentrations (Ryan, 1985). These studies suggest that a rise in pulsatile LH secretion occurs after exposure to long day photoperiods and may drive maturational changes in ovarian function.

The true contribution of follicle stimulating hormone (FSH) to follicular development and maturation in the ferret is unknown. Plasma FSH concentrations are greater in anestrous than estrous female domestic ferrets (Donovan and ter Haar, 1977a; Gledhill and Donovan, 1981; Donovan et al., 1983). Immature ferrets respond to pregnant mares' serum gonadotropin (PMSG) by vulval edema (Chang, 1950), indicating elevated plasma estradiol levels and the potential presence of FSH receptors within the ovary. However, exposure to longer days (16 hr light: 8 hr dark) causes a decline in FSH levels, which is accelerated during the proestrual rise of estradiol titres (Donovan, et al., 1983). Therefore, FSH may only be responsible for priming early follicular development

required for future ovarian activity observed under long day stimuli (Ryan, 1985; Ryan et al., 1988).

Mechanisms governing the regulation of gonadotropin secretion during different stages of the reproductive cycle have been partially defined for the domestic ferret. The FSH and LH secretory response of the anestrous ferret to gonadotropin releasing hormone (GnRH) injection is much greater than that of the estrous domestic ferret (Donovan and ter Haar, 1977a). Castration and hormonal replacement studies suggest estradiol produced by the ovaries is the most important factor restraining LH secretion (Donovan and ter Haar, 1977a; Ryan, 1984; Ryan et al., 1985; Carroll and Baum, 1989). Little is known of FSH secretion, since concentrations either have remained unchanged or were not studied. The major sites of estradiol negative feedback in anestrous (Ryan et al., 1985) and estrous (Tritt et al., 1986) female domestic ferrets are the hypothalamus and pituitary, respectively. Additionally, experimentation utilizing antiserum to estradiol has demonstrated that the increased frequency of LH episodic secretion during sexual maturation is due to the decreased efficacy of estradiol negative feedback (Ryan et al., 1988). Such a mechanism may also be plausible for the onset of estrous ovarian activity in the adult female ferret.

Vaginal and Vulval Changes

Vaginal cytology effectively has been used to detect estrus in a number of carnivores, including dogs (Olson et al., 1984), cats (Michael, 1961), and ferrets (Hamilton and Gould, 1940; Carpenter and Hillman, 1978; Hillman and Carpenter, 1984 ; Mead et al., 1990; Carvalho et al., 1991; Williams et al., 1992). In 1940, Hamilton and Gould demonstrated a correlation between estrous cycle stages in the domestic ferret with both vaginal cytology and genital tract histology. Vaginal cytological characteristics of the black-footed and domestic ferret have been reported as similar (Carpenter and Hillman, 1978). More recently, a thorough description of the epithelial cell types present in lavages during different periods of the estrous cycle for domestic ferrets, Siberian polecats

and black-footed ferrets has been compiled (Williams et al., 1992). These three species have virtually identical vaginal cytology, with the percentage of superficial keratinized cells in anestrus being minimal (i.e. BFF $\leq$ 40%, SP and DF $\leq$ 30%). The percentage of superficial keratinized cells present within the lavage gradually increases through proestrus to peak at greater that 90% during estrus. Following copulation and reflex ovulation, the percentage of superficial keratinized cells will decrease to less than 70% within 4 - 10 days, after which a further reduction in keratinized cells characteristic of anestrus occurs. Prior to mating, changes in percentage cornification are highly correlated with urinary estrone conjugates in the Siberian polecat (Mead et al., 1990) and fecal estradiol in the black-footed ferret (Brown, 1997).

Unlike the cat, the vulval labia of the ferret increase dramatically in size during proestus. Maximal vulval edema (> 10 mm width) traditionally has been used as an indicator for 'heat' and breeding readiness of the female ferret. In association with vulval tumescence and turgidity, a serous or mucoid-like secretion becomes apparent during estrus (Hammond and Marshall, 1930; Carpenter and Hillman, 1978). The vulva of the estrous domestic ferret develops to a greater extent than either the Siberian polecat or black-footed ferret (Carpenter and Hillman, 1978; Williams et al., 1992), reaching 50X its anestrual size (Hammond and Marshall, 1930). Carvalho et al. (1991) observed only a 25-fold total area increase for black-footed ferrets progressing from anestrus through to copulation. Vulval edema increases in parallel with elevated serum or plasma estradiol concentrations (Donovan et al., 1983; Ryan, 1984), supporting the observation that differences in vulval size are related to follicular enlargement (Hammond and Marshall, 1930). The vulva of the domestic ferret will remain turgid for 30 - 36 hr after copulation and becomes flaccid by 60 hr (Hammond and Walton, 1934). Similarly, a comparison of the three different ferret species found that the vulva required 3 - 9 days before it regressed in both size and turgidity (Williams et al., 1992). Anestrual morphology returns 2 -3 weeks following mating (Lagerkvist, 1992a).

Ovulation

Robinson (1918) was the first to demonstrate that the female domestic ferret was a reflex ovulator, requiring coitus to ensure the release of oocytes from ovarian follicles and the formation of corpora lutea (CL). Similar to the rabbit and cat, the physical act of mating by the domestic ferret triggers the transmission of neurogenic stimuli to higher brain centers (Carroll et al., 1987), where hormonal pathways responsible for inducing ovulation are activated. In mammals, GnRH released from the hypothalamus into the portal circulation secures the release of hypophyseal LH (Ganong, 1991; Guyton, 1991). The ovulatory surge of LH can be mimicked by infusion of exogenous GnRH in the domestic ferret (Donovan and ter Haar, 1977a; Gledhill and Donovan, 1981; Carroll et al., 1987) or a single injection of hCG in the domestic and black-footed ferret (Chang, 1965; Mead et al., 1988; J.G. Howard, personal communication).

Electrical stimulation of the domestic ferret's medial hypothalamus consistently yields the largest LH response (Donovan and ter Haar, 1997b), suggesting that this region may be responsible for interpreting the neurogenic signal. The speed of the neuronal and humoral mechanisms which terminate with the LH surge are extremely rapid, since hypophysectomy 110 min after the start of copulation fails to inhibit ovulation (Hill and Parkes, 1932).

As previously discussed, the responsiveness of the hypophysis to GnRH is reduced in the estrous domestic ferret in comparison with that of anestrous animals. It has been proposed that specific steroids, factors or peptides are released to prime the hypothalamo-hypophyseal-ovarian axis of induced ovulators to future stimulation that will eventually culminate with ovulation (Ramirez and Beyer, 1988). Such a factor or peptide may be released during the pre-intromission period of the domestic ferret to increase the sensitivity of the hypophysis to GnRH (Carroll et al., 1985). Evidence for this comes from the fact that both masculine copulatory behaviors (i.e. neck gripping, mounting and pelvic thrusts) and penile intromission are required concurrently to achieve

ovulation. Carroll et al. (1985) demonstrated that receiving solely masculine behaviours was insufficient stimulus to elevate plasma LH in estrual domestic females. However, when masculine behaviours were provided in combination with penetration by the male, the female ovulated following a surge of plasma LH. Similarly, cervical stimulation with a glass rod fails to induce the LH surge required for ovulation in the estrous female (Baum, 1988a, M.J. Baum, personal communication). But when provided in combination with the neck grip and mounted pelvic thrusts of a male, an ovulatory LH surge was achieved. A single intromission, even as short as 1 min in duration, is sufficient to induce ovulation in the domestic ferret 100% of the time (Carroll et al., 1985).

Changes in plasma LH concentrations of the estrual domestic ferret following mating were initially evaluated by radioimmunoassay of serial blood samples (Carroll et al., 1985). Within 1.5 hr of intromission, mean (± SEM) plasma LH values became significantly elevated above those of pre-intromission (2.35 ± 0.21 ng/ml vs. 0.74 ± 0.08 ng/ml). Mean (± SEM) plasma LH concentrations peaked at 3.80 ± 0.80 ng/ml by 6 hr following intromission and gradually declined to values not significantly different from those of pre-intromission by 10 hr post coitus (p.c.). Subsequent studies also have demonstrated an increase in episodic or pulsatile LH secretion following intromission (Ryan et al., 1985; Carroll et al., 1987) and a persistence of LH elevations for up to 12 hr p.c. (Carroll et al. 1987).

Ovulation was originally estimated to occur as early as 30 hr following copulation, with an observed range of 30 - 36 hr in individual ferrets (Robinson, 1918; Hammond and Walton, 1934). More recently, similar results of 30 - 40 hr p.c. were obtained by Chang and Yanagimachi (1963). Upon histological evaluation of fixed ovaries, Donovan (1963) determined that an average of 10.9 (range 8 -17) CL had been formed in response to the ovulatory surge of LH.

There is no correlation between the duration of intromission and the number of CL formed or area under the LH response curve (Carroll et al., 1985). Additionally, there is

no relationship between the level of hypophyseal secretion and the final CL size, since CL always develop to a similar size irregardless of the number within the ovary (Donovan, 1963).

Hormonal Induction of Estrus and Ovulation, Artificial Insemination

A number of experiments have explored the efficacy of exogenous hormones to alter ovarian activity in the domestic ferret. Early studies demonstrated the ability of crude pituitary extracts to induce estrus in anestrous ferrets (Hill and Parkes, 1930) and ovulation in estrous ferrets (Chang 1965). Infusion of 4.1 μg GnRH over a 200 min period was shown to produce ovulation in 2 females and luteinization in a third (Donovan and ter Haar, 1977a). However, single and repeated daily injections of either GnRH (4 μg/day) or long-acting GnRH analogues (1μg/day) alter plasma LH and FSH levels, but fail to instigate ovulation (Gledhill and Donovan, 1981). Histological evaluation suggested these treatments lacked the ability to stimulate follicular development in anestrus and to cause the degeneration of healthy follicles during estrus.

Utilization of gonadotropins rather than releasing factors also has been investigated in the domestic ferret. Subcutaneous injections of PMSG (40 to 60 IU) at 2 - 4 day intervals for several weeks during anestrus causes vulval swelling and stimulates follicular growth (Chang, 1950). A final injection of 200 IU PMSG approximately 2 weeks after the start of treatment induces ovulation (Chang, 1950). The administration of 0.25 mg FSH 2X daily until estrus, supplemented with 0.5 IU hCG 1X daily when vaginal cytology exceeded 73% cornification has been demonstrated to effectively induce vulval swelling and sexual receptivity (Mead and Neirinckx, 1989). The majority of females (80%) treated by these regimens ovulate when bred by a male. Although dosages of 50 - 300 IU hCG stimulate ovulation, Mead et al. (1988) determined 100 IU hCG to be optimal, producing 14.1 ± 4.8 CL (mean ± SD). These results are similar to the average number of oocytes (14.5) recovered from the oviduct in ferrets treated with 90 IU hCG (Chang, 1965).

Since all ferret species are reflex ovulators, the induction of ovulation by exogenous gonadotropins finds practical use in the application of artificial insemination. The deposition of epididymal sperm (Chang, 1965), electroejaculated fresh semen (Wildt et al., 1989) or frozen-thawed semen (Howard et al., 1991) within the uterine horn of the domestic ferret, has yielded fertilized oocytes and pregnancies. In each of these studies, 90 IU hCG was administered to induce ovulation. There was no significant difference in the number of pregnancies produced between groups of inseminated ferrets administered hCG at -24 hr or 0 hr, where 0 hr was the time of laparoscopic insemination (Wildt et al., 1989). Chang (1965) determined that the greatest number of fertilized eggs occurred when gonadotropin treatment was concurrent with intra-uterine insemination at laparotomy and decreased when administered at progressive periods following insemination. Injection of 90 IU hCG 18 - 24 hr pre-laparoscopic uterine insemination has been found to be most effective at inducing ovulation and subsequent pregnancy when employed with black-footed ferrets (J.G. Howard, personal communication).

In contrast to uterine deposition of spermatozoa, attempts to vaginally inseminate domestic ferrets have met with little success (Hammond and Walton, 1934; Wildt et al., 1989). Failure of this method primarily has been attributed to the inability of sperm to both pass through the cervix and ascend the uterus (Hammond and Walton, 1934; Chang, 1965; Wildt et al., 1989).

Pregnancy and Pseudopregnacy

General Overview

Approximately 30 hr after coitus (Hammond and Walton, 1934), ova are shed into a space around the ovary, which is delimited by a bursa. The ovarian bursa or capsule protects the ovary and ensures the passage of virtually all ova into the oviduct (Robinson, 1918). After insemination, spermatozoa have a fertilizing life of no more than 126 hr (Chang, 1965) and require 3.5 - 11.5 hr within the reproductive tract to reach capacitation

(Chang and Yanagimachi, 1963). Spermatozoa first appear in the distal uterine horn 3 hr after coitus, but do not reach the ovarian capsule until approximately 6 hr following coitus (Hammond and Walton, 1934). After 36 hr, spermatozoa will again be absent from the ovarian capsule even though they are still present within the uterus (Chang, 1965).

Domestic ferret ova remain viable for 30 hr after ovulation, with reduced litter sizes being produced when fertilization occurs between 18 and 30 hr (Hammond and Walton, 1934). The exact region of the ferret reproductive tract in which fertilization occurs has not been well defined. Hammond and Walton (1934) suggested that fertilization occurred within the ovarian capsule, while Robinson (1918) speculated that the site was in the middle third of the oviduct.

On day 6 p.c. embryos emerge from the oviduct to enter the uterus as morulae and blastocysts (Chang, 1965). While within the uterus, embryos readily migrate between horns (Hammond and Marshall, 1930) via the small uterine body (0.22 cm; An and Evans, 1988) located cranial to the cervix. Implantation occurrs in the 24 hr period between days 12 and 13 (Daniel, 1970; Enders and Schlafke, 1972). Expansion of the blastocyst facilitates adhesion to the uterine endothelium and penetration proceeds by the intrusion of the trophoblast between adjacent uterine luminal epithelial cells. Although the ferret, like other carnivores is adeciduate (Beck, 1974; Rose et al., 1993), an increased vascular permeablility of blood vessels has been demonstrated in the vicinity of blastocysts on the morning of day 12 (Mead et al., 1988). The developing placentae of the ferret is zonary and endothelialchorial in nature (Morrow, 1980). Pregnancy can be detected by ultrasonography (Peter et al., 1990) and abdominal palpation (Conalty, 1967) as early as days 12 and 16 after mating, respectively.

The period of post-implanational gestation is 30 days in the domestic ferret (Murphy, 1989), with parturition occurring approximately 6 weeks or 42 days after copulation for all three ferret species (Hammond and Marshall, 1930; Stroganov, 1962; Mead and Neirinchx, 1989; Mead et al., 1990; Carvalho et al., 1991; Williams et al.,

1991). Gestational ranges reported by Mead et al., (1990) and Williams et al., (1991) suggest a tendency for Siberian polecats to deliver prior to 42 days. The number of kits per litter varies with species, but in general the black-footed ferret produces the fewest kits. Domestic ferrets are known to average 8 kits (Fox, 1988), while their free-ranging European polecat cousin has a mean of 5 (Stroganov, 1962). The mean number of kits produced by Siberian polecats has varied in reports: 8.5 for free-ranging (Stroganov, 1962), and 6.8 (Mead et al., 1990) and 8.4 (Williams et al., 1991) for captive colonies. The black-footed ferret produces 3-4 kits on average (3.3 ± 0.5; Carvalho et al., 1991; 3.0 ± 1.4; Williams et al., 1991).

Failure of fertilization following mating-induced ovulation results in a phantom pregnancy, termed pseudopregnancy, of the same duration as a true pregnancy (Hammond and Marshall, 1930). Females entering either of these two states exhibit identical behavioral, hormonal and uterine histological changes (Hammond and Marshall, 1930; Hamilton and Gould, 1940; Carlson and Rust, 1969; Blatchley and Donovan, 1972; Heap and Hammond, 1974; Blatchley and Donovan, 1976; Daniel, 1976; Williams et al., 1992; Porter and Brown, 1993; Brown, 1997). The example which best exemplifies the similar endocrinology of pregnant and pseuodpregnant diestrus statuses is that both experience hypertrophy of mammary glands 3 to 5 weeks following copulation (Hammond and Marshall, 1930). The lifespan of the corpora lutea is 6 weeks regardlesss of the presence or absence of embryos within the the uterine horns (Deanesly, 1967; Blachtchley and Donovan, 1976). Furthermore, early studies involving hysterectomy suggest placental and uterine factors contribute little to the hormonal environment and maintenence of the corpora lutea (Deanesly and Parkes, 1933; McPhail, 1935; Donovan, 1963; Galil, 1965; Deanesly, 1967).

Endocrinology

The longitudinal study profiles of plasma ovarian steroid concentrations during diestrus have been the subject of detailed study in the domestic ferret (Carlson and Rust,

1969; Blatchley and Donovan, 1972; Heap and Hammond, 1974; Blatchley and Donovan, 1976; Daniel, 1976; Rose et al., 1993). Fluorimetry, competitive protein-binding and radioimmunoassay all have been used as analysis techniques to quantify steroid hormones. The consensus has been that both hormonal profile patterns and concentrations during pseudopregnancy in the domestic ferret parallel those of pregnancy. By day 7 p.c., plasma estrogens have declined to low levels and fluctuate between 5 - 15 pg/ml until the end of diestrus. An increase in estrogen levels concurrent with parturition has not been observed in the ferret. In contrast, plasma progestin levels, particulary progesterone, undergo a dramatic rise and fall during diestrus. A rise in peripheral progesterone values above the negligible levels of anestrus and estrus (< 2.5 ng/ml) first occurs 5 - 6 days following mating. Concentrations steadily increase to peak at days 12 - 14, where they remain elevated until day 21. Maximal progesterone concentrations recorded during the plateau stage have ranged between 17 - 35 ng/ml. These differences were due to both the sensitivity of different analysis techniques and their subsequent refinement with time. Following day 21, progesterone concentrations decline fairly rapidly to approximately 4 ng/ml by day 35 and 2.5 ng/ml by day 42.

Embryonic and Implantational Requirements

Extensive experimentation has detailed the relative importance of several hormonal steroids and factors to the processes of pre-implantation development of the ferret embryo and implantation. Progesterone is the only progestin manufactured by luteal tissue (Heap and Hammond, 1974; Kintner and Mead, 1983) and is obligatory for embryonic survival and delivery of live offspring. Bilateral ovariectomy prior to day 10 p.c results in the failure of blastocyst expansion and implantation (Buchanan, 1969; Wu and Chang, 1972, Wu and Chang, 1973; McRae, 1992). Ovariectomy after day 10 p.c. permits implantation but is followed by resorption (Buchanan, 1969; Wu and Chang; 1973). Similarly, immunoneutralization of progesterone by injection of antiserum for 3 days following mating, created an oviductal envrionment unfavorable to cleavage and

arrested embryonic growth at the 4-cell stage (Rider and Heap, 1986). However, progesterone replacement therapies will support implantation and further fetal development in ferrets ovariectomized on day 8, 10 and 13 (Wu and Chang, 1973; Foresman and Mead, 1978).

In addition to progesterone, an unknown factor produced during days 6 - 8 p.c. is crucial to nidation (Wu and Chang; 1972; Wu and Chang, 1973; Foresman and Mead, 1978). Research by Foresman and Mead (1978) involving ovariectomy and autotransplantation of follicles on day 1 p.c. suggests that the corpora lutea are the source of this critical factor. Although granulosa cells contain the enzymatic capacity to synthesize estradiol, administration of this hormone to progesterone-primed females during the critical period of 6 - 8 days p.c. failed to permit implantation (Wu and Chang, 1972; Foresman and Mead, 1978; Mead and Swannack, 1980; Mead and McRae, 1982; Kintner and Mead, 1983). Furthermore, injecting mated ferrets with antiserum against estrogens on days 3 or 4 - 13 p.c. does not disrupt trophoblastic attachment or fetal development (Murphy and Mead, 1976).

A recent study suggests that the unknown luteal factor is a protein with a molecular weight greater than 50,000 kDa (Mead et al., 1988). Luteal extracts injected into mated females ovariectomized on day 6 that receieved Silastic implants containing progesterone permitted blastocyst attachment and epithelia penetration. In contrast, blastocyst implantation failed when luteal extracts were incubated with a broad-spectrum protease prior to treatments.

Luteal Maintenance and Lifespan

Both the development and maintenance of luteal tissue within the ferret ovary are solely dependent upon the hypophysis. Removal of the pituitary gland within 15 hr after mating arrests the growth of the corpora lutea (Hill and Parkes, 1932), while removal 4, 5, 6 or 21 days p.c. leads to luteal regression, fetal resorption and/or abortion (McPhail, 1935; Donovan, 1963; Donovan 1967; Murphy 1979). The fetus, placenta and pregnant

uterine horn do not contribute to luteal lifespan, since their removal fails to impair CL structure or function (Deanesly, 1967; Blatchley and Donovan, 1976).

Several studies have demonstrated that the hormone prolactin has luteotrophic properties. The administration of exogenous prolactin to hypophysectomized ferrets maintains the morphology of the CL (Donovan, 1967) and its ability to synthesize progesterone (Murphy, 1979) during the first half of gestation. McKibbin et al. (1984) demonstrated that the addition of prolactin to ferret luteal cell cultures increased progesterone output when in the presence of but not absence of low-density lipoproteins. They further suggested the luteotropic effect of prolactin was to enhance the uptake or liberation of cholesterol from lipoproteins, thereby increasing the resources available for steroidogenesis.

In contrast, in vivo hormonal therapies with exogenous LH, FSH, growth hormone or thyrotropic hormone do not prevent morphological regression of the CL within hypophysectomized pseudopregnant ferrets (Donovan, 1967). Nor is the coincubation of luteal cell cultures with ovine LH adequate to stimulate progesterone secretion (McKibbin et al., 1984).

More recently, it has been suggested that prolactin and LH act synergistically as a luteotropic complex to maintain the corpora lutuea during the first half of gestation. Experimentation with pseudopregnant ferrets provides several lines of evidence to support this postulate: 1) inoculation with antibodies against GnRH from days 2 - 20 reduced plasma concentrations of LH and progesterone, 2) daily administration of 100 IU hCG, known to down-regulate LH receptors in the rat (Catt et al., 1979), significantly reduced circulating plasma progesterone levels, 3) passive immunization against LH also lowered plasma progesterone concentrations (Agu et al., 1986).

The temporal patterns of circulating progesterone during the first half of gestation correspond to changes in luteal activity (Blatchley and Donovan, 1972; Hammond and Head, 1974). For 2 weeks following copulation, the increase in corpora lutea size is

matched by a rise in peripheral progesterone concentrations. The maximal luteal dimension of 2 mm (Hammond and Marshall, 1930; Hill and Parkes, 1932) coincides with the elevated plateau of plasma progesterone between days 14 and 28. However, while corpora lutea size decreases only slightly from days 28 - 42 (Hammond and Marshall, 1930; Hill and Parkes, 1932), progesterone concentrations decline dramatically. Atrophy of the corpora lutea finally occurs about 5.5 weeks p.c. in pregnant (3 - 4 days before parturition) and pseudopregnant animals (Hammond and Marshall, 1930), when progesterone levels are quite low. Blatchley and Donovan (1972) state this disjunction was due to functional and structural luteolysis not coinciding in the ferret.

The exact mechanisms which induce luteolysis and the onset of parturition in the ferret are unknown. Since both mammary development and lactation require hypophyseal support (Hammond and Marshall, 1930; McPhail, 1935), the withdrawal of prolactin can not be involved. Fetal and placental signals are discounted from a possible lytic mechanism because the process occurs to the same extent in both pregnancy and pseudopregnancy (Hammond and Marshall, 1930). Similarly, the uterus is unlikely to be involved since both hysterectomy (Deanesly and Parkes, 1932) and exogenous administration of prostaglandins (PGF 2α; Blatchley and Donovan; 1972) fail to cause premature morphological regression of the corpora lutea. Two plausible mechanisms are: 1) prolactin may lose its luteotropic effect during the second half of gestation (Murphy, 1979) or 2) the cor*pora lutea may have an inherent lifespan after which it ceases to function (Heap and Hammond, 1974). Further investigation is required to understand both luteolysis and parturition in the ferret.

Stress and Reproductive Function

In the last decade, the negative impact which acute and chronic stress have upon the reproduction of laboratory and domestic animals has been extensively reviewed (Collu et al., 1984; Moberg, 1985a; Armstrong, 1986; Liptrap, 1993; deCatanzaro & MacNiven,

1992). Numerous studies have demonstrated that environmental factors can alter the estrous cycle and ovulation pattern in mammals. Exposure to severe cold, wind, and rain has been found to reduce the incidence of estrus in ewes (MacKenzie et al., 1975). Social stress from overcrowding shortens estrous cycles in dairy cattle (MacMillan and Watson, 1971; Wagon et al., 1972) and decreases the frequency of estrus in mice (Pennycuik, 1972). Elevation of adrenocorticosteroids, a characteristic associated with stress, has been demonstrated to inhibit female sexual receptivity in mice (deCatanzaro et al., 1985) and rats (deCatanzaro, 1987).

Evidence suggests that psychological stressors during early pregnancy can impede intrauterine implantation or induce fetal resorption and abortion. Daily transport and excessive dog herding significantly decreases the number of viable pregnancies in ewes (Doney et al., 1976). Heat stress applied to cows increases the incidence of embryonic mortality and/or developmental abnormalities (Putney et al., 1988; Liptrap, 1993). Diverse stressors such as human handling (Runner, 1959; Kvetnansky et al., 1978), exposure to predators (deCatanzaro, 1988), exposure to strange males and their odors (Bruce, 1959) and physical restraint (Euker & Riegle, 1973) have a similar influence on pregnancy rates in laboratory rodents.

Stress also impacts on the sexual activity and reproductive success of male animals. Moberg (1985a) suggests the greatest effect of acute or chronic stress on males is to alter their ability to evince sexual behaviour. Physical restraint of bulls (Johnson et al., 1981) and chemical immobilization of baboons (Sapolsky, 1985) suppresses serum titers of testosterone, an androgen of critical importance to spermatogenesis and sexual behaviour in males. In vitro and in vivo studies have shown that testicular hyposensitivity to gonadotropins is associated with stressful events in rats (Charpenet et. al, 1982), which is likely the result of elevated plasma glucocorticoid levels reducing LH receptor numbers on Leydig cells (Bambino and Hseuh, 1981).

Behaviors and endocrine physiology are frequently used to define the level of 'stress' experienced by an animal. Behavioral assessment is plagued by numerous problems including variability between species and between individuals of the same species with respect to activities during stressful periods. Differences also exist between what is recorded by observers as stress-related behaviors. On the other hand, increases in plasma concentrations of corticosteroids can be successfully used as physiological indices for stress (Moberg, 1985a; Wildt et al., 1984; Wildt et al., 1986; Graham & Brown, 1996). The mechanism by which 'stress' elevates plasma adrenocorticosteroids has been partially determined (Jones, 1979; Guyton, 1981). Internal and external stressors, via the central nervous system and hypothalamus, stimulate increased adrenocorticotropic hormone (ACTH) release from the hypothalamic-adenohypophysis axis into the general circulation. Raised concentrations of ACTH in the blood heighten adrenocortical activity, increasing both the synthesis and secretion of corticosteroids. Stress-induced elevations of serum cortisol or corticosterone, two major corticosteroids, have been described in the white-tailed deer (Wesson et al., 1975), pig (Hemsworth et al., 1981), sheep (Hargreaves and Hutson, 1990), red fox (Hartley et al., 1994), and impala, zebra and giraffe (Morton et al., 1995).

The administration of exogenous ACTH is routinely performed to evaluate adrenal function in dogs, cats, humans (Peterson et al., 1984; Rosenthal et al., 1993) and domestic ferrets (Garibaldi et al., 1988; Heard et al., 1990). This procedure has become a particularly useful tool for diagnosis of hypo- and hyperadrenocorticisms in both man and animals (Peterson et al., 1984; Rosenthal et al., 1993). In stress-related research, ACTH challenges have been used to characterize behavioral and physiological responses to both acute and chronic stressors. The type of treatment regimen determines which of these stressors is mimicked. Intramuscular injections of long-acting ACTH gel (e.g. Acthar Gel®) more accurately reflect chronic stressors, while injections of synthetic

ACTH suspensions (e.g. Cosyntropin®) elicit responses similar to acute stressors (Peterson et al., 1984).

Mechanisms of Stress Impact

There is a voluminous amount of data concerning the possible mechanisms by which stress affects reproduction. Of critical importance to captive management programs for exotic species such as the black-footed ferret is how stress impacts on the female's reproductive physiology during the peri-copulatory interval and pregnancy.

Since the hypothalamic-pituitary-adrenal axis is central to the stress response, there is reason to believe that classical hormones of stress (i.e. ACTH and glucocorticoids) are involved with pregnancy disruption. Administration of ACTH to rodents and lagomorphs at various times of pregnancy has been linked to fetal loss through increased incidence of abortion and resorption as well as reduced fetal growth and litter size (Robson and Sharaf, 1952; Velardo et al., 1956; Kittinger et al., 1980). Adrenocorticotropic hormone and glucocorticoids such as cortisol injected in the first few days of pregnancy also decrease the number of embryos which successfully implant (Bitman and Cecil, 1967; Yang et al., 1969; Schlough, 1971). There are several possible criticisms for the classical stress hormones having a primary role in pregnancy demise: (1) earlier studies pre-dated purification of these hormones, since more recent works have suggested dissimilar results during early gestation (deCatanzaro et al., 1991; deCatanzaro and MacNiven, 1992), (2) inconsistent findings from the administration of these hormones to adrenalectomized (Snyder and Taggert, 1967; Sahu and Dominic, 1981) as well as ovariectomized animals given estrogen/progesterone (Hensleigh and Johnson, 1971) are suggestive of an alternative mechanism for stress induced pregnancy failure.

More recent evidence points clearly to a role of excessive androgens and estrogens in stress-induced failures of intrauterine implantation (deCatanzaro et al., 1991). Androstendione and dehydroepiandrosterone released from the adrenal glands during stress (Fenske, 1986; Fuller et al., 1984), cause the expulsion of fertilized ova from the

reproductive tract and decrease litter sizes (Harper, 1967; Harper, 1969). These androgens are thought to act upon the female's reproductive tract mainly through their metabolic conversion to estrogens (Fenske, 1986; deCatanzaro and MacNiven, 1992). Exogenous elevations of androgens and estrogens completely disrupt early pregnancy in a variety of mammalian species, including rabbits, hamsters, guinea pigs, rats and mice (Deanesly, 1963; Stone, 1964; Chang and Yanagimachi, 1965; Greenwald, 1965; Smith and Biggers, 1968; Huet and Dey, 1987; deCatanzaro et al., 1991).

Convergent evidence suggests that administration of ACTH can increase endogenous estrogen levels (Arai et al., 1972; Strott et al., 1975) and that circulating estrogens may rise significantly during early pregnancy in response to physical restraint stress (MacNiven et al., 1992). However, injections of a monoclonal antibody to estradiol will prevent pregnancy loss in mice incurred following restraint (deCatanzaro et al., 1994).

Precise ratios of ovarian steroids are crucial to both the development and implantation of the mammalian embryo (Smith and Biggers, 1968; Roblero and Garavango, 1979). Alterations of this balance have been documented as the main reason for pregnancy loss (Gidley-Baird, 1981; Gidley-Baird, et al., 1986) Thus, disruptions of the maternal hormone environment by stress-induced elevation of androgens and estrogens could easily contribute to the disruption of early pregnancy.

Non-invasive Hormonal Analysis

Hormonal analyses have been widely utilized for inquiries into reproductive processes and general clinical reproductive well-being. These simple techniques to quantify steroid content provide both an immediate and accurate picture of endocrine status. Prior to the late 1970's, urine samples and blood collected via venipuncture were the only substrates readily available in which to quantify hormone levels. This method worked well with human beings and docile domestic species, but proved more difficult

with less tractable subjects such as wildlife species. In the latter case, manual restraint and/or chemical immobilization became a necessity to facilitate adequate sample collection.

The advent and refinement of hormonal analysis from excreta, specifically urine and feces, has revolutionized endocrine studies. There are several advantages to monitoring randomly collected urinary or fecal steroid concentrations which make them particularly inviting for use with wildlife species (Lasely and Kirkpartrick, 1991): 1) prolonged, longitudinal monitoring can occur without manipulation, restraint or even stress of the subject animal, 2) a wide range of assay systems may be employed since concentrations of the steroids and their metabolites are 2 - 4 orders of magnitude higher than circulating vascular steroid forms, 3) samples may be collected under the 'most adverse conditions' and from the 'most pugnacious' animals, with little risk to either the subject or investigator. The fact that concentrations of urinary and fecal steroids accurately reflect those circulating within the blood has justified the appropriateness of non-invasive monitoring (Monfort et al., 1990; Gross et al., 1992; Larter et al., 1994; Hay, 1996; Berkeley et al., 1997).

Non-invasive hormonal analysis has been utilized in reproductive studies of captive and free-ranging wildlife species. One of its first applications was for sex determination in the Hispaniolan parrot (Amazona ventralis), a monomorphic bird (Bercovitz et al., 1979). Aspects of the estrous cycle, reproductive seasonality, and pregnancy have been characterized by urinary and/or fecal steroid analysis for several herbivores including wood bison (Bison bison athabascae; Matsuda et al., 1996), Eld's deer (Cervus eldi thamin; Monfort et al., 1990), Dall's sheep (Ovis dalli dalli; Goodrowe et al., 1996), moose (Alces alces; Monfort et al., 1993), musk-oxen (Ovibus moschatus; Desaulniers et al., 1989) and black rhino (Diceros bicornis; Schwarzenberger et al., 1996; Berkeley et al., 1997). Ovarian function also has been investigated by similar urinary or fecal steroid analyses in a host of carnivores, including the maned wolf (Chrysocon

brachyurus; Wasser et al., 1995), domestic cat (Felis catus; Shille et al., 1990; Graham et al., 1995), cheetah (Acinonyx jabatus; Czekala et al., 1994; Graham et al., 1995; Brown et al., 1996) and clouded leopard (Neofelis nebulosa; Brown et al., 1995a), and primates such as the yellow baboon (Papio cynocephalus cynocephalus; Wasser et al., 1994), cynomolgus monkey (Macaca fascicularis; Shideler et al., 1993), and lowland gorilla (Gorilla gorilla; Bellem et al., 1995).

The employment of non-invasive hormonal analyses is not solely restricted to monitoring steroids of an ovarian, testicular or placental origin. Similar techniques may be applied to investigating the excretion patterns of corticosteroids; thereby, providing detailed information in regards to the effect of stress on adrenocortical activity. This method is particularly suited for wildlife species since it obviates the elevation of peripheral cortisol frequently observed when physical restraint and/or chemical immobilization are used in combination with venipuncture (Reinhardt et al., 1991; Morton et al., 1995) Levels of corticosteroids within urine and feces have been successfully monitored in domestic and small, nondomestic felids (Carlstead et al., 1992; Graham and Brown, 1996; Jurke et al., 1997), elephant (Elephas maximus, Loxodonta africana; Brown et al., 1995b), mule deer (Odocoileus hemionus; Saltz and White, 1991), Rocky Mountain bighorn sheep (Ovis canadensis canadensis; Miller et al., 1991), timber wolf (Canis lupus; McLeod et al., 1996) and African wild dog (Lycaon pictus; Monfort et al., 1997).

Mustelid Fecal Steroid Analysis

In the last few years, a number of studies have assessed ovarian steroid concentrations within the feces of several mustelids, including the Siberian polecat and black-footed ferret (Gross et al., 1991; Gross, 1992; Brown, 1997), Asian small-clawed and river otters (Aonyx cinera, Lutra canadensis; Gross, 1992) and mink (Mustela vison; Möstl et al., 1993).

Möstl et al. (1993) effectively monitored corpora lutea activity during mink pregnancy with a 20-oxo-gestagen enzyme immunoassay. High performance liquid chromatography (HPLC) determined the immunoreactive steroid was not progesterone, but rather several metabolites with polarities similar to unconjugated monohydroxylated progestagens. Similarly, in the black-footed ferret, only 8% of the progestagen immunoreactivity will co-elute with progesterone (Brown, 1997). The remainder corresponded to a single conjugated and several unconjugated steroid forms. However, greater than 90% estrogen immunoreactivity for the black-footed ferret coincides with unconjugated estradiol-17β.

Several small studies have longitudinally assessed ovarian activity during the breeding season of the black-footed ferret by radioimmunoassay (Gross et al., 1991; Gross 1992, Brown, 1997). These studies have evaluated only a minimal number of females; 7 pregnancies and 9 pseudopregnancies (Brown, 1997) and ≤ 5 pregnancies and ≤ 3 pseudopregnancies (Gross et al., 1991, Gross, 1992). Fecal estrogen concentrations increased through proestrus to peak during estrus. Following mating, there were differences in the excretion patterns of estrogens between these studies. While Gross et al. (1991) and Gross (1992) demonstrated estrogens levels decreasing dramatically by 1- 2 weeks following mating, Brown (1997) reported a more gradual decline with levels reaching minimal values concurrently with parturition. Both studies were similar in respect to progesterone excretion patterns during diestrus. Progesterone concentrations rise following mating, remain elevated until 4 - 5 weeks after breeding and then decline to parturition. Although pre-breeding fecal estrogen concentrations were significantly lower in pseudopregnant than pregnant estrous females, there was no distinguishable difference between progesterone concentrations either pre- or post-mating.

Infusion of radiolabelled isotopes for estradiol and progesterone into the Siberian polecat indicates that > 90% of each steroid is excreted into the feces (Gross, 1992). The ratio of unconjugated:conjugated metabolites for estradiol and progesterone were 61:39

and 66:34, respectively. It was determined that the extraction of unconjugated (i.e. free) steroids could be further enhanced by the addition of enzymes to hydrolyze conjugated forms.

RATIONALE

In the past few years, serious questions have been raised about the ability of the captive BFF breeding program to provide sufficient animals to meet reintroduction demands and achieve recovery goals. Over the 1992-1994 breeding seasons, the captive population exhibited a reduced fecundity. There were 2 contributing factors: (1) a decrease in the percentage of bred females that successfully whelped, and (2) a decline in the number of kits surviving until weaning (Thorne, 1995). Even though the recent pregnancy rate of the population has improved from the 1994 low (63% vs. 41%; Thorne, 1996), there is great concern about the variation of success across the individual institutions involved with the BFF's captive propagation (Hutchins et al., 1996). For instance, in 1995 the Metro Toronto Zoo and Omaha's Henry Doorly Zoo bred 9 and 15 females, but had only 2 and 12 pregnancies, respectively.

Another major concern is the effect that certain aspects of captive management and their associated levels of physiological stress have upon the BFF at critical times of the reproductive cycle (Hutchins, et al., 1996). In the current breeding program, a 'hands-off' approach is taken to maintain the BFF in as 'wild' or natural state as possible, primarily for future release considerations. However, during the breeding season (February-April) regular restraint episodes are conducted to evaluate vaginal cytology and testicular growth, thereby providing pertinent information regarding female receptivity and optimal breeding time. The frequency of these restraint episodes progresses from weekly in mid-February to daily when the optimal time of breeding readiness approaches (i.e. estrus). In some instances, vaginal lavages are conducted following copulation to detect the presence or absence of spermatozoa. Additionally, environmental disturbances (e.g. noise and cage maintenance) following copulation are thought to have negative implications on pregnancy in the BFF (Williams et al., 1991; Hutchins et al., 1996).

In light of these concerns expressed by the American Zoo and Aquarium Association, this laboratory proposed a series of endocrine studies which would not only

enhance understanding of BFF basic biology, but would also potentially improve its reproductive success. Since non-invasive hormonal monitoring of ovarian and adrenal function had proven invaluable to the acquisition of knowledge for other non-tractable carnivores (Carlstead et al., 1992, Brown et al., 1995a; Graham et al., 1995; Wasser et al., 1995; Jurke et al., 1997), the primary focus was to apply similar techniques to the BFF. Although previous studies of a similar nature have been conducted on the BFF to evaluate ovarian function (Gross et al., 1991; Gross, 1992; Brown, 1997), relatively few animals were monitored. By increasing the number of subjects, the accuracy and precision of the BFF reproductive database would be greatly improved. The hypothesis was that ovarian steroids and adrenocorticoids could be measured quantitatively and qualitatively in the feces of the BFF by enzyme immunoassay.

The first objective was to develop and validate an extraction protocol to measure fecal estradiol, progesterone and cortisol by enzyme immunoassay. The second objective was to elucidate the relationship between fecal estradiol levels and physical changes (i.e. vulval tumescence and increasing vaginal cornification) experienced by the BFF female during proestrus/estrus. If the correlation between these attributes was strong, fecal hormone analysis might be used to replace the more invasive methods of timing male/female introduction. The third objective was to describe the endocrine changes which occurred following copulation for pregnant and pseudopregnant female BFFs. Potentially, fecal hormone (i.e. estradiol and progesterone) profile patterns, concentrations or ratios might differ between these 2 groups, thereby permitting early pregnancy detection or provide insight into failed conception. From a management perspective, the ability to detect pregnancy would facilitate the provision of special care to these animals. The fourth objective was to determine the level of 'stress' that restraint episodes placed upon the BFF. To accomplish this, fecal cortisol levels would be used as physiological indices of stress following restraint and an ACTH challenge test would be conducted to ensure adrenocortical activity was being appropriately reflected in the

hormonal analyses. Collectively, the information gained from this study would have direct application towards the improvement of husbandry techniques and the general reproductive success for this species.

METHODS AND MATERIALS

Animals

Adult black-footed ferrets were maintained in the quarantine facilities of the Metro Toronto Zoo, Ontario, Canada and Omaha's Henry Doorly Zoo, Nebraska, USA over 3 years (1994 - 1996). Lighting was provided by large windows and full spectrum fluorescent fixtures. Fluorescent lighting was adjusted to natural ambient photoperiod for each institution, automatically turning on 10 - 30 min prior to sunrise and turning off 10 - 30 min after sunset. Room temperature was monitored daily and adjusted manually by thermostat when required to a controlled range of 15 - 19°C. The relative geographical locations of the Metro Toronto Zoo (MTZ) and Henry Doorly Zoo (HDZ) are 44°N 79° W and 42°N 96°W (Pleva, 1963), respectively. Ferrets were housed individually in either concrete floor cages (3 x 1.5 x 2.4 m) covered with mulch substrate or wooden freestanding cages (2.4 x 0.9 x 1.7 m). Each cage included 2 nest boxes as denning quarters and several lengths of large diameter tubing for behavioral enrichment. A 'hands-off' policy was implemented to ensure that the ferrets remained in a 'wild' or natural state, primarily for release consideration. Therefore, animals were subjected to a minimal number of disturbances other than cage maintenance or feeding. The daily diet consisted of mink chow (59.2%), ground rabbit meat (39.4%), and blood meal (0.4%), supplemented with vitamin E (1.0%). Each animal also received 2 live mice per week. All ferrets had access to water ad libitum.

Study 1: Reproductive Cycle

Breeding

Adult female black-footed ferrets housed in the quarantine facilites of both the MTZ (n=31, age range 1- 6 yrs) and HDZ (n=42, age range 1 - 7 yrs) during the breeding seasons of 1994 - 1996 and 1995 - 1996, respectively, were utilized in this study. The methods for evaluating reproductive status and protocols governing breeding management

for female black-footed ferrets in this study were similar to those sanctioned by the BFF SSP© and reported by previous works (Carvalho et al., 1991; Williams et al., 1992). Slight differences existed in the commencement and extent of breeding evaluation between the 2 institutions in this study. Beginning in February, MTZ female ferrets were restrained once weekly in small vinyl-coated mesh cages (37x12x12cm) to measure vulval size (length and width) with a plastic ruler. Once vulval intumescence had exceeded 5mm x 5mm, vaginal lavages also were conducted to assess vaginal cytology. Restraint episodes were initially conducted once weekly, but increased to 3X per week and then daily when the percentage of keratinized epithelial cells in the lavage exceeded 70% and 80%, respectively. Ferrets at the HDZ were observed in their pens daily for signs of vulval enlargement commencing in late February, but were not restrained for measurement or vaginal lavage until extensive swelling occurred. In general, first retraints occurred after the vulva attained a 5 mm x 5mm size. The methods of cytological evaluation were similar between the 2 instituitons. A plastic pipette tip was inserted 1.0 to 1.5 cm into the vaginal vault until it met slight resistance, and 50 µl sterile saline (0.9 % w/v NaCl) were then flushed into the vagina and aspirated twice. The contents of the pipette tip were then expelled onto a clean labeled slide, spread, and allowed to dry. Within 1 h, each smear was stained using a modified version of the Papanicolaou method (Sheehan and Hrapchak, 1980). In brief, this involved passing slides through the following series of solutions: 95% ethanol (v/v, 30 sec), tap water (60 sec), Harris' haematoxylin (4 min), tap water (30 sec), alkaline 80% ethanol (v/v; 1.5% NH_4OH, 15 sec), tap water (30 sec), 95% ethanol (60 sec), Orange G6 (5 min), 95% ethanol X2 (30 sec each), Eosin-azure 65 (2 min), 95% ethanol (60 sec), 100% ethanol (60 sec), xylene X2 (45 sec each). After staining, a glass cover slip was fixed to each slide with mounting medium.

The cytological contents of the smears were evaluated under light microscopy at 100 and 400X magnification. Percentage of cornification for each slide was established by averaging the results of three 100 cell counts in which vaginal epithelial cells were

classified as either keratinized or non-keratinized. The latter group consisted of all epithelial cells that were parbasal, intermediate, and superficial intermediate, while the former included only anuclear superficial cells (Olson et al., 1984, Carvalho et al., 1991, Williams et al., 1992). Figure 1 depicts the staining affinity and morphology of the various vaginal epithelial cell types during proestrus and estrus of the black-footed ferret. When the percentage of cornification in vaginal lavages exceeded 90%, the female was deemed to be at the optimal period of breeding readiness and was introduced to a sexually active male 3 nights later. The chosen male for each female was based on BFF SSP© recommendations and had the least coefficient of inbreeding with the respective female. Interactions between the pair were monitored by video-camera for up to 4 hr in the evening. Fifteen to thirty minutes after mating, the female was removed and restrained for a vaginal lavage. The wet smear was cover slipped and evaluated for the presence of sperm under phase contrast microscopy at 400X magnification. If sperm were present, the female was removed from breeding; however, if no sperm were found she was reintroduced to the male. The pair was then left together overnight for several days while their interactions were recorded on video-tape. Each morning the video-tape was reviewed to observe any breeding attempts of the previous night. Sperm-checks also would be repeated in the morning. Additional post-copulatory vaginal lavages were conducted for up to 4 days to evaluate vaginal cytology. When the percentage of cornification was observed to steadily decline from 90%, it was concluded that the female had ovulated and was no longer paired for breeding. If the female failed to ovulate or the male's breeding attempts were unsuccessful, an alternate male would be chosen and the pairing process was begun again until ovulation or sperm were confirmed by vaginal lavage.

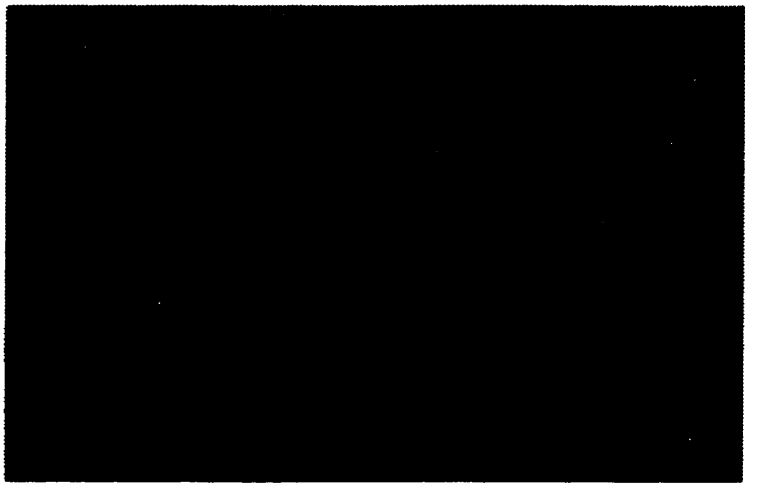
Fecal Sample Collection

Fecal samples were collected between 0800 - 1000 h from each female during the daily husbandry routine. Approximately 2.5 g of the total preceding 24 h excrement was

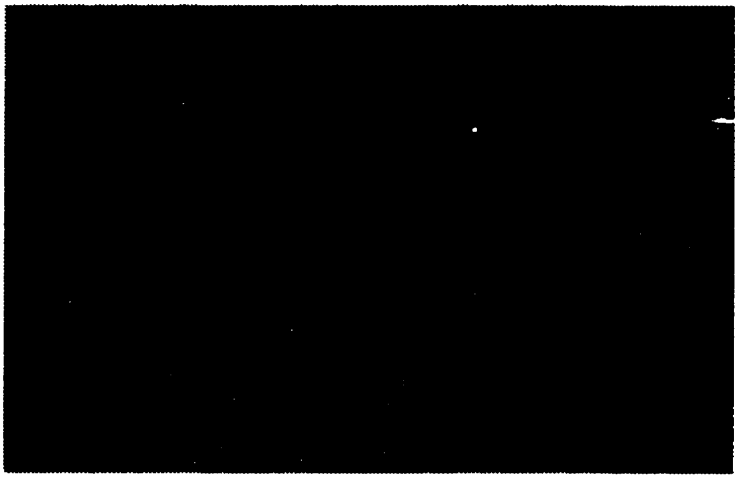
Figure 1. Vaginal epithelial cells from black-footed ferrets. Intermediate (A), superficial intermediate (B) and superficial (C) cells. Panels A and B are typical of proestrus and C is typical of estrus. Vaginal smears were stained using a modified Papanicolaou method. Microscopic slides for the panels were photographed at 400X (A) and 250X (B and C) magnification.

A

B



C



stored in a small polypropylene tube at -20°C until steroid extraction. For purposes of identification, tubes bore the collection date and ferret studbook number. Collections extended from the middle of February until 42 days past the last day of breeding.

Study 2: Physical Restraint and ACTH Challenge

Treatment Episodes

Adult male (n=4) and female (n=6) black-footed ferrets, aged 2 - 5 yrs, housed in the quarantine facility of the MTZ between mid-November 1996 to mid-January 1997 were utilized in this study. Ferrets were administered an ACTH challenge to determine the feasibility of detecting changes in adrenocortical activity by fecal cortisol analysis. During the course of this study, all ferrets received each of 4 different treatment regimens, designed to elicit different levels of adrenocortical response and to allow each animal to act as its own control. The different regimens comprised: 1) cage restraint only, 2) 0.2 ml saline injection (0.9% NaCl), 3) 20 μg ACTH liquid injection, and 4) 20 μg ACTH gelatin injection. Cage restraint was required to facilitate the intra-muscular injections of saline and ACTH to the upper hind leg. R. Knapp (Front Royal, Virginia) provided both liquid and gelatin ACTH preparations by suspending porcine ACTH in saline (0.9% NaCl) and methyl-cellulose gelatin, respectively. To account for possible acclimatization to the 'stressing' episodes, treatments were administered in a randomized order among the ferrets, with at least 14 days between treatments. The treatment dates were November 21st, December 13th, December 27th and January 10th. Mean dosages by ferret body weight of liquid and gelatin ACTH were 21.2 ± 0.5 μg/g and 21.4 ± 0.4 μg/g , respectively, for males and 23.9 ± 1.1 μg/g and 26.4 ± 1.2 μg/g, respectively for females.

Fecal Sample Collection

Although fecal sample collection times were similar to those of the reproductive cycle study, the specifics differed. The entire mass of feces excreted within a 24 hr period was collected daily from individual ferrets into plastic bags pre-labeled with the

appropriate studbook number and date. Collections started on November 21st, 8 days prior to first stress treatment episode and ended on January 18th, 8 days after the final treatment. Samples were stored at -20°C until steroid extraction was performed.

Fecal Steroid Extraction

Progesterone and Estradiol

Method 1

Fecal progesterone and estradiol were extracted from samples collected from 4 pregnant and 7 pseudopregnant females maintained at the MTZ (1995) according to a method modified from Shideler et al. (1993). After the 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% w/v BSA, 10% v/v methanol and 0.2% v/v 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 tube and stored at -20°C until analysis.

Method 2

The fecal steroid extraction procedure used in preparation for hormonal analysis was a modification of the methods applied by Graham et al. (1995) to monitor ovarian function in domestic and exotic felid species. Daily fecal samples collected from 14 pregnant and 12 pseudopregnant females (MTZ n=2 and 8, HDZ n=12 and 4, respectively) were processed in this manner. After thawing, 0.5 g wet feces were combined with 0.5 ml distilled water, 4.0 ml methanol and 1.0 g aluminum oxide in a clean 15 ml tube. Aluminum oxide was added since it previously had been shown to ensure uniform sample breakdown and remove background fecal pigments (Lucas et al. 1991; Graham et al., 1995). Following initial mixing with a stir rod, a teflon-lined cap was screwed onto the tube and the sample was vortexed at high speed for 1 min. Samples

were shaken for 1 h on a rotator at room temperature and then centrifuged for 10 min at 3500 rpm. The methanol fraction was decanted into a labeled polypropylene tube and stored at -20°C until hormonal analysis.

Cortisol

The protocol for extraction of cortisol differed from that of ovarian steroids because the extraction efficiency observed in a small pilot study utilizing method 2 was approximately 10-15%. Removal of the aluminum oxide step greatly improved the extraction efficiency, therefore the following extraction protocol was developed. To ensure uniformity of the feces, total daily fecal samples were thawed and thoroughly mixed within their plastic sample bags. For extraction, 0.5 g wet feces were added to 2.5 ml of 90% methanol (v/v) in a 5 ml polypropylene tube. The alcohol and fecal combination was immediately mixed with a stir rod, capped and vortexed for 1 min. Sample tubes were transferred to a mechanical rotator and shaken for 2 h, after which they were centrifuged for 10 min at 3500 rpm. The supernatant was then transferred to a clean, labeled polypropylene tube for storage at -20°C. Prior to hormonal analysis, 50 µl of extract were aliquotted into a 5 ml glass tube, submersed in a 34°C waterbath and taken to dryness under a stream of nitrogen gas. The lyophilent was stored for a maximum of 12 h at -20°C, before resuspension in 50 µl enzyme immunoassay (EIA) buffer and dilution for hormonal analysis.

Fecal Steroid Hormonal Analyses

Enzyme Immunoassays

Fecal sample extracts were analyzed for steroid hormone concentrations by enzyme immunoassay as previously described (Munro and Stabenfeldt, 1985; Munro et al., 1991) and as adapted for use in our laboratory with wood bison (Matsuda et al., 1996; Othen, 1997). Feces collected during the reproductive cycle of the black-footed ferret

were quantified for total progesterone and estradiol content, while those from the ACTH challenge were analyzed solely for cortsiol.

Progesterone

The polyclonal antiserum to progesterone (R4861) raised in rabbits was provided by C.J. Munro (University of California, Davis). The cross-reactivities for the antibody were: progesterone, 100%; 11α-hydroxyprogesterone, 40%; 5α-pregnone-3,20-dione, 12.19%; 17α-hydroxyprogesterone, 0.38%; 20α-hydroxyprogesterone, 0.13%; pregnandiol, < 0.01%; pregnenolone, 0.12%; estradiol-17β, estrone, and testosterone, <0.01% and cortisol, < 0.04% (Munro and Stabenfeldt, 1984). A primary antibody stock of 1:10 was prepared by dilution in 50 mM sodium bicarbonate coating buffer (pH 9.6) and stored at - 20°C until required.

Progesterone standards, controls and sample extracts were all prepared prior to assay by dilution in EIA buffer (0.1 M phosphate buffered, 0.1% bovine serum albumin, pH 7.0). Sample extracts were diluted 1:13 or 1:16 for use in the assay, while 2 internal controls were prepared by diluting the stock control 1:10 and 1:100. In our laboratory, progesterone control stock used was the supernatant collected after extracting feces from the luteal phase of a pregnant lowland gorilla by the extraction protocol of Schideler et al. (1993). The concentrations of the progesterone solutions used in the standard curve ranged from 0 - 2500 pg/well. Diluted extracts and controls were pipetted in duplicate and standards in triplicate; a pair at the beginning of the microtiter plate and a third at the end to help account for pipetting time lag and drift across the plate.

Prior to use in the EIA, the primary antibody stock was further diluted to a working stock of 1:6,000 with additional coating buffer. An aliquot of 50 µl was used to coat each well of a microtiter plate, which was then covered with an acetate plate sealer and incubated overnight (14 - 18 h) at 4°C. Plates were rinsed 5X with a 0.15 mM NaCl wash solution (containing 0.05% Tween) in a Dynatech Ultrawash II microplate washer to remove unbound antibody and then blotted dry to ensure the removal of excess wash

solution. After washing, 50 µl volumes of progesterone standards, controls and samples were added to the plate and immediately followed by the addition of 50 µl progesterone-horseradish peroxidase (HRP; 1:60,000 dilution; supplied by C.J. Munro, Univerisity of California, Davis) to each well. Two wells of the plate, loaded with HRP only, served as blanks for calibration. Plates were covered with an acetate plate sealer and incubated 2 h at room temperature. After incubation, unbound HRP was removed from the plate wells by washing 5X with 0.15 M NaCl wash solution and patting to dryness. Immediately after preparation, 100 µl substrate buffer [0.4mM azino-bis(3-ethylbenzthiaxoline-6-sulfonic acid) with 1.6 mM H_2O_2 in 0.05 M citrate solution, pH 4.0] were pipetted into each well. Plates were tapped slightly to settle the substrate, sealed with acetate plate sealers and shaken on a rotator at room temperature for approximately 30 min. The optical densities were read at 405 nm using a Dynatech 700 plate reader interfaced with a MacIntosh computer.

Estradiol

The polyclonal antibody to estradiol-17β (R4972) and HRP conjugate were supplied by C.J. Munro (University of California, Davis). The antiserum cross-reacted with estradiol-17β, 100%; estrone, 3.3%; progesterone, 0.8%; testosterone, 1.0%; androstenedione, 1.0% and had negligible reactivity with other steroids tested (<1.0%; C.J. Munro, personal communication). The antibody and HRP conjugate were diluted 1:10,000 and 1:50,000, respectively, with EIA coating buffer. Standards were prepared by serial dilution to concentrations of 500, 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9, 1.95, and 0 pg/well estradiol in EIA buffer. Fecal sample extracts were diluted 1:3 with EIA buffer, while internal controls were diluted from house stocks by 1:10 and 1:50. Similar to the progesterone procedure, the estradiol control stock was supernatant collected after extracting feces from the luteal phase of a pregnant lowland gorilla by the extraction protocol of Schideler et al. (1993). With one exception, the estradiol assay protocol was identical to that of the progesterone assay: following the initial wash to remove unbound

antibody, microtiter plates were coated with EIA buffer (50 µl/well) and incubated for 2 h at room temperature before standards, controls, diluted samples and HRP were added.

Cortisol

Fecal corticosteroids were quanitified with EIA, using a polyclonal antibody to cortisol raised in rabbits (R4866; supplied by C.J. Munro, University of California, Davis). The cross-reactivities of this antibody were cortisol, 100%; prednisolone, 9.9%; prednisone, 6.3%; cortisone, 5.0%; corticosterone, 0.7%; deoxycorticosterone, 0.3%; 21-deoxycortisone, 0.5%; and 11-desoxycortisol, 0.2% (C.J. Munro, personal communication). Antibody and HRP were diluted 1:8,500 and 1:20,000, respectively. Standards prepared in EIA buffer ranged from 0 - 1000 pg , while controls were diluted to 1:4 and 1:20. The cortisol control stock used in our laboratory was neat urine collected from a single male wood bison during the fall rut. A dilution of 1:10 with EIA buffer prepared extracts for use. This assay was conducted in a manner similar to the progesterone assay except that plates were incubated for only 1 h at room temperature following loading with standards and samples.

Assay Validation

Endogenous Steroid Extraction Comparison

Fecal samples (n=26) collected during the pericopulatory interval for a single female BFF were extracted by 3 separate protocols to compare the relative efficiencies of these methods to liberate endogenous ovarian steroids from feces. These protocols included: method 1, method 2 and the fecal extraction procedure orginally used by Graham et al. (1995). The last differred from method 2 by the addition of 3.0 ml petroleum ether prior to centrifugation and collection of the methanol phase as the supernatant. Extracts were assayed for progesterone and estradiol content as described previously.

Extraction Efficiency

Feces collected from undisturbed, anestrous ferrets (n=5) were pooled and used to assess steroidal extraction efficiency of the various protocols. Increasing levels of steroid standards were added to 0.5 g aliquots of feces prior to extraction. For extraction method 1, the concentrations of progesterone and estradiol obtained after spiking were 25, 50, 75, 100, 150, 200, 300, 400, 600 pg/well and 20, 40, 60, 80, 100, 140, 200, 260, 360 pg/well, respectively. Final steroid levels obtained after spiking for extraction method 2 were: 1.95, 3.9, 7.8, 15.6, 62.5, 125, 250, 500 pg estradiol/well and 9.75, 19.5, 39, 78, 156, 312, 625, 1250, 2500 pg progesterone/well. Concentrations obtained following spiking for the cortisol extraction method were 7.8, 15.6, 31.2, 62.5, 125, and 500 pg/well. Sample duplicates were assayed for the appropriate steroids as previously described above.

Percent Recovery

Separate pools of fecal extract from the cortisol protocol and extraction method 2 were created to examine the percent recovery of cortisol and ovarian steroids, respectively. Pools were produced by combining the supernatant collected from 6 separate 0.5 g fecal samples from method 2 extraction and 10 separate 0.5 g fecal samples from cortisol protocol extractions. In both cases, the feces used came from single samples collected from 4 anestrous ferrets. One ml aliquots from the appropriate pool were spiked to obtain increasing amounts of progesterone (9.75, 19.5, 39, 78, 156, 312, 625, 1250, 2500 pg/well), estradiol (1.95, 3.9, 7.8, 15.6, 62.5, 125, 250, 500, 1000 pg/well) or cortisol (3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500, 1000 pg/well). Samples were then assayed in duplicate as described above.

Parallelism

Fecal extracts (20 µl from every sample of 4 female ferrets) were pooled over the collection periods of each study to evalute parallelism with the standard curve. Using EIA buffer, serial two-fold dilutions were made from the fecal extract pool to yield a range from neat (undiluted) to 1:512. Dilutions from the reproductive cycle were assayed

separately against estradiol and progesterone standards, while those from the ACTH challenge test were assayed against cortisol standards. In all cases, mean percentage binding data generated from the standard curve were plotted against their logarithmically transformed doses on the x-axis.

Reproductive Characteristics

Records gathered from the MTZ (1994-1996) and HDZ (1995-1996) were analyzed for population parameters in order to examine BFF colony demographics. Data included number of breeding days for each female, females' ages, pregnancy rate, gestation length, number of kits whelped and kit survival until weaning.

Statistical Analyses

Correlation of estradiol and progesterone concentrations for each extraction protocol was determined by using a linear model from the SAS statistics package (SAS, 1985).

Study 1

Only data from extraction method 2, the preferred protocol, was used in this study because it had greater extraction efficiencies for endogenous and exogenous ovarian steroids. Samples for individual ferrets were aligned by the last day of breeding when categorized as pregnant and pseudopregnant status. Since fecal sampling was often infrequent, hormone levels were pooled in groups of 4 and 3 days starting with the day of last breeding to facilitate analysis of proestrual and gestational data, respectively. Changes in estradiol and progesterone levels for pregnant and pseudopregnant animals both pre- and post-mating were determined by ANOVA for repeated measures using StatView (Version 4.5, Abacus Concepts, Inc., Berkley CA). Differences between pre- and post-mating overall mean estradiol concentrations for both pregnant and pseudopregnant animals were analyzed by using Student's t-test. The correlation

between physical changes during proestrus and estradiol concentrations as well as area under the estradiol and progesterone curves with litter size also were determined by using StatView.

Study 2

Mean basal cortisol concentrations of the two adrenocortical activities (high and low) were analyzed for differences by using a Student's t-test. Baseline cortisol levels for each ferret prior to each treatment were calculated by averaging values from day -3 to day 0. Significant changes in hormone concentrations for cortisol following treatment were determined by ANOVA for repeated measures using StatView. Differences in mean cortisol concentrations between various stages of the physical restraint/ACTH challenge study were analyzed by using a Student's t-test.

Reproductive Characteristics

Each ferret's age was calculated by rounding up to the closest full year (e.g. 3 yrs 6 months became 4 yrs). Gestation lengths were determined from day of last breeding until the day in which kits were found within the nest box.

RESULTS

Assay Validation

Parallelism

The curves generated by analyses of serially diluted fecal extract pools for estradiol and progesterone were parallel with their respective standard curves for both ovarian steroid extraction protocols (Figures 2 and 3). For cortisol, the serially diluted fecal extract pool was parallel with a standard curve of cortisol. However, failure to dry-down and resuspend corticosteroid extracts in EIA buffer produced a curve which did not exhibit parallelism with the standard curve (Figure 4).

Endogenous Extraction Comparison

Longitudinal profiles comparing recovery of endogenous ovarian steroids by three separate extraction protocols [method 1, method 2, method of Graham et al., (1995)] appear in Figure 5. The correlation analysis describing the relationships of these protocols appears in Table I. For estradiol and progesterone, method 2 and the method of Graham demonstrated profiles which closely mimicked each other over time, both qualitatively and quantitatively. While method 1 demonstrated similar patterns, it appeared to extract only ~66% and ~80% of the estradiol and progesterone present, respectively, than was measured by the other methods.

Extraction Efficiency

The mean efficiencies of extraction method 1 and method 2 to remove progesterone from the feces were 53.5% (range 26.0%-73.3%) and 85.0% (range 66.4%-96.3%), respectively. For estradiol, the mean extraction efficiencies by these two protocols were 60.3% (method 1, range 39.8%-81.6%) and 77.7% (method 2, range 72.7%-89.7%). Based on these efficiencies and the endogenous extraction results, method 2 was selected for use in this study to monitor ovarian activity in the black-footed ferret.

The mean extraction efficiency of cortisol from feces was 83.7% (range 68.1%-99.8%).

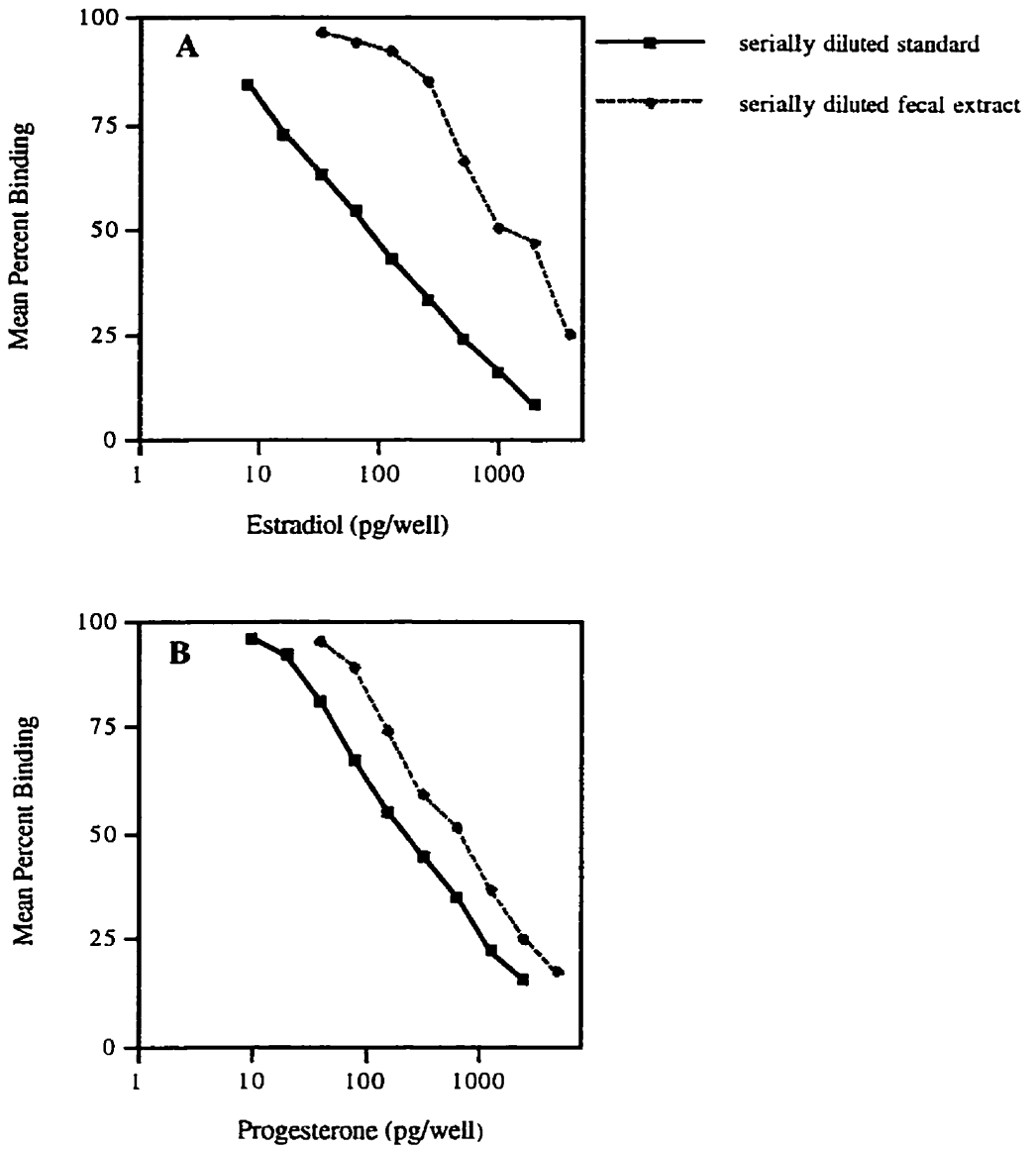


Figure 2. Parallelism curves resulting from analysis of serially diluted estradiol (A) and progesterone (B) standards and pooled fecal extracts using extraction method 1.

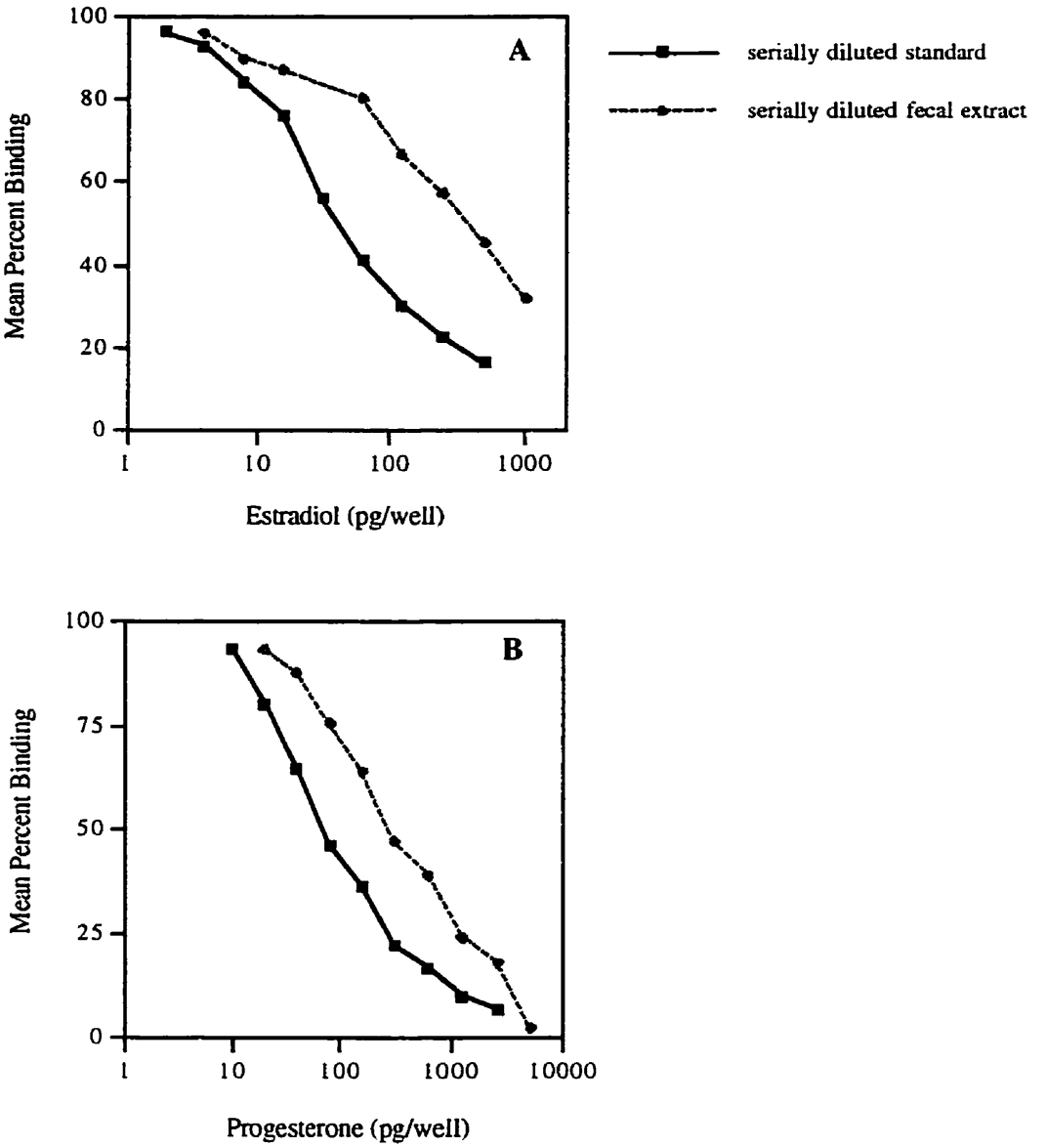


Figure 3. Parallelism curves resulting from analysis of serially diluted estradiol (A) and progesterone (B) standards and pooled fecal extracts using extraction method 2.

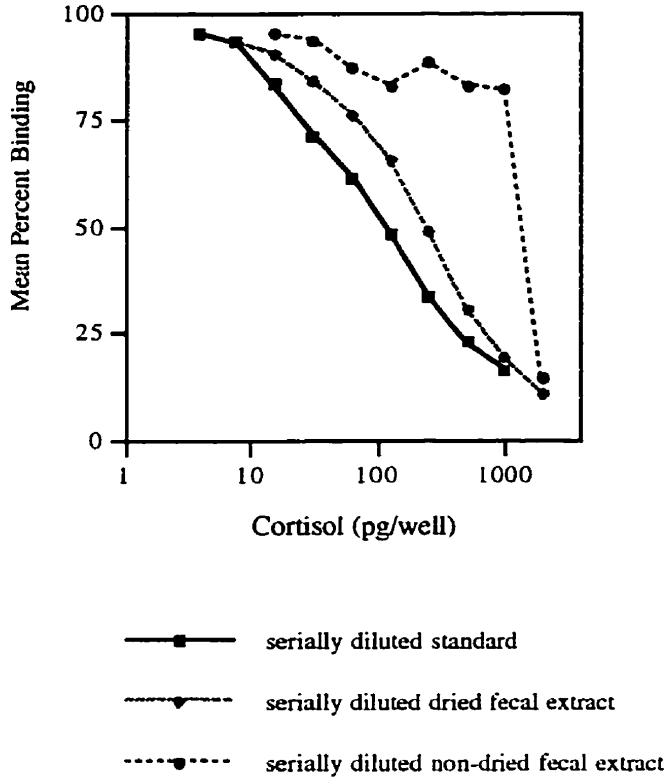


Figure 4. Parallelism curves resulting from analysis of serially diluted cortisol standards, dried/EIA resuspended and non-dried pooled fecal extracts.

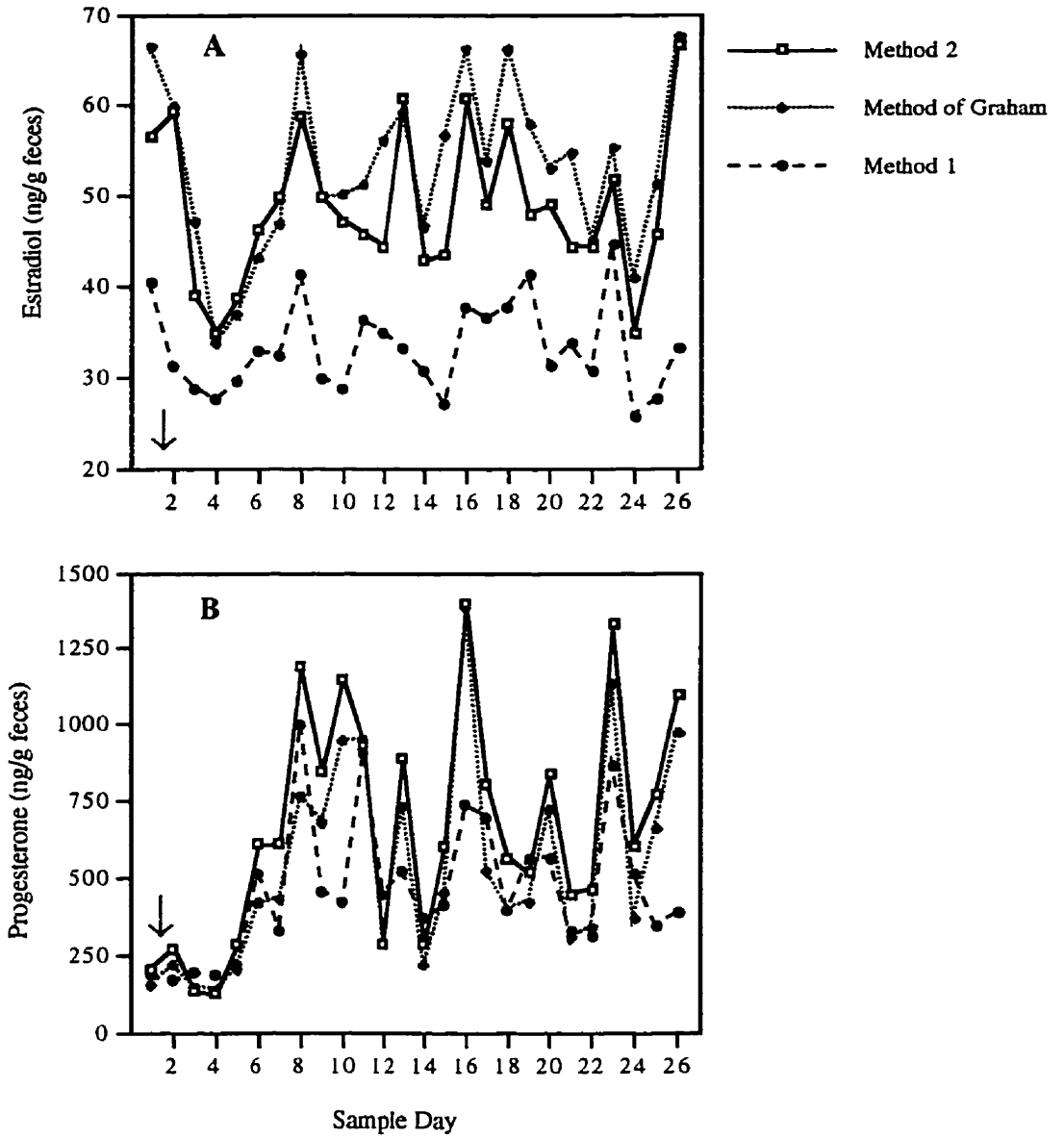


Figure 5. Composite profiles detailing relative quantities of endogenous estradiol (A) and progesterone (B) removed from feces by three extraction protocols using samples collected during the peri-copulatory period from one female black-footed ferret. Arrow indicates time of mating.

Table I. Correlation between extraction protocols for steroid yield.

Steroid			Correlation Coefficient	P-Value
Estradiol	Method 2	Method 1	0.545	0.0040
	Method 2	Graham Method	0.863	0.0001
	Method 1	Graham Method	0.620	0.0007
Progesterone	Method 2	Method 1	0.765	0.0001
	Method 2	Graham Method	0.964	0.0001
	Method 1	Graham Method	0.709	0.0001

Percent Recovery

The progesterone and estradiol assays detected a mean of 82.7% (range 68.1%-99.0%) and 80.2% (range 67.0%-97.1%), respectively, of the appropriate standard added to anestrous fecal extracts. The cortisol assay measured a mean of 92.6% (range 80.4%-105.5%) of added cortisol standard.

Assay Variation

Two lots of controls were used to monitor variation of the progesterone assay. The inter-assay coefficients of variation for lot 1 were 16.3% and 16.7% (n=28), at mean percent bindings of 29.6% and 56.2%, respectively, and for lot 2, 14.2% and 16.4% (n=95) at average percent bindings of 37.6% and 69.9%, respectively. The intra-assay coefficients of variation for 2 separate samples analyzed on a single plate were 6.4% (n=15) and 4.1% (n=11), at mean percent bindings of 31.8% and 62.0%, respectively.

Similarly, two separate lots of controls were used to monitor variation of the estradiol assay. For lot 1, the inter-assay coefficients of variation were 15.0% and 17.2% (n=71), at mean percent bindings of 46.4% and 78.9%, respectively. The inter-assay coefficients of variation for lot 2 were 15.2% and 13.6% (n=72), at mean percent bindings of 39.4% and 79.0%, respectively. The intra-assay coefficient of variation of a single sample was 5.3% (n=13), at a mean percent binding of 72.1%.

The inter-assay coefficients of variation for the cortisol assay were 7.9% and 13.9% (n=33), at mean percent bindings of 30.9% and 77.5%, respectively. A single sample analyzed with a mean percent binding 61.5% had a 6.0% (n=13) intra-assay coefficient of variation.

Estrous Cycle Characteristics

Proestrus

Physical Changes

Percent cornification and vulval square area (length x width) were examined for 12 BFFs with excellent breeding readiness records. A similar trend appears in mean profiles for both physical changes; increasing during proestrus to reach peak values just prior to or at first breeding and then declining following breeding (Figure 6). Vulval size plateaued from -18 days to breeding (day 0 = first breeding), with a mean (± SEM) area of 82.2 ± 7.3 mm^2 at first breeding. The largest and smallest vulval areas for individual ferrets at first breeding were 110 mm^2 and 54 mm^2, respectively. Mean (± SEM) percent cornification peaked at 90.6% ± 2.7% 3 days prior to first breeding. and was significantly correlated with vulval area (r^2=0.474, $p<0.0001$).

Estradiol

Fecal estradiol concentrations on days of breeding readiness evaluation for the 12 BFF females were compared with vulva size and vaginal cytology. Similar to the physical changes observed, mean fecal estradiol increased during proestrus, but failed to demonstrate a definite peak associated with estrus or breeding (Appendix IIA). Concentrations of estradiol on the day of breeding readiness evaluation were significantly correlated with vulval area and percent cornification (Table II). However, correlation coefficients and their associated significance were greatly improved when a 4 day average of estradiol values (3 days prior plus day of cytological evaluation) were used (Table II). Figure 6C depicts the mean 4 day average of estradiol concentrations during proestrus until 10 days following first breeding and demonstrates an increasing trend followed by a fall after breeding. Single day and 4 day average estradiol concentrations were significantly correlated with each other (r^2=0.707, $p<0.001$).

Figure 6. Mean changes (+ SEM) in vulval size (A), vaginal cytology (B) and mean 4 day estradiol concentrations (C) for black-footed ferret females (n=12) prior to and following breeding.

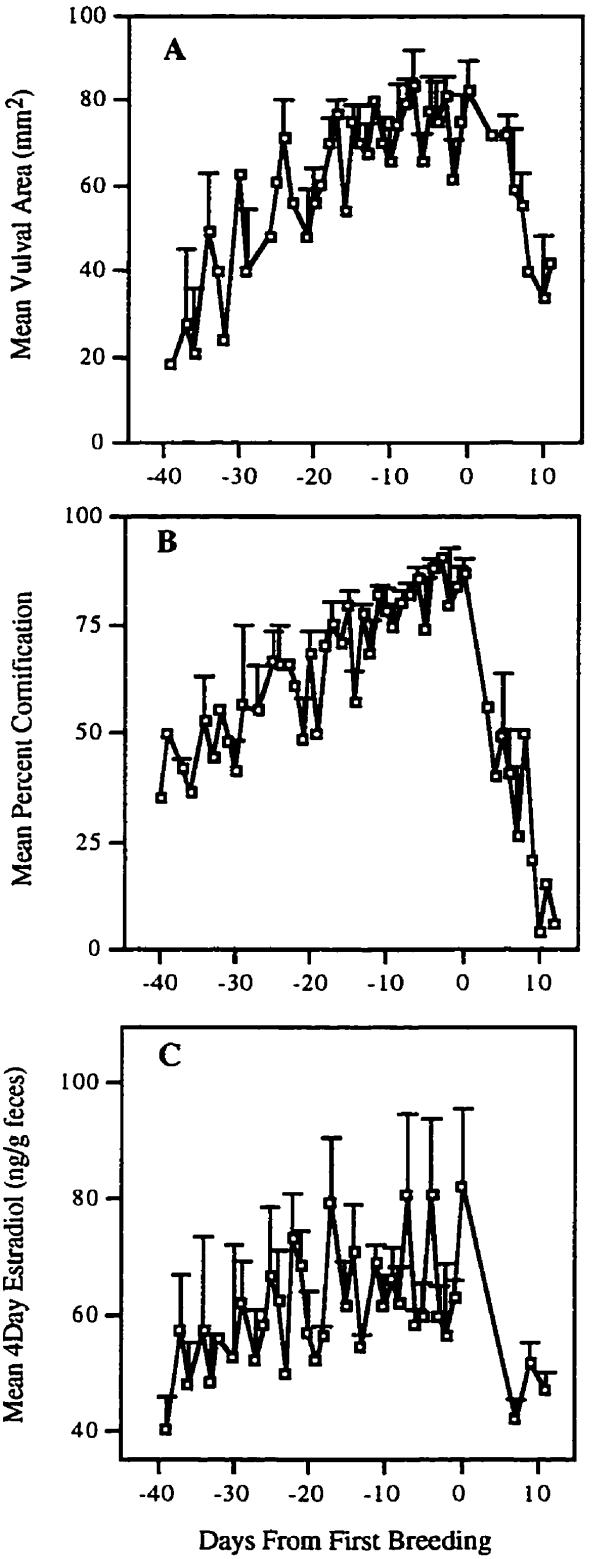

A
Mean Vulval Area (mm²)
100
80
60
40
20
0
-40
-30
-20
-10
0
10
B
Mean Percent Cornification
100
75
50
25
0
C
Mean 4Day Estradiol (ng/g feces)
100
80
60
40
Days From First Breeding

Table II. Relationship between hormonal and physical changes prior to and following breeding.

		Correlation Coefficient	P-Value
Single Day Estradiol	Vaginal Cytology	0.186	0.0269
4 Day Estradiol Average	Vaginal Cytology	0.287	0.0004
Single Day Estradiol	Vulval Area	0.180	0.0362
4 Day Estradiol Average	Vulval Area	0.330	<0.0001

Longitudinal Assessment

Composite profiles of the daily temporal endocrine patterns for pregnant (n=14) and pseudopregnant (n=12) female BFFs over the reproductive cycle are shown in Figure 7. A similar mean profile for 4 pregnant and 7 pseudopregnant female BFFs extracted by method 1 and aligned by last day of breeding is depicted in Appendix IIB. The daily ovarian steroids during the reproductive cylce of a single female induced to ovulate with 110 IU hCG appears in Appendix IIC.

Estradiol

For pregnant and pseudopregnant ferrets, mean (± SEM) estradiol concentrations 30 days prior to the last day of breeding did not differ ($p>0.05$) and were 56.4 ± 4.9 ng/g feces and 55.7 ± 4.3 ng/g feces, respectively, and increased to 91.5 ± 11.0 ng/g feces and 86.1 ± 12.7 ng/g feces during breeding, again with no differences between the two groups. The declining trend in estradiol values during the time of breeding (Figure 7A) represents a limited number of samples since collections were infrequent in this time period. It is likely not an artifact since a similar trend appears in the endogenous estradiol extraction comparison (Figure 5A) and the profile of a single BFF injected with hCG (Appendix IIC). Composite pregnant and pseudopregnant profiles for both estradiol and progesterone appeared to diverge between days 18 - 25, with values for pseudopregnant ferrets being lower. Mean estradiol concentrations at day 42 following breeding were 54.2 ± 17.6 ng/g feces and 50.9 ± 6.2 ng/g feces for pseudopregnant and pregnant ferrets, respectively. There were no significant differences between estradiol values for pregnant and pseudopregnant ferrets in either pre-breeding or post-breeding time periods when an ANOVA for repeated measures was performed ($p>0.05$). Overall mean (± SEM) pre-breeding estradiol concentrations (days -30 to -7) for pregnant and pseudopregnant ferrets were 68.8 ± 2.2 ng/g feces (n=305) and 74.3 ± 2.5 ng/g feces (n=259), respectively. These values were significantly higher than their associated post-breeding (days 0 to 42) counter parts: 59.9 ± 1.3 ng/g feces (n=467) and 52.8 ± 1.2 ng/g feces (n=400, $p<0.0001$),

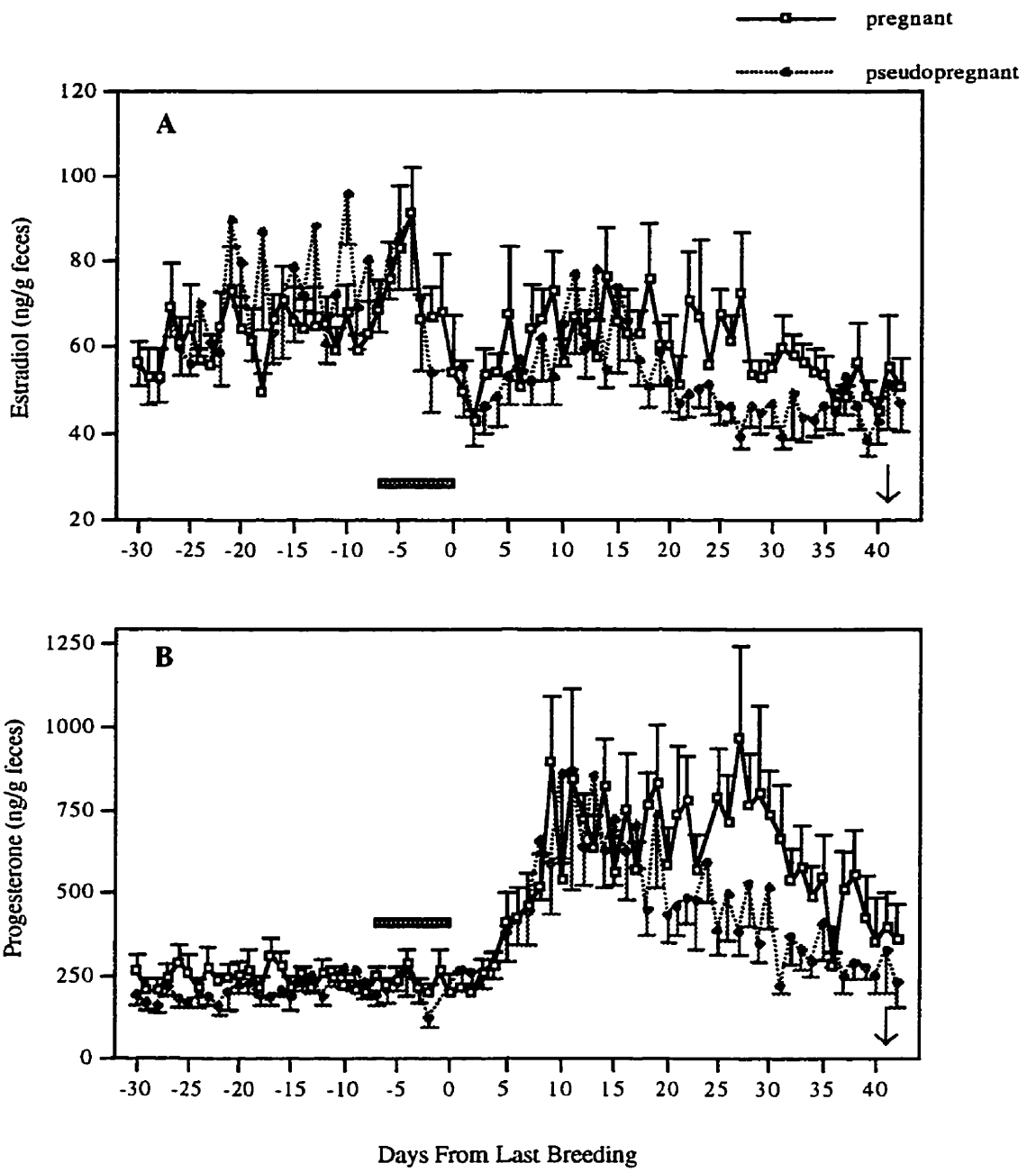


Figure 7. Longitudinal profiles of mean fecal estradiol (A) and progesterone (B) during the estrous cycles and post breeding of pregnant (n=14) and pseudopregnant (n=12) black-footed ferrets. Horizontal bars denote days of breeding. Arrows indicate mean day of parturition for pregnant females. Vertical lines indicate + or - SEM.

respectively. For pregnant females, the area under the individual estradiol curves from day 15 to 35 had a correlation coefficient of 0.560 with litter size (p=0.0356).

Progesterone

Mean progesterone concentrations for the 30 days prior to the last day of breeding for pregnant (247.0 ± 7.0 ng/g feces, n=302) and pseudopregnant (212.2 ± 7.1 ng/g feces, n=6.98) females were relatively low and did not differ (p>0.05, Figure 7B). Progesterone levels rose above the pre-breeding baseline for both groups by 5 days following the last day of breeding, when mean values were 382.6 ± 88.7 ng/g feces and 414.0 ± 86.1 ng/g feces for pseudopregnant and pregnant animals, respectively. Values then increased dramatically to day 10 and plateaued until days 18 and 33 for pseudopregnant and pregnant females, respectively. Based on ANOVA by repeated measures, progesterone concentrations did not differ between days 5 and 14 after breeding for the two groups (p>0.05). However, after day 15, the hormone profiles diverged and were significantly different between days 15 - 32 when examined by an ANOVA for repeated measures (p=0.0293). Mean progesterone concentrations declined to 232.1 ± 76.1 ng/g feces and 356.0 ± 104.5 for pseudopregnant and pregnant animals, respectively, at day 42 following last breeding. The area under the individual progesterone curves did not correlate significantly with litter size following day 15 after breeding (r^2=0.383, p=0.181). There were no significant differences for ratios of progesterone:estradiol between pregnant and pseudopregnant females either pre- or post-breeding when tested with ANOVA by repeated measures.

Physical Restraint and ACTH Challenge

Cortisol

To determine if differences existed in basal adrenocortical activity of the BFF (n=10), 9 days of fecal cortisol concentrations prior to the commencement of the stress study were averaged. Based on these mean cortisol values, ferrets were arbitrarily

assigned to either low or high activity categories. Five fell into each category, with mean cortisol values for individuals ranging from 45.0 ± 10.4 to 59.5 ± 6.1 ng/g feces (low group) and 78.0 ± 12.2 to 115.1 ±14.4 ng/g feces (high group). Within each group, there were no significant differences between mean cortisol values of individual ferrets ($p>0.05$). The overall mean (± SEM) cortisol levels for low and high activity categories were 51.5 ± 3.0 ng/g feces and 87.4 ± 5.5 ng/g feces, respectively, with a significant difference in mean basal cortisol concentrations existing between the categories ($p<0.0001$).

Composite cortisol profiles for cage restraint, saline injection, liquid ACTH injection and gelatin ACTH injection treatments administered to 10 BFF ferrets are shown in Figure 8. To create a fecal cortisol baseline value for each of the 4 treatments regimens, 4 days (3 days prior to plus day of treatment) were grouped together. Mean fecal cortisol levels during baseline periods prior to cage restraint (72.8 ± 5.3 ng/g feces), saline (81.55 ± 5.9 ng/g feces), liquid ACTH (73.7 ± 3.7 ng/g feces), and gel ACTH (68.9 ± 4.0 ng/g feces) treatments episodes were not different (range 32 - 177 ng/g feces, $p>0.05$). Following cage restraint, although an elevation was observed, mean fecal cortisol concentrations were not significantly different from pre-treatment baseline values ($p>0.05$), and peaked at 96.0 ± 12.3 ng/g feces on day 2. Mean peak cortisol levels one day after saline were 114.1 ± 26.9 ng/g feces, with these levels approaching significance from the pre-treatment baseline ($p=0.0792$). Post-treatment mean cortisol concentrations of liquid and gelatin ACTH treatments were different from their pre-treatment baselines ($p=0.0004$ and $p=0.0463$, respectively). Mean peak values appeared at days 1 (114.4 ± 17.6 ng/g feces) and 2 (142.3 ± 17.5 ng/g feces) for gelatin and liquid ACTH regimens, respectively. After both ACTH treatments, mean cortisol concentrations returned to values not different from pre-treatment baseline values by the first day after the treatment peak ($p>0.05$).

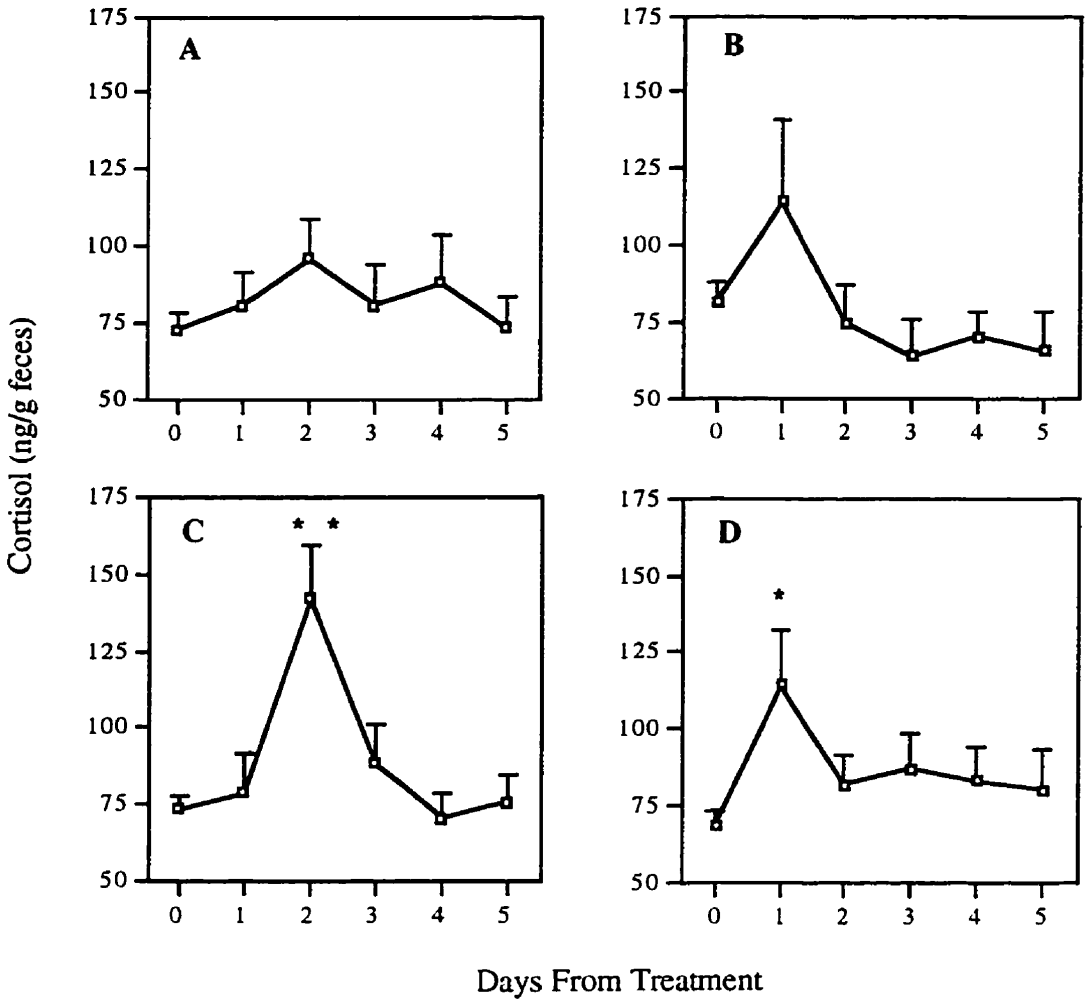


Figure 8. Mean fecal cortisol profiles for 10 black-footed ferrets following cage restraint (A), saline IM (B), liquid ACTH IM (C), and gelatin ACTH IM (D). Day 0 = average of 4 pretreatment days. Asterisks indicate significant elevation above day 0 value (* p=0.0463, ** p=0.0004). Vertical lines indicate + SEM.

Overall, there were no differences in fecal cortisol excretion patterns between the 4 different treatment regimens ($p>0.05$) of all 10 ferrets when compared using ANOVA by repeated measures. Subdivision of data by either sex (male/female) or adrenocortical activity (low/high) failed to demonstrate statistical differences among the treatments ($p>0.05$).

Levels of fecal cortisol excretion were compared at two time periods of the stress experiment: pre- and post-study (Figure 9). In each case, values represented the average cortisol concentrations for 3 consecutive days from all 10 BFFs. Pre-, and post-study time periods were 3 - 5 days prior to the first, and 6 - 8 days post final treatment episodes, respectively. Average (±SEM) cortisol concentrations were 66.6 ± 5.9 ng/g feces (pre) and 82.9 ± 6.9 ng/g feces (post). Post-study cortisol values were significantly greater than those of the pre-study period ($p=0.0384$).

Reproductive Characteristics

A total of 73 female BFFs were maintained at the 2 institutions over a 3 year period: 31 at MTZ (1994-1996) and 42 at HDZ (1995-1996). Sixty of these animals (82.2%) were successfully bred and deemed to have ovulated based on vaginal cytology. The remainder were removed from the breeding program due to health complications ($n=1$), hCG induction of ovulation ($n=3$), or failure to exhibit signs of reproductive activity ($n=7$). For statistical purposes, 2 females were eliminated from anaylses of this data set since it was not known whether they were truly pseudopregnant or had cannibalized their newborn young. Blood was found in their cages following the days of predicted parturition. Additionally, 3 females (2 at HDZ and 1 at MTZ) entered a second estrus, 2 following the end of pseudopregnancy and 1 following the loss of her litter. Two of these females were re-bred in the beginning of June, but failed to become

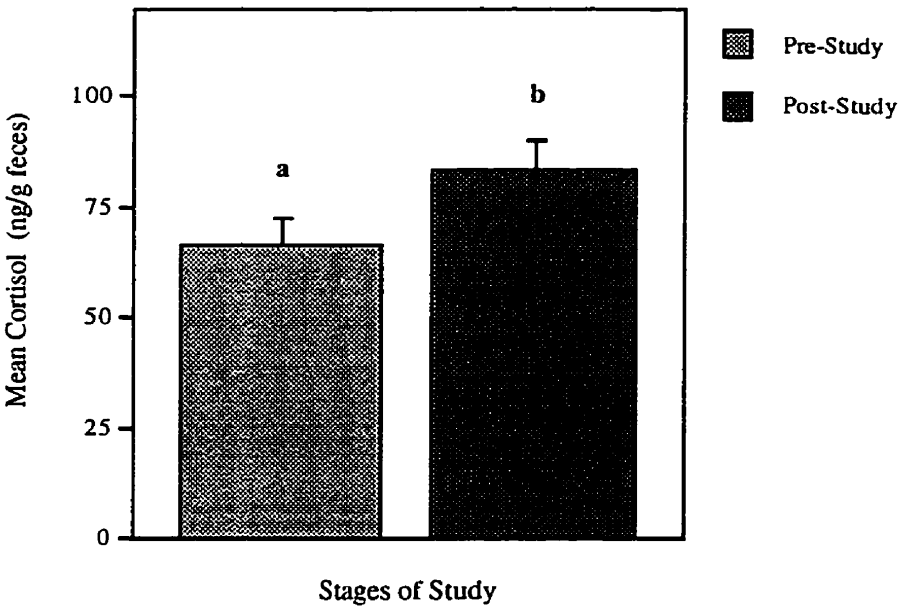


Figure 9. Basal adrenocortical activity during two time periods of stress study for 10 black-footed ferrets. Stages with different letters were significantly different ($p<0.05$).

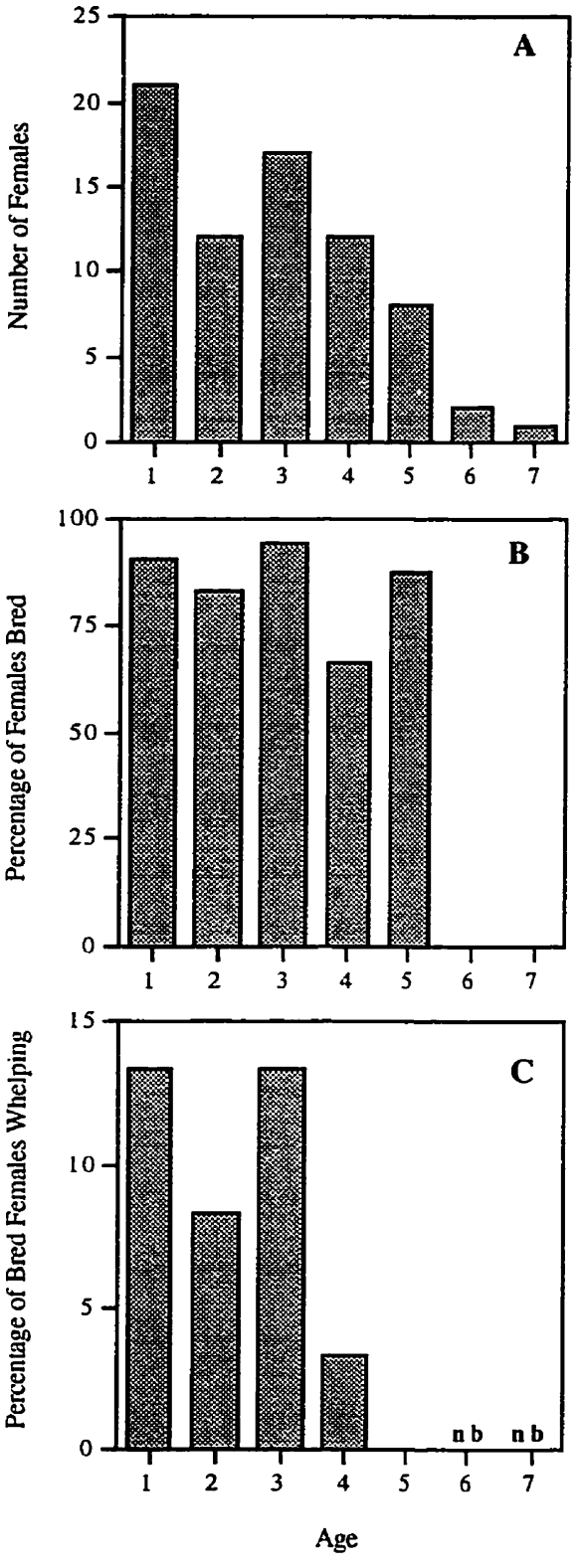
pregnant. The third cycled out of estrus in mid-July prior to attempts at breeding. Mean duration of continuous estrus for 3 females prior to ovulation induction with hCG (110 IU) was 31 ± 6.2 days (individual lengths = 19, 34 and 40 days).

The overall pregnancy rate of females bred was 38.3%, with individual institutional rates being 18.5% (n=5) and 54.6% (n=18) for the MTZ and HDZ, respectively. Mean (± SEM) gestation length from date of last breeding was 41.5 ± 0.5 days (range 37-48 days, n=23). A total of 92 kits were whelped from these pregnancies, with 18 from the MTZ and 74 from the HDZ. Average litter sizes for the MTZ and HDZ were 3.6 ± 0.6 (range 2-5) and 4.1 ± 0.4 (range 2-7), respectively, with a combined average of 4.0 ± 0.3 kits. The overall kit survival rate was 39.2%. There were differences in kit survival between institutions as demonstrated by 61.1% and 39.8% survival post-weaning at the MTZ and HDZ, respectively. Finally, females < 3 yrs of age had a greater kit survival rate than females ≥3 yrs (43.6% vs. 32.4%), but produced similar numbers of kits per female on average (4.2 vs. 3.7, $p>0.05$).

There was a significant difference in the number of physical restraint episodes conducted at the 2 institutions to evaluate vulval size and vaginal cytology prior to breeding. On average, the MTZ (n=28) and HDZ (n=35) conducted 20.1 ± 1.1 and 7.1 ± 0.4 episodes, respectively, during the time of this study ($p<0.0001$).

The number of female BFFs maintained at the 2 institutions declined with age (Figure 10A), with the MTZ generally having older animals (Figure 11). One exception to this trend was that there were fewer 2 yr old than 3 and 4 yr old animals. Percentages of females bred per age class varied (Figure 10B). The number of bred females that successfully whelped also varied by age class (Figure 10C). However, the percentage of bred animals whelping after 3 yrs of age was dramatically lower than those in the age range of 1-3 yrs. Additionally, 5 females failing to exhibit estrous activity (no evidence of vulval swelling or increasing percent cornification) were in the range of 4-7 yrs.

Figure 10. Black-footed ferret population demographics and reproductive success (n=73). Age distribution over 3 breeding seasons (A), percentage of total females bred by age group (B) and percentage of females successfully whelping by age group (C).


A
Number of Females
25
20
15
10
5
0
1
2
3
4
5
6
7
B
Percentage of Females Bred
100
75
50
25
0
C
Percentage of Bred Females Whelping
15
10
5
0
n b
n b
Age

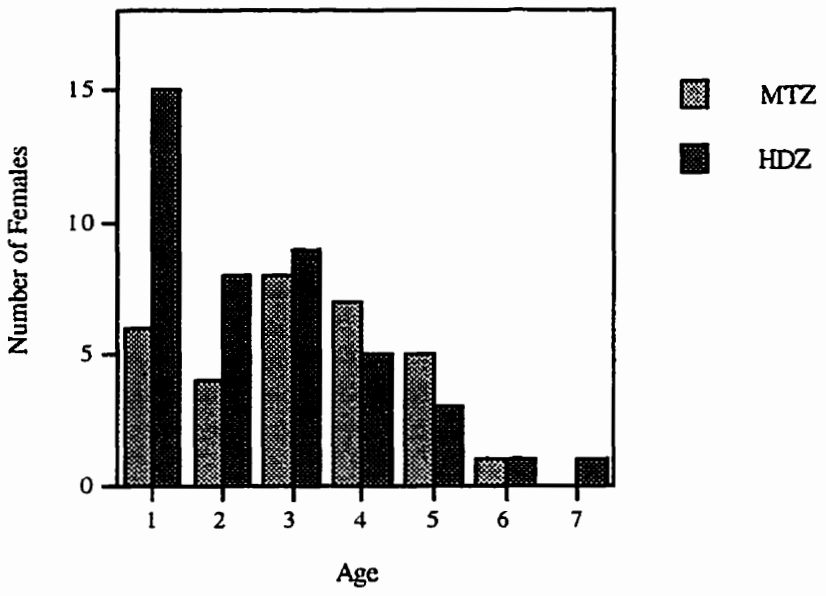


Figure 11. Age distribution of black-footed ferret colonies maintained at 2 institutions. MTZ indicates Metro Toronto Zoo (n=31). HDZ indicates Henry Doorly Zoo (n=42).

DISCUSSION

Validation

The first objective of this study was to develop or adopt a method for non-invasively monitoring the reproductive status and adrenocortical activity of the black-footed ferret. Progestins and estrogens have been successfully measured for a host of carnivores in urine (mustelids, Mead et al., 1990; Madej et al., 1992) and feces (felids, Brown et al., 1994; Brown et al., 1995a; Graham et al., 1995; canids, Wasser et al., 1995; Hay, 1996; mustelids, Gross et al., 1991; Gross, 1992; Brown, 1997), with feces as the predominant choice. Similarly, corticosteroids have been quantified in urine and/or feces for felids (Carlstead et al., 1992; Carlstead, et al., 1993; Graham et al., 1996; Jurke et al., 1997) and mustelids (Madej et al., 1992). There are several reasons why feces were chosen as the substrate for hormonal analyses in this study: 1) feces were readily available and easily collected during the daily husbandry routine, while urine was often difficult to find since it was absorbed by the mulch covering cage floors, and 2) approximately 93% of infused radiolabelled ovarian steroids (estradiol and progesterone) were detected in feces of the related Siberian polecat (Gross, 1992).

Achievement of the first objective required the adaptation and validation of fecal extraction procedures for use with our laboratory's established enzyme immunoassays. Data from the dose-response experiments indicated that ovarian hormone (methods 1 and 2) and corticosteroid extraction protocols were effective at removing progesterone/estradiol and cortisol, respectively, from the feces of the black-footed ferret. Tests of parallelism suggested that extracted steroids behaved in an immunologically similar manner to steroids used in the standard curve and were present in quantities which were measurable by the assay systems. Ovarian method 2 (~90% methanol) was utilized for evaluation of the female black-footed ferret's reproductive cycle since it provided consistently higher steroid yields than method 1 (~10% methanol) in endogenous and exogenous extraction comparisons. A similar finding has been reported by Palme et al.

(1996; 1997), who demonstrated improved recovery of radiolabelled progesterone with increasing concentrations of alcohol, specifically methanol, in the extraction medium. These results are to be expected since lipid-like molecules such as steroids readily dissolve in organic solvents. By increasing the percentage of alcohol the extraction efficiency also should be enhanced. Poor extraction efficiencies of cortisol in the pilot study (~10 - 15%) can be attributed to these polar steroids remaining bound to the aluminum oxide, rather than precipitating into the supernatant upon centrifugation.

Although these results establish the feasibility of fecal steroid monitoring for the non-invasive assessment of ovarian and adrenocortical function in the black-footed ferret by enzyme immunoassay, there is still room for refinement of this technique. One such area would be the choice of antisera for the immunoassay. Previous studies, facilitated by HPLC, have shown that fecal estrogen immunoreactivity is predominately associated with estradiol-17β in an assortment of felids (Brown et al. 1994; Graham et al., 1995) and the black-footed ferret (Brown, 1997). In contrast, the majority of progesterone- (Möstl et al., 1993; Brown et al., 1994; Brown, 1997) and cortisol-like (Graham and Brown, 1996; Monfort et al., 1997) immunoreactivity in carnivore feces is not from the native steroid, but rather from several metabolite forms. Hence, for detecting follicular steroidogenesis it is best to use antisera specific for estradiol, but for luteal and adrenocortical activities, antisera with broader spectrums of cross-reactivity would be more favorable. A similar strategy for evaluating reproductive function by fecal steroid analysis has been proposed by several authors (Schwarzenberger et al., 1996; Brown, 1997).

Stages of the Reproductive Cycle

Pre-breeding

The second objective of this study was to describe the relationship between physical changes in vaginal cytology and vulval size with ovarian hormone levels

experienced prior to mating. Similar to previous works, the percentage of cornified cells in vaginal lavages and vulval size (length x width) of the black-footed ferret increase during proestrus to reach maximal values during estrus (Carvalho et al., 1990; Williams et al., 1992; Brown, 1997). In the dog (Olson et al., 1984), cat (Michael, 1961; Shille et al. 1979), domestic ferret (Donovan et al., 1983; Ryan, 1984; Mead et al., 1988) and wolverine (Mead et al., 1991) changes in vaginal epithelial cell morphology and/or vulval tumescence during proestrus are clearly correlated with increased secretion of estradiol from the developing follicles. Due largely to the up take of steroid hormones by the liver for metabolism and excretion in bile, fecal steroid levels have been shown to be reflective of their counterparts circulating within the vascular system (Adlercreutz et al., 1979; Kohlmeier et al., 1986; Shille et al., 1990; Lasley and Kirkpatrick, 1991; Hay, 1996; Schwarzenberger et al., 1996). Therefore, it can be expected that fecal estradiol concentrations in the black-footed ferret would bear a strong relationship to serum levels during proestrus/estrus and in turn changes in both physical attributes examined in this study. The improvement of the correlation between the physical changes and fecal estradiol values when considering the average concentration of 4 days indicates that the cumulative effects of blood estrogens over time rather than single day levels are largely responsible for the gradual development of the physical pre-breeding characteristics.

The measurement of estradiol concentrations in the feces of the black-footed ferret has practical application for non-invasively detecting the onset of the reproductive cycle and follicular activity. In general, fecal estradiol concentrations were higher during proestrus and estrus than diestrus or anestrus in the black-footed ferret. Mating occurs concurrently with an estrogen peak in plasma of the domestic cat (Shille et al., 1979) and mink (Lagerkvist et al., 1992b) and feces of the cheetah (Brown et al., 1994; Brown et al., 1996). Analysis of estrone conjugates in the steppe polecat indicated that the majority of females (60%) bred when estrone conjugate concentrations peaked in urine, while the remainder mated prior to the peak (Mead et al., 1990). Brown (1997) suggests that fecal

estradiol in the black-footed ferret reaches maximal values 7 days following first breeding. Data from this study indicate that the mean estradiol concentration in the feces is higher during estrus but is not significantly different from random elevations which occur throughout proestrus. Even within individual profiles, animals failed to show a significant association between time of breeding and peak concentrations of fecal estradiol. Hence, fecal estradiol analysis could not serve as a complete replacement for the traditional invasive monitoring techniques used in predicting the optimal period of female receptivity (i.e. estrus) and timing introductions to males.

The results of this investigation support the previous contention that the black-footed ferret, like other ferret species is an induced ovulator (Robinson, 1918; Hammond and Marshall, 1930; Mead et al., 1990; Carvalho et al., 1991; Williams et al., 1991; Williams et al., 1992). Females failing to successfully breed exhibited a prolonged estrus (19-42 days). Similar to plasma concentrations in the domestic ferret (Heap and Hammond, 1974; Donovan et al., 1983), progesterone levels are minimal prior to mating and do not rise significantly above baseline until 5 days following intromission.

Post-breeding

Contrary to previous reports on diestrus in domestic and black-footed ferrets (Carlson and Rust, 1969; Heap and Hammond, 1974; Blatchley and Donovan, 1976; Daniel, 1976; Brown, 1997), this study demonstrated a difference between the hormonal profiles of pregnancy and pseudopregnancy. For days 15 - 32 of diestrus inclusive (2 days following implantation in the domestic ferret; Enders and Schlafke, 1972), fecal progesterone concentrations of pregnant females were significantly higher than their pseudopregnant counterparts. These results are suggestive of an embryonic or placental influence over the maternal hormonal environment, which increase levels above those provided solely by CL secretion in pseudopregnancy. There are several plausible sources for this additional contribution: 1) embryonic signals may directly increase steroidogenesis of the CL, 2) embryonic and/or placental signals may heighten the release

of luteotrophic complex hormones (i.e. prolactin and LH) from the hypophysis and thereby indirectly alter CL activity, 3) the placenta may manufacture and secrete progesterone directly into the maternal circulation.

In many mammals, ranging from primates to bovids to rodents, the placenta has at least some steroidogenic capabilities (Albrecht and Pepe, 1990; Conley et al., 1992; Strauss et al., 1992). However, the contributions of the carnivore placenta to gestation are poorly understood. Verhage et al. (1976) proposed that the placenta of the domestic cat was responsible for differences in progesterone concentrations and profile lengths between pregnant and pseudopregnant queens. Support for this hypothesis came from in vitro studies demonstrating the presence of enzymes within the felid placenta that converted pregnenolone to progesterone (Malassiné and Ferré, 1979). However, a more recent in vivo study suggests that the felid CL is solely responsible for progesterone secretion during the second half of pregnancy, since the placenta fails to produce progesterone in any significant quantity (Verstegen et al., 1993). Additionally, molecular investigations of the mink provide strong evidence that the placenta does not synthesize progesterone (Douglas et al., 1997). There currently is little reliable information on steroidogenesis in the ferret placenta.

Although statistically there was no significant variation between estradiol concentrations of pregnancy and pseudopregnancy in the black-footed ferret, the divergence of fecal profiles following day 15 is suggestive of differences in biological function between these two states. A trend for estrogens to peak at implantation and decline through gestation also has been reported in mink (Tauson, 1991; Lagerkvist et al., 1992b) and the Siberian polecat (Mead et al., 1990). Significant correlation between estradiol curve area and litter size in the black-footed ferret supports the postulate of a placental or embryonic contribution to the maternal hormone environment. The secretion of estrogens would be expected to bear close relation to the number of embryos present in the uterus. Similarly, Tauson (1991) demonstrated a relationship between plasma

estradiol levels and CL number in the mink and suggested that estradiol concentrations in late gestation were of placental or fetal origin. The failure of the progesterone curve area to correlate with litter size in the black-footed ferret may be attributed to CL numbers not accurately reflecting the number of observed oocytes or embryos in the domestic ferret (Mead et al., 1988). Further research is necessary in mustelids and carnivores in general to elucidate the source and function of estrogens during diestrus.

There is a paucity of information in the ferret concerning the existence of embryonic/placental signals during pregnancy and their possible impact upon ovarian or hypophyseal activity. One plausible mechanism by which signals could indirectly alter the maternal hormone envrionment is through the regulation of luteotrophic factors. In the domestic ferret, CL morphology and steroidogenesis are dependent upon both LH and prolactin (Donovan, 1967; Murphy, 1979; Agu et al., 1986). It is possible that signals of embryonic or placental origin could increase hypophyseal secretion of prolactin and/or LH and in turn enhance progesterone secretion. A recent study in the domestic dog has demonstrated significant differences in diestrual plasma prolactin concentrations for pregnant and pseudopregnant bitches (Onclin and Verstegen, 1997). Prolactin levels remained uniformly low in pseudopregnancy (5 ng/ml), while they rose gradually during pregnancy to peak values of 40 ng/ml at parturition. These authors suggest that pregnancy-specific luteal regulation, possibly through embryonic or placental signals, maintains the prolactin secretion which is responsible for the observed differences in pregnancy and pseudopregnancy. They also propose prolactin analysis as an accurate means for pregnancy diagnosis in the dog. This strategy should be evaluated for the ferret, since present knowledge concerning temporal prolactin secretion extends from a single study in which plasma prolactin levels remained relatively constant throughout pseudopregnancy in the domestic ferret (range 19 - 23 ng/ ml, Rose et al., 1993). Potentially, a sensitive immunoassay (RIA or EIA) or an elaborate bioassay could be

validated to non-invasively monitor urinary prolactin, similar to previous success with FSH (Monfort et al., 1989).

Adrenocortical Activity

The final objective of this study was to determine the endocrine level of physiological 'stress' which restraint episodes elicited in the black-footed ferret. Variations in baseline levels of cortisol prior to experimentation indicated that differences in basal adrenocortical activities exist between animals and these formed 2 distinctive groups: high and low cortisol output. These distinctions were not specific to a particular sex, nor did they alter the degree of response to applied 'stressing' treatment episodes during the physical restraint/ACTH challenge. A previous study with the domestic ferret found considerable variation in the basal levels of plasma cortisol between individuals, reporting overlapping ranges of 5.5 - 18.4 ng/ml and 1.3 - 27.0 ng/ml for males and females, respectively (Garibaldi et al., 1988). Analyses of urinary and fecal corticosteroids in domestic and non-domestic felids also have demonstrated considerable differences in baseline concentrations (Carlstead et al. 1993, Graham and Brown, 1996) Keeping in mind that there is great inter-animal variability in physiological responses to stress, coping ability and individual perception of what is stressful (Collu, 1984; Moberg, 1985b; Moberg, 1987), it is then plausible to suggest that individual black-footed ferrets may interpret their surroundings differently. High adrenocortical activity animals may view the captive environment, with strange odors and frequent disturbances by husbandry routines, as threatening and more stressful than their low cortisol output counterparts. In addition, differential activation of the hypothalamic-pituitary-adrenocortical system by stress has been related to genetic background (Collu et a., 1984). Since a mother and daughter pair of black-footed ferrets were both classified in the high cortisol output category, there may be some heritable predisposition for levels of adrenocortical activity.

The biological relevance of cortisol immunoreactivity detected in the feces of the black-footed ferret was established by the ACTH challenge. Injection regimens of gelatin and liquid preparations of ACTH were followed 1-2 days later by a significant rise in cortisol excretion. These results indicate that the cortisol detected in fecal extracts by the 'highly specific' cortisol EIA (i.e. low cross-reactivity for other corticosteroids) reflected heightened adrenocortical activity caused by the ACTH. This is in contrast with previous work on carnivores, which suggest there is no native cortisol in feces, but rather other adrenocorticoids or metabolites which are immunoreactive with corticosterone antisera (Graham and Brown, 1996; Monfort et al., 1997). Fractionation of extracts by HPLC and further hormonal analysis would help elucidate the true nature of corticosteroids within the feces of the black-footed ferret.

Adrenal function tests, such as an ACTH challenge, also serve as a method by which to evaluate the caliber of physiological stress which manipulation imposes upon exotic species (Wildt et al., 1984; Wildt et al., 1986; Carlstead et al., 1992). Based on a greater elevation of fecal cortisol levels, the administration of a 20 μg dose of ACTH may in fact be representative of the effects of more severe stressors upon the black-footed ferret. Single episodes of restraint and saline injection did not significantly elevate adrenocortical activity above baseline levels of fecal cortisol excretion. Hence, they are perceived by the black-footed ferret as events that are mildly stressful, with restraint being the lesser of the two. In comparison with the ACTH regimens, it is also noted that restraint and particularly saline injection produce submaximal activation of the adrenal cortex.

The temporal difference in mean peak elevation of fecal cortisol between the 4 treatment episodes is intriguing. Mean peak concentrations from saline and gelatin ACTH administration were detected on day 1 following treatment administration, while those of restraint and liquid ACTH were on day 2. The reason for this discrepancy in unknown. However, injection of synthetic ACTH to domestic cats also demonstrated a

differential excretion pattern of cortisol metabolites over time, with maximal and distinct rises for individuals appearing at either 24 or 48 hrs after administration (Graham and Brown, 1996). These authors offered no explanation for their findings. One possibility in the present study arises from the timing of fecal collections. The period of collection on day 1 was actually much shorter than the following days (18 vs. 24 hrs), due to treatment administration in the late afternoon of day 0 to accommodate husbandry practices for the ferrets. Since the quantity of feces collected would be less on day 1 than suceeding days and varied from day to day between animals in general, apparent daily excretion of fecal steroids may not have been uniform over time. Additionally, closer examination of individual profiles for each animal revealed peak concentrations occurring on either day 1 or 2 for all treatments, with some skewing of the mean profiles in the presented results (Figure 8). To minimize these sources of variation in fecal corticosteroid monitoring, daily hormone levels should be adjusted for the total amount of feces excreted on the day of collection. For example, analysis of a single fecal sample from ferrets A and B determined the concentrations of cortisol to be 200 ng/g feces and 100 ng/g feces, respectively. Adjusting for the total amount of feces excreted on the collection day for ferrets A (5 g feces) and B (10 g feces) yields 1000 ng of cortisol per day for each. Although initial analyses suggest these animals have different levels of adrenocortical activity, they are similar upon final calculation.

It already has been established by this study that single restraint episodes failed to significantly 'stress' the black-footed ferret based on cortisol excretion. In contrast, multiple restraints (n=4) to facilitate manipulation of the black-footed ferret over approximately 1.5 months significantly increased basal levels of adrenocortical activity and hence 'stressed' these animals. Since it is well documented that 'stressors' have dramatic repercussions upon reproductive function of many mammals (see review Collu et al., 1984; Moberg, 1985a; Armstrong, 1985; Liptrap, 1993; deCatanzaro and MacNiven, 1992), these findings suggest that frequent restraint of the black-footed ferret

during the peri-copulatory period may have a causative role in its poor reproductive success.

Reproductive Characteristics

The mean gestation length of 41.5 ± 0.5 days for black-footed ferrets maintained at the MTZ and HDZ was not unlike that reported for other captive colonies (42.4 ± 0.2 days, Carvalho et al., 1991; 42.7 ± 0.7 days, Williams et al., 1991; 42.6 ± 0.5 days, Brown, 1997). Similarly, the average litter sizes for the MTZ (3.6 ± 0.3 kits) and HDZ (4.1 ± 0.4 kits) colonies were consistent with findings from the Sybille Wildlife Research Center (3.0 ± 1.4 kits, Williams et al., 1991) and Conservation Research Center (3.3 ± 0.5 kits, Carvalho et al., 1991). Williams et al. (1991) suggested that wild black-footed ferret litter sizes are more accurately reflected by the survival of kits to weaning and reported an 80% (2.4 ± 1.7) survival rate of litter mates, which is smaller than estimated litter sizes of free-ranging ferrets in Wyoming (mean = 3, Forrest et al., 1988). Findings from Carvalho et al. (1991) and this study have much smaller sizes of litters post weaning, with 69.6% and 39.2% of the kits surviving, respectively. A number of factors account for the loss of kits prior to weaning in captivity, including stillbirths, deaths shortly after birth due to underdevelopment, accidental injury by excessive transport and neglect, and maternal cannibalism (Carvalho et al., 1991; Carpenter and Hillman, 1978; Hutchins et al., 1996; D. Olson, personal communication).

The overall pregnancy rate of females in this study (38.3%) was lower than the mean rate for the entire captive black-footed ferret population in 1994 (41%, Thorne, 1995) and 1995 (63%, Thorne, 1996). Gross variability in these rates of overall whelping as well as individual rates between the MTZ (18.5%) and HDZ (54.5%) directly address AZA concerns with institutional variation in reproductive success (Hutchins et al., 1996). One possible explanation may reside with the ages of females maintained at the different institutions. Results from this study suggest that animals > 3 yrs of age are less likely to

conceive following mating (Figure 10), have lower rates of kit survival (32.4%) and greater chances of ovarian inactivity during the breeding season. Williams et al. (1991) demonstrated similar findings, with a 31.5% pregnancy rate for animals aged 4 - 7 yrs.

In 1994, black-footed ferret females in the SSP© captive breeding program < 3 yrs and ≥ 3 yrs of age weaned 1.54 and 0.26 kits, respectively, suggesting that animals ≥ 3 yrs were relatively nonproductive (Thorne, 1995). This study concurred, demonstrating 43.6% and 32.4% of kits surviving for females aged <3 yrs and ≥ 3 yrs, respectively, but also indicates that females aged 3 yrs are as equally capable of produce offspring as 1 and 2 yrs old animals. Therefore, 3 yr old animals should not be dismissed as potential contributors to kit production in the black-footed ferret breeding program.

A major area of investigation for the BFF SSP© should be in developing methods to improve neonatal survival. This study indicates that over a period of 3 years, only 39.1% of the kits whelped at the MTZ and HDZ survived past the stage of weaning. The overall captive breeding program rates of kit survival were relatively stable at 64 - 66% from 1994-1995, (Thorne, 1995; Thorne, 1996). A recent publication analyzing the results of the BFF Recovery Program, recommends that even though the captive black-footed ferret population experiences rates of kit mortality comparable with that of domestic ferrets and mink, steps should be taken to determine if the rates may be decreased (Hutchins et al., 1996). Several factors may be responsible for the present rates of kit mortality: 1) improper levels of maternal nutrition have been isolated as a major cause of neonatal deaths for carnivores (Tauson, 1992), and 2) inbreeding depression is associated with the death of offspring in mammals (Ralls and Ballou, 1979; Ralls and Ballou, 1982) and becomes more severe in stressful environments (Miller, 1994).

Management Application and Future Considerations

Overall, this study demonstrates the ability to non-invasively monitor ovarian and adrenal endocrine function in the black-footed ferret. The knowledge gained from this

innovative technology greatly improves our understanding of the basic endocrinology of this small endangered carnivore and has direct application towards its in situ and ex situ management.

Through a combination of fecal estradiol and progesterone hormonal analyses, follicular and luteal activities may be evaluated and monitored. Utilization of this technique in captive and free-ranging black-footed ferrets would assist with detecting cyclicity and distinguishing the relative stage of the reproductive cycle for these animals. For free-ranging ferrets, it would act as an excellent indicator for determining the number of females which have successfully bred in wild release sites and the time of year in which reproduction occurs. Additionally, diagnosis of diestrus in as few as 5 days following copulation provides captive colony managers with an alternate means of post-breeding evaluation as opposed to the invasive vaginal cytology methods traditionally used.

One of the most significant findings from this study was that repetitive restraint episodes significantly heighten adrenocortical activity in the black-footed ferret. Implications of chronic stress in mammalian species have already been discussed. The stressful nature of current breeding evaluation procedures to the black-footed ferret may play a crucial role in its poor reproductive success under captive conditions. In the present study for instance, the MTZ restrained its ferrets 3 times more often than the HDZ and had only one third the number of pregnancies. Gross variances in the relative breeding success between institutions could then be directly related to the frequency and techniques used to evaluate breeding readiness in the black-footed ferret. Further investigations should evaluate adrenocortical activity during the breeding season and contrast profiles of pregnancy with those of pseudopregnancy. One would hypothesize a strong correlation between higher levels of adrenocortical activity and pseudopregnancy. A recent study with the cheetah actually has demonstrated that females with higher levels of fecal corticoids fail to reproduce (Jurke et al., 1997).

Additionally, fecal corticosteroid analysis could be instrumental in determining environmental factors of daily management which distress the black-footed ferret. Hormonal profiles could easily be compared with levels established by the ACTH challenge test of this study as an means to distinguish which practices are stressful and should be altered. Carlstead et al. (1992; 1993) determined that simple changes in husbandry routines and the provision of behavioral enrichment affected the well-being of the domestic and leopard cat, a small exotic felid.

SUMMARY AND CONCLUSION

In light of recent concerns over reduced fecundity within the captive black-footed ferret population and the inability of the current breeding program to meet reintroduction demands and recovery goals, this investigation was launched primarily as a means to improve our basic knowledge of this species' reproductive biology. In addition, it was hoped that fundamental biological data would be accumulated, which could prove effective at refining management techniques and improving the breeding success of this small, endangered carnivore.

To facilitate the non-invasive monitoring of ovarian steroids and adrenocorticoids within the feces of the black-footed ferret, several different extraction protocols were evaluated for use with enzyme immunoassay. Validations were performed by conducting tests of dose response and parallelism with known standard curves for estradiol, progesterone and cortisol. Although all methods of extraction were effective at removing ovarian steroids and cortisol in measurable quantities, the protocols selected for use in further studies provided the highest yields of endogenous and exogenous hormones.

The traditional husbandry technique for evaluating a female black-footed ferret's readiness for breeding (i.e. optimal period of receptivity or estrus) involves physical restraint for measurement of vulval size and determination of percentage cornification of the vaginal epithelial cells. Females are bred when the percentage of cornified cells in vaginal lavages reach 90%. During proestrus the vulval area and percentage of cornified epithelial cells steadily increase to reach maximal values at estrus. Concurrent with these physical changes is the elevation of estradiol levels within the feces of the black-footed ferret. Fecal estradiol concentrations positively correlate with both vulval area and vaginal cytology during the peri-copulatory interval, with a stronger relationship occurring when a 4 day mean of consecutive fecal estradiol values are considered. Unfortunately, there was no definitive peak in fecal estradiol concentrations coinciding with breeding of the female ferrets, thereby inhibiting the use of non-invasive hormonal

monitoring as a potential replacement for the more invasive techniques involving physical restraint. Area under the estradiol curve correlated significantly with litter size following day 15 after breeding (r^2=0.560, p=0.0356)

Longitudinal monitoring of ovarian steroids was conducted on fecal samples collected during the reproductive cycles of female black-footed ferrets maintained at the Metro Toronto Zoo (MTZ) and Omaha's Henry Doorly Zoo (HDZ). Mean fecal estradiol concentrations of proestrus/estrus were significantly higher ($p<0.0001$) than values of diestrus. Although mean estradiol profiles between pregnant and pseudopregnant females appeared to diverge at days 15 - 35 following last breeding, there were no statistical differences between estradiol levels of pregnant or pseudopregnant animals in either the pre- or post-breeding periods ($p>0.05$). In contrast, fecal progesterone concentrations remained uniformly low prior to mating for both pregnant and pseudopregnant females and did not rise significantly above these baseline values until 5 days after the last day of breeding. Elevations in progesterone excretion were maintained throughout diestrus and differed statistically between pregnant and pseudopregnant females for days 15 - 32 ($p=0.0293$). Mean fecal progesterone concentrations were consistently higher in pregnant animals during this period.

A combined physical restraint and ACTH challenge was performed on male and female ferrets maintained at the MTZ during the winter of 1996 - 1997. Analysis of fecal cortisol levels prior to experimentation defined 2 groups of basal adrenocortical activity (high and low cortisol output), which differed statistically ($p<0.0001$). Restraint and saline injection treatment episodes failed to significantly elevate fecal cortisol levels above pre-treatment baselines ($p>0.05$). In contrast, liquid and gelatin ACTH injections produced significant elevations in cortisol values on days 2 ($p=0.0004$) and 1 ($p=0.0463$), respectively, following administration. Overall, there were no differences in fecal cortisol excretion patterns between the 4 different treatment regimens ($p>0.05$). Mean cortisol concentrations post-study were statistically greater than pre-study values, suggesting that

repetitive restraint episodes (n=4) over the 1.5 months of the challenge had heightened adrenocortical activity of the black-footed ferret and were perceived as a chronic stressor. It should then be noted that physical restraint of estrous animals, especially during the peri-copulatory interval may in turn have a negative impact upon the reproductive success of this mustelid.

Mean gestation length and average litter size for the female black-footed ferrets in this study were 41.5 ± 0.5 days and 4.0 ± 0.3 kits, respectively. Although the overall pregnancy and kit survival rates were 38.3 % and 39.2%, respectively, they differed significantly between institutions (MTZ 18.5% and 61.1%; HDZ 54.6% and 39.8%, respectively). This study alluded to 2 possible sources of the variation in institutional reproductive success. First, population age demographics may be a factor, since animals >3 yrs of age consistently have lower pregnancy rates and higher kit mortality. Second, the frequency of restraint episodes to evaluate readiness for breeding may be an important factor. The MTZ conducted 3 times as many evaluations as the HDZ but had about 1/3 the pregnancy rate. In any event, these are areas for further consideration if management of the captive breeding program is to be improved.

In conclusion, this study has demonstrated the efficacy of non-invasively monitoring ovarian and adrenal function in the black-footed ferret through fecal steroid analysis. It has greatly enhanced our knowledge base of the hormonal events which occur during the reproductive cycle of these animals and provided exciting new evidence of potential causes of their poor breeding success. Future investigations should focus on developing new husbandry techniques which are less invasive and stressful to the black-footed ferret, such as decreasing the number of restraint episodes and examining cortisol profiles and fecundity compared to those of animals receiving more frequent restraint. Improving the management practices of the captive breeding program, could greatly increase the production of new progeny for reintroduction to the wild and ensure the black-footed ferret's survival in perpetuity.

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APPENDIX I.A

CHEMICALS

ACTH (liquid and gelatin)	R.Knapp, The Medicine Shop, Front Royal, Virginia, USA
ABTS 2,2' - Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)	Fisher Scientific, Unionville Ont., Canada
Aluminum Oxide	BDH Ltd., Burlington, Ont., Canada
Ammonium Hydroxide	Fisher Scientific, Unionville, 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
Eosin-Azure 65	Fisher Scientific, Unionville, Ont., Canada
Estradiol-17β antiserum (R4972)	C.J. Munro, University of California, Davis, USA
Estradiol-17β standard	C.J. Munro, University of California, Davis, USA
Estradiol-17β /horseradish peroxidase	C.J. Munro, University of California, Davis, USA
Harris' Haematoxylin	Fisher Scientific, Unionville, Ont., Canada
Hydrocortisone standard	Sigma Chemical Co., St. Louis, MO, USA
Hydrogen peroxide	Fisher Scientific, Unionville Ont., Canada
Methanol	BDH Inc., Toronto, Ont., Canada
Orange G6	Fisher Scientific, Unionville, Ont., Canada
Permount Mounting Medium	Fisher Scientific, Unionville, Ont., Canada
Petroleum Ether	Fisher Scientific, Unionville, 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	Sigma Chemical Co., St. Louis, MO, USA
Reagent Alcohol (~90% Ethanol)	Fisher Scientific, Unionville, Ont., Canada
Saline (sterile)	Veterinary Teaching Hospital, Ontario Veterinary College, University of Guelph, Guelph, Ont., Canada
Sodium carbonate	Fisher Scientific, Unionville, Ont., Canada
Sodium chloride	Fisher Scientific, Unionville, Ont., Canada
Sodium hydrogen carbonate	Fisher Scientific, Unionville, Ont., Canada
Sodium phosphate monobasic	BDH Ltd., Burlington, Ont., Canada
Sodium phosphate dibasic	BDH Ltd., Burlington, Ont., Canada
Tween 20	Sigma Chemical Co., St. Louis, MO, USA
Xylene	Fisher Scientific, Unionville, Ont., Canada

APPENDIX I.B

EQUIPMENT

Acetate Plate Sealers	Baxter/Canlab, Mississauga, Ont., Canada
Dynatech Ultrawash II Microplate Washer	Baxter/Canlab, Mississauga, Ont., Canada
Dynatech 700 Plate Reader	Baxter/Canlab, Mississauga, Ont., Canada
Fisher Platelet Mixer Model 348	Fisher Scientific, Unionville, Ont., Canada
Gilson P200 Diamond tips	Mandel Scientific Co., Ltd., Guelph, Ont., Canada
Kimax Borosilicate Glass Tubes (16 x 125 mm) with PTFE-faced ribber liner	Fisher Scientific, Unionville, Ont., Canada
Microscope Slides, Frosted End	VWR Canlab, Mississauga, Ont., Canada
Nunc-Immuno 96F Maxisorp plates	Mandel Scientific Co., Ltd., Guelph, Ont., Canada
Polypropylene Tubes (12 x 75 mm)	VWR Canlab, Mississauga, Ont., Canada
Rotator American Instruments R4140	Baxter/Canlab, Mississauga, Ont., Canada

APPENDIX I.C

ENZYME IMMUNOASSAY SOLUTIONS

Coating Buffer

Na_2CO_3	1.52g	
$NaHCO_3$	2.93g	
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

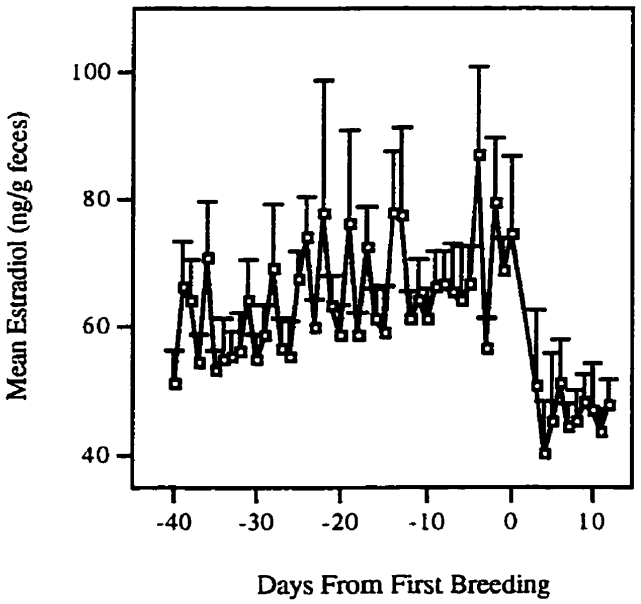
Wash Solution Concentrate

NaCl	87.66 g
Tween 20	5.0 ml
dH_2O	1000 ml

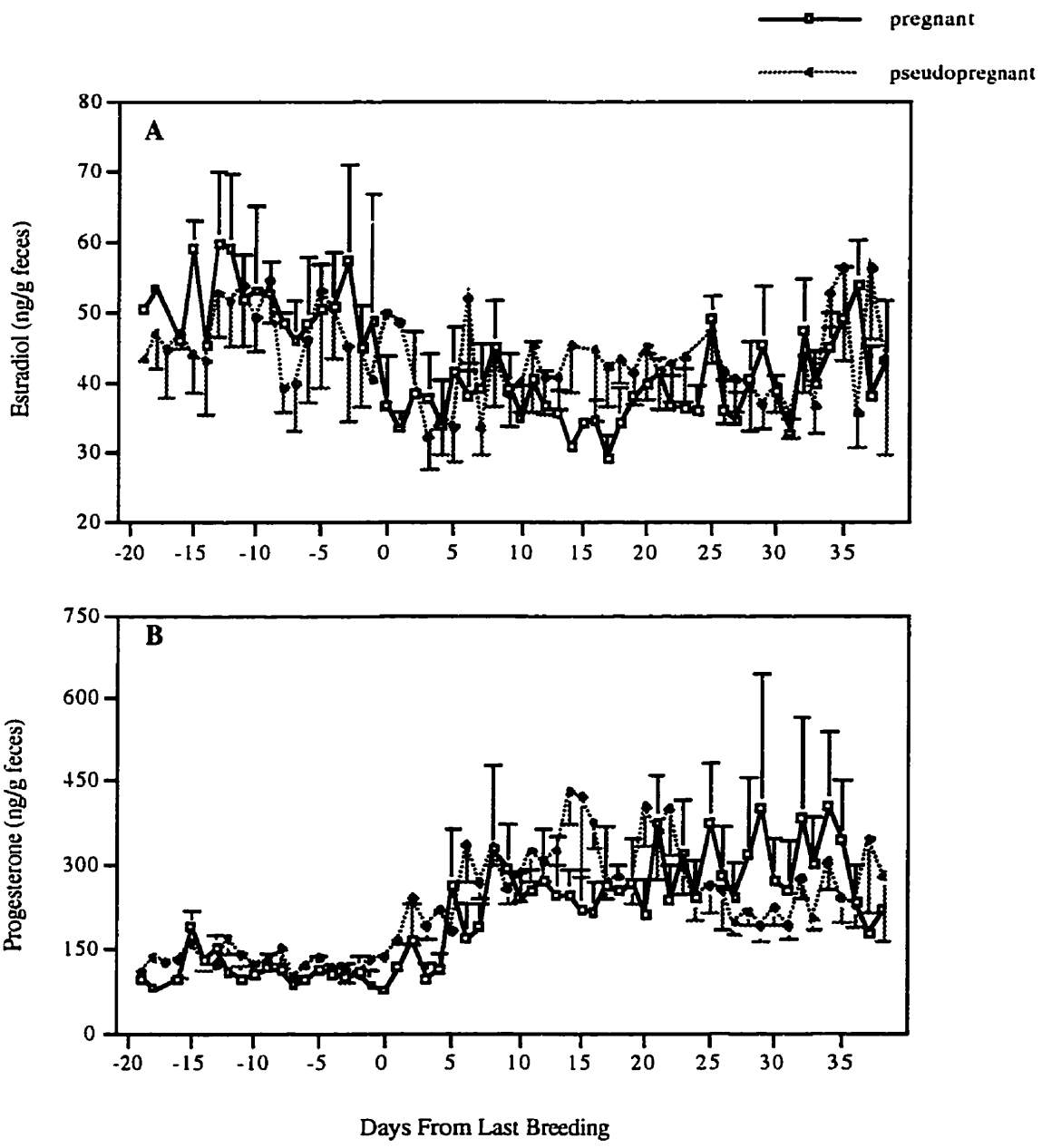
Substrate Buffer

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	

APPENDIX II.A Mean (+SEM) daily fecal estradiol profile for 12 black-footed ferrets prior to and following breeding.



APPENDIX II.B Longitudinal profiles of mean fecal estradiol (A) and progesterone (B) during the reproductive cycles of pregnant (n=4) and pseudopregnant (n=7) black-footed ferrets. Extraction method 1 was used to recover steroids from fecal samples. Vertical lines indicate + or - SEM.



APPENDIX II.C Longitudinal profile of ovarian steroids and vaginal cytology for a single black-footed ferret induced to ovulate by intra-muscular injection of 100 IU hCG. Horizontal bar denotes failed breeding attempts with a male.

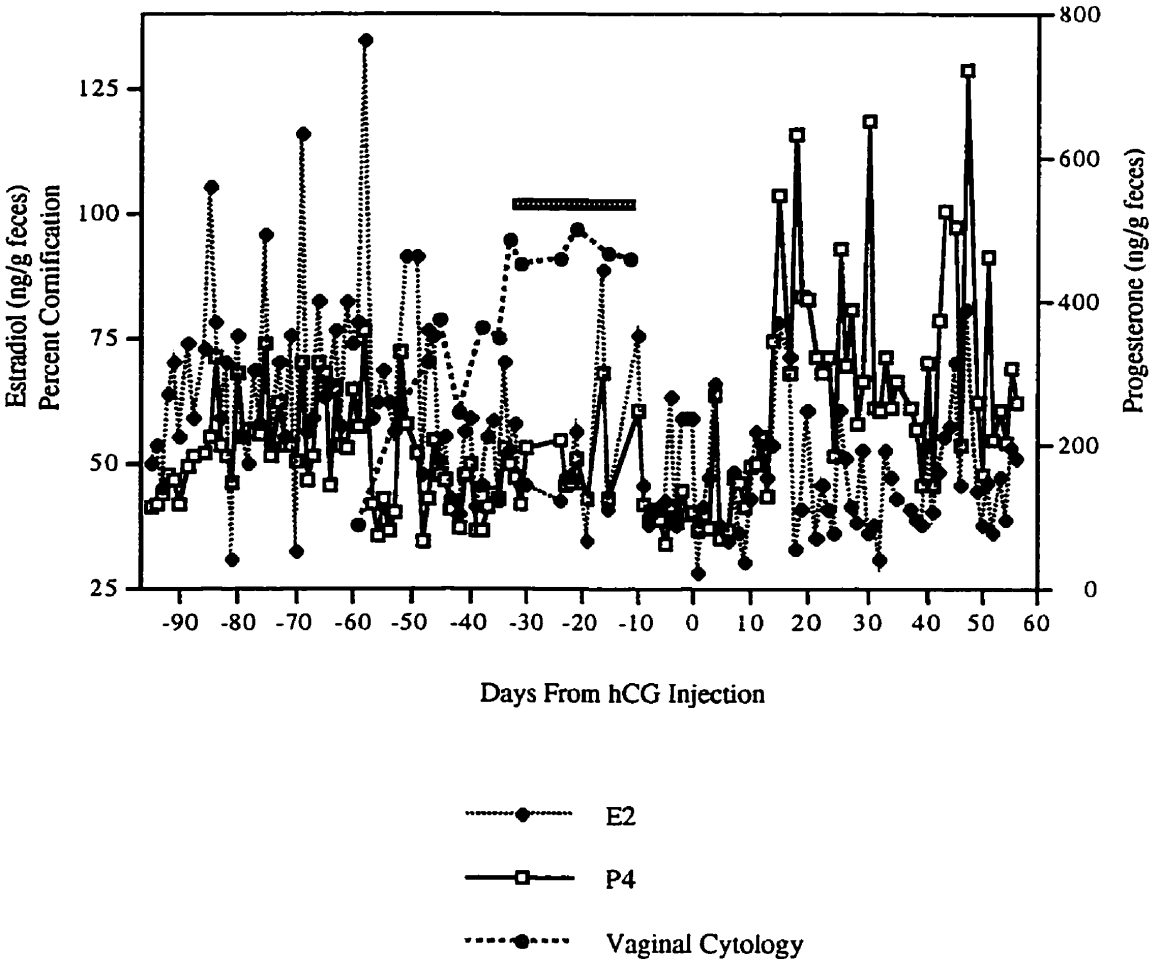
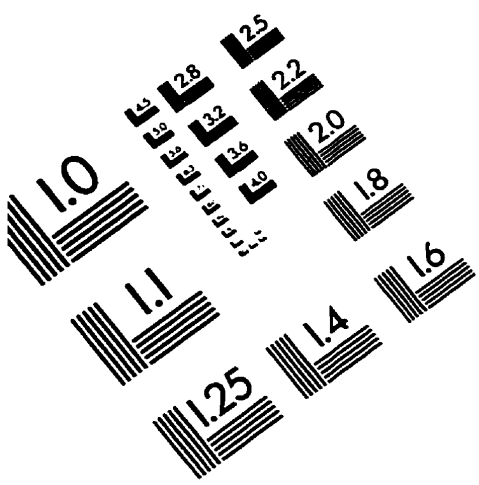
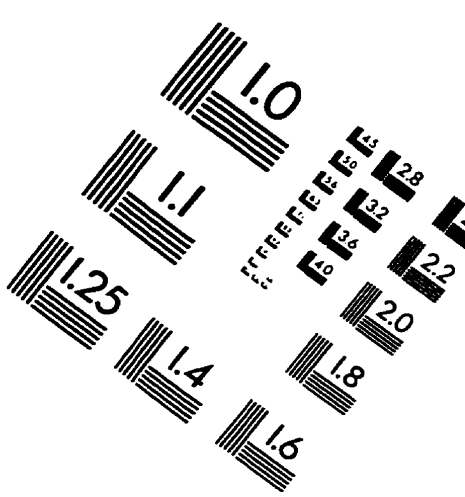
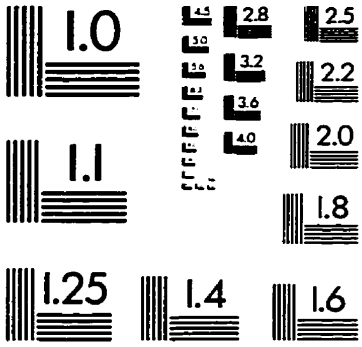
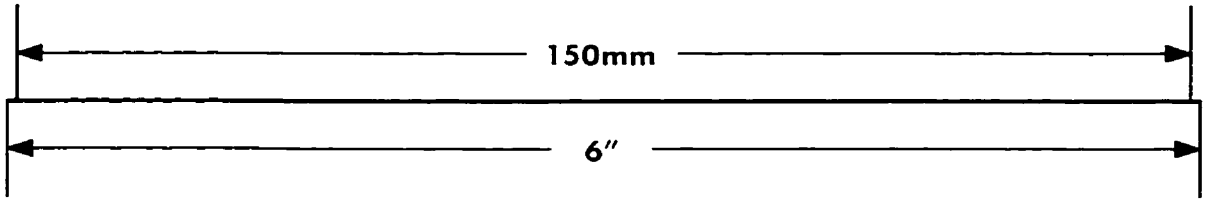


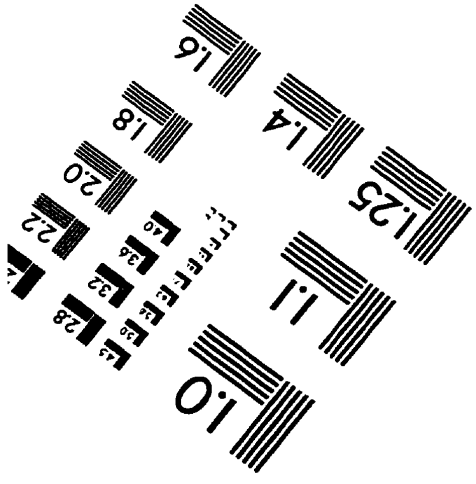
IMAGE EVALUATION
TEST TARGET (QA-3)











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