

## ABSTRACT

Title of dissertation: PRIMING WITH ORAL PROGESTIN BEFORE OVULATION INDUCTION FACILITATES OVARIAN FUNCTION IN THE CAT (*FELIS CATUS*)

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Artificial insemination (AI) has been developed in multiple felid species as a tool for retaining gene diversity in threatened or endangered populations. Yet, pregnancy success remains low (< 5%) following AI in most felids, particularly in species that spontaneously ovulate. This failure has been attributed to variable ovarian status at the time of insemination and adverse residual effects caused by exogenous gonadotropins used to induce ovulation. Using the domestic cat as a research model, a new AI regimen that incorporated short-term ovarian suppression with oral progestin (altrenogest; ALT) before ovulation induction was investigated. The hypothesis was that oral progestin priming would prevent spontaneous ovulation, improve ovarian responsiveness to exogenous gonadotropins and mitigate adverse effects caused by persistent gonadotropin actions. Specific objectives were to: (1) increase fundamental understanding of the mechanisms controlling ovarian function; and (2) characterize how oral progestin priming prior to exogenous gonadotropin treatment influences ovarian responsiveness, fertilization, early embryonic development and luteal function in the cat.

Fecal hormone monitoring was used to establish an ALT dosage that provides rapid, reversible ovarian suppression with no residual effects on estrous cyclicity. With this information, the influence of progestin priming on ovarian responsiveness to exogenous gonadotropin dosage was investigated. Priming increased ovarian sensitivity

to gonadotropins, supporting the use of lower dosages for ovulation induction. Next, *in vivo* fertilization success and *in vitro* early embryonic development was characterized following laparoscopic, intrauterine AI in cats treated with ALT. Progestin-primed females demonstrated a good ovarian response to ovulation induction and more consistent embryonic development, compared to cats treated with gonadotropins alone. Furthermore, endocrine data revealed that normal luteal progesterone levels were maintained only in queens primed with the oral progestin. Finally, histology and quantitative RT-PCR were used to characterize the differential effects on luteal function observed. Aberrant CL progesterone production was not associated with changes in ovarian morphology, or the expression of six specific genes associated with luteal function and progesterone biosynthesis. Overall, these studies increased knowledge of domestic cat reproductive physiology and improved understanding of ovarian suppression for enhanced AI efficiency in felids.

PRIMING WITH ORAL PROGESTIN BEFORE OVULATION INDUCTION FACILITATES  
OVARIAN FUNCTION IN THE CAT (*FELIS CATUS*)

by

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For my parents, who believed in me every step of the way.

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## LIST OF ABBREVIATIONS

3 $\beta$ -HSD	3-beta hydroxysteroid dehydrogenase
AI	artificial insemination
ALT	altrenogest
BSA	bovine serum albumin
cDNA	copy deoxyribonucleic acid
CH	corpora hemorrhagica
CL	corpus luteum or corpora lutea
CYP11A1	cytochrome P450, family 11, subfamily A, polypeptide 1; scc
DNA	deoxyribonucleic acid
E	estradiol or estrogens
E1S	estrone sulfate
EC	estrogen conjugate
eCG	equine chorionic gonadotropin
EIA	enzyme immunoassay
ER $\alpha$	estrogen receptor alpha
ET	embryo transfer
FCS	fetal calf serum
FSH	follicle stimulating hormone
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GnRH	gonadotropin releasing hormone
i.m.	intra-muscular
IU	international unit
IVF	<i>in vitro</i> fertilization
LH	luteinizing hormone

LHR	luteinizing hormone receptor
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
OD	optical density
P	progesterone or progestins
PBS	phosphate-buffered saline
PG	pregnane
PR	progesterone receptor
PRLR	prolactin receptor
qRT-PCR	quantitative real-time reverse transcriptase polymerase chain reaction
RIA	radioimmunoassay
RT-PCR	reverse transcriptase polymerase chain reaction
scc	side chain cleavage enzyme
SEM	standard error of the mean
sSTAR	steroidogenic acute regulatory protein
UFO	unfertilized oocyte
UTJ	uterotubal junction
VEGF	vascular endothelial growth factor

## CHAPTER 1

### INTRODUCTION AND LITERATURE REVIEW

#### Overview

The domestic cat (*Felis catus*) is a valuable research model for understanding complex reproductive mechanisms and developing assisted breeding techniques for endangered felids. The cat also plays an important role in human biomedical and genetics studies. Indeed, domestic cat research provides a unique opportunity to document previously uncharacterized physiological processes and apply these data in applications relevant to felid reproduction, animal conservation and human health.

Developing safe and effective approaches to sustaining populations of endangered cats is a priority of conservation biologists and the zoological community (Wildt and Roth, 1997; Swanson, 2006). Equally important is the value in maintaining feline biomedical models that exhibit reduced reproductive capacity (Howard et al., 1992c; Critser and Russell, 2000). In cats, prominent methods used to assist reproduction include artificial insemination (AI) and *in vitro* fertilization (IVF) followed by embryo transfer (ET). These techniques circumvent mate incompatibility and poor breeding performance, reduce the need for costly animal transfers and provide the potential for introducing new genes from genetically valuable individuals into breeding populations (Howard, 1999).

Offspring have been produced in the cheetah (Howard et al., 1992b), ocelot (Swanson et al., 1996b), clouded leopard (Howard et al., 1996), tiger (Donoghue et al., 1993), puma (Barone et al., 1994b), leopard cat (Howard, 1991), snow leopard (Roth et al., 1997a) and tigrina (Swanson and Brown, 2004) using a minimally-invasive laparoscopic intrauterine AI technique first developed in the domestic cat (Howard et al.,

1992a). IVF and ET also have been successful in felids, resulting in births in the tiger (Donoghue et al., 1990), African wild cat (Pope et al., 2000), ocelot (Swanson and Brown, 2004), caracal (Pope et al., 2001), fishing cat (Pope et al., 2006a) and domestic cat (Goodrowe et al., 1988b). These assisted breeding techniques also have been used to develop and refine methods for maintaining populations of domestic cat models for hereditary disease (Swanson et al., 2000; Magarey et al., 2006).

Despite success with assisted reproduction in felids, incidence of pregnancy following AI and IVF/ET remains inconsistent (Pelican et al., 2006b). Much of this variability can be related to the two ovulation mechanisms observed in individuals of the Felidae: induced or spontaneous ovulation. Spontaneous ovulation can be continuous (e.g. ovulation following each follicular phase) or intermittent. The cheetah and ocelot are induced ovulators and demonstrate AI efficiencies of approximately 45% and 25%, respectively (Swanson et al., 1996b; Howard et al., 1997). In contrast, AI success is less than 5% in clouded leopards and 0% after eight attempts in the fishing cat, both spontaneous ovulators (Howard et al., 1996; Bauer et al., 2004). IVF/ET in fishing cats has provided only marginally better success (< 10%) (Pope et al., 2006a).

Spontaneous ovulation frequently leads to an inconsistent ovarian response after exogenous gonadotropin stimulation for AI or oocyte retrieval. Subsequently, establishment of pregnancy is more difficult to achieve. However, other factors also must be considered when uncovering the etiology of pregnancy failure following ovulation induction and assisted reproduction, demonstrated by low (< 5%) AI success in the tiger, an induced ovulator (Graham et al., 2006). In addition to inconsistent ovarian response, causes for pregnancy failure can include poor oocyte and/or sperm quality, ovarian hyperstimulation, ancillary folliculogenesis, abnormal endocrine dynamics and procedural errors.

Short-term ovarian suppression before ovulation induction may improve assisted reproduction in felids. This approach has been used in humans (Burry et al., 1991; Barbieri and Hornstein, 1999), marine mammals (Robeck et al., 2004; Robeck et al., 2005), the cow (Patterson et al., 1997; Xu and Burton, 1999), horse (Lofstedt, 1988), pig (Wood et al., 1992) and sheep (Deligiannis et al., 2005). The temporary down-regulation of follicular activity can enable a more uniform ovarian response at the time of insemination, oocyte retrieval or embryo transfer. There also is evidence that agents used for ovarian suppression mitigate adverse effects caused by gonadotropin administration (Kol, 2004; Oshima et al., 2004). Several exogenous hormones have been used in mammals to down-regulate follicular activity, including progestins, prostaglandins and GnRH analogs. In the cat, progestins are optimal for ovarian suppression, compared to GnRH analogs or prostaglandins (Wildt et al., 1979b; Pelican et al., 2005). Furthermore, progestins have been used successfully to suppress ovarian activity before assisted breeding without negatively affecting subsequent ovarian response to exogenous gonadotropins or oocyte quality (Pelican et al., 2001; Pelican et al., 2007). However, the influence of progestin priming on ovarian sensitivity to gonadotropin dosage, *in vivo* early embryonic development and implantation following AI has not been previously studied in the cat.

Understanding the factors regulating pregnancy establishment in the cat following progestin priming, ovulation induction and assisted reproduction could improve these techniques. This research project employed a multi-disciplinary approach aimed at investigating these techniques more closely in the context of four distinct studies. In Study 1, the effect of different dosages of an oral progestin (altrenogest; ALT) on ovarian activity was evaluated in the domestic cat. In Study 2, the influence of progestin priming on ovarian sensitivity to exogenous gonadotropins was investigated through assessments of ovarian responsiveness, endocrine dynamics and corpora lutea (CL)



function. Study 3 focused on characterizing *in vivo* fertilization efficiency, subsequent early embryonic development, and ovarian morphology and function following progestin priming and AI. In Study 4, archived reproductive tissues were assessed to understand differences in ovarian form and function using histology and gene expression analyses. The overall goal of these studies was to increase understanding of fundamental feline reproductive physiology and provide the data necessary to develop refined hormone regimens for ovulation induction in the cat. Results from these studies also can be used to modify assisted reproduction techniques in both wild felids and domestic cat biomedical models.

### **The Domestic Cat Model**

*Biomedical research.* The domestic cat is a research model for understanding mechanisms of disease and developing appropriate therapies. Cats exhibit numerous physiological abnormalities that parallel human conditions, such as obesity (Hoenig, 2006), retinal degeneration (Seeliger and Narfstrom, 2000), islet amyloidosis (Hoenig et al., 2000), cardiomyopathy (Fox et al., 2000), pulmonary fibrosis (Williams et al., 2004), filariasis (Grenfell et al., 1991), drug-induced hypersensitivity reactions (Utrecht, 2005), asthma (Norris Reinero et al., 2004; Kurucz and Szelenyi, 2006) and cancer (Rohn et al., 1996; McNiel, 2001; Porrello et al., 2006). Furthermore, they serve as a model for at least 40 heritable human disorders (O'Brien et al., 2002), including diabetes mellitus (Rijnberk et al., 2003; Henson and O'Brien, 2006), mucopolysaccharidosis (Haskins et al., 1983), spinal muscular atrophy (He et al., 2005), polycystic kidney disease (Lyons et al., 2004), mucopolipidosis (Mazrier et al., 2003), glycogen storage disease (Fyfe et al., 1992) and Niemann-Pick disease (Somers et al., 2003). Feline models also have been utilized for the development and evaluation of novel disease treatments including bone

marrow transplantation (Simonaro et al., 1999), gene therapy (Ellinwood et al., 2004; Vite et al., 2005; Casal and Haskins, 2006) and enzyme replacement (Byers et al., 2000).

The domestic cat has been studied extensively because it can contract an acquired immunodeficiency syndrome (AIDS) similar to that in humans (Willett et al., 1997). The progressive decline in immune function associated with this disease is the result of infection with feline immunodeficiency virus (FIV), a complex lentivirus that shares similar transmission and pathogenesis characteristics with human immunodeficiency virus (HIV) (Burkhard and Dean, 2003). The FIV model has enabled numerous *in vivo* investigations that are relevant to HIV/AIDS research, including studies on cytokine modulation (Dean et al., 2006), vaccine development (Dunham, 2006), perinatal transmission (Johnson et al., 2001; Weaver et al., 2005; Jayaraman and Haigwood, 2006) and transmission via semen and artificial insemination (Jordan et al., 1996; Jordan et al., 1998). Feline infectious peritonitis virus (FIP) and feline leukemia virus (FeLV), which are prevalent in feral cat populations, also have been studied to provide increased understanding of viral pathogenesis and immune response (Hardy et al., 1980; Weiss and Scott, 1981; Miyazawa, 2002; de Groot-Mijnes et al., 2005). The discovery that domestic cats are susceptible to avian influenza A (H5N1), either through consumption of infected birds or direct contact with an infected conspecific, has sparked interest in uncovering the role cats play in H5N1 transmission among poultry farms and from poultry to humans (Kuiken et al., 2004; Rimmelzwaan et al., 2006). The cat also serves as a research model for severe acute respiratory syndrome (SARS) and may be useful for testing new antiviral drugs under development to combat this corona virus (Martina et al., 2003).

*Genetics research.* The cat is an important model for interpreting the function and regulation of the human genome (O'Brien et al., 1999; O'Brien et al., 2001). While murine models have been the focus of much genetic research, the cat genome is three to four times less rearranged than the mouse or rat genome, compared to humans (O'Brien et al., 2002). As a result, the feline genome displays high levels of syntenic conservation relative to humans (Murphy et al., 2000) and serves as a useful reference for comparative genome analyses (Murphy et al., 2007). Cats also have been the subject of extensive molecular and evolutionary study of the major histocompatibility complex (MHC) (O'Brien and Yuhki, 1999), a cluster of loci encoding for immune response on the surface of most cell types. The high levels of DNA sequence homology between the human, murine and feline MHC are striking (Yuhki et al., 2003). Equally interesting are comparisons in MHC diversity among different species of the Felidae family (O'Brien, 1994). Studies suggest that reduced allelic diversity at the MHC (via inbreeding) is correlated with increased susceptibility to infectious disease and congenital defects in the cheetah (*Acinonyx jubatus*), Asiatic lion (*Panthera leo persica*) and Florida panther (*Puma concolor coryi*) (O'Brien and Yuhki, 1999).

Genetic analyses have been used to characterize population dynamics and genetic variation in highly elusive species or subspecies where limited information exists. In the case of the Tanzanian leopard (*Panthera pardus*), microsatellite analyses of pelt samples revealed high genetic variation and a stable effective population size, indicating that this species was actually less susceptible to ecological disruptions that had plagued other carnivores in the same habitat (Spong et al., 2000). The development of feline microsatellite maps for genetic studies (Menotti-Raymond et al., 2003) also has led to interesting applications in the field of human forensics (O'Brien et al., 2002). In one case, a murder suspect was implicated because his pet cat's hair was positively identified on a leather jacket stained with the victim's blood (Menotti-Raymond et al.,

1997). Advances in genetic techniques also have enabled a more comprehensive understanding of the phylogeny and divergence of living species within the Felidae family (Mattern and McLennan, 2000; Johnson et al., 2006).

*Infertility research.* There is evidence that domestic cat research could increase understanding of certain human infertility disorders. Their placental morphology is different, but cats share similar mechanisms of trophoblast invasion during the early stages of implantation, making them an interesting model for understanding the requirements of human pregnancy establishment (Carson et al., 2000). The cat placenta, like the human placenta, is capable of explant regrowth in culture and could prove useful for studying placental function (Jones et al., 2005). Cats also serve as an animal model for studying lentivirus-induced reproductive failure (Coats, 2005; Weaver et al., 2005). Additionally, following ovulation induction with exogenous gonadotropins the queen can exhibit poor embryo quality, ovarian hyperstimulation and alterations in oviductal transport, similar to human females (Roth et al., 1997b; Graham et al., 2000).

In males, domestic and non-domestic cats can display teratospermia, a phenomenon also observed in humans, where greater than 40% of sperm in serial ejaculates are structurally abnormal (Howard, 1993; Pukazhenthil et al., 2001). The etiology and implications of teratospermia in felids have been assessed at both the macrocellular and subcellular level, providing a useful database of information on this disorder (Howard et al., 1990; Howard et al., 1991; Howard et al., 1993a; Pukazhenthil et al., 1996; Pukazhenthil et al., 1998a; Pukazhenthil et al., 1998b; Pukazhenthil et al., 1999; Penfold et al., 2003). Efforts to characterize molecular mechanisms regulating spermatogenesis and spermiogenesis in normo- versus teratospermic cats have been initiated and are yielding valuable information (Pukazhenthil et al., 2006b). Additionally, technologies aimed at restoring fertility, such as testis xenografting (Snedaker et al.,

2004) and spermatogonial stem cell transplantation (Kim et al., 2006), are under development (Pukazhenti et al., 2006a). Xenografting of testis tissue from a domestic cat donor to an immunodeficient mouse host has resulted in complete feline spermatogenesis (Snedaker et al., 2004). Such advances could benefit similar research in humans to identify options for safeguarding fertility before cancer therapy.

*Zoological applications.* Most of the 39 wild cat species are classified as endangered in all or part of their native range (Wozencraft, 2005). Wild felid populations are declining primarily due to habitat loss, poaching, pollution and viral epidemics (Nowell and Jackson, 1996). One result of such decline is poor gene flow, which leads to inbreeding depression and, ultimately, risk of extinction (O'Brien, 1994). An extensively studied example of this phenomenon is the cheetah, which underwent a significant population bottleneck ~10,000 years ago. Today, the cheetah exhibits low genetic heterozygosity (O'Brien et al., 1983) and males consistently produce a high proportion of structurally abnormal sperm (> 70% per ejaculate) (Wildt et al., 1993; Crosier et al., 2007). Similar evidence of inbreeding depression and teratospermia is observed in the Florida panther, a subpopulation of the puma, which has low levels of genetic diversity due to concurrent geographic isolation and significant population decline (Barone et al., 1994a). Conservation attention also has been directed at the critically-endangered Iberian lynx (*Lynx pardinus*), which has suffered the effects of a severe population bottleneck coupled with rapid reductions in population size (Johnson et al., 2004).

The domestic cat is an important model for improving the reproduction, management and conservation of wild felids. Basic research in the domestic cat can be valuable when a limited number of non-domestic individuals are available for study (Wildt and Roth, 1997). Additionally, this research is often necessary to ensure the

safety and efficacy of new techniques before they are applied in endangered individuals. Accordingly, domestic cats have been studied extensively to evaluate techniques, including gamete/embryo cryopreservation, AI, IVF/ET and non-invasive fecal hormone monitoring (Brown, 2006; Swanson, 2006), which ultimately enhance reproductive success in a host of wild felids.

## **Felid Reproductive Physiology**

*Female domestic cats.* Free-ranging queens are seasonally polyestrous, long-day breeders with onset of puberty generally occurring at seven to 10 months of age (Concannon, 1991; Tsutsui et al., 2004b). Photoperiod, geographic location, breed and environment have been implicated in regulating pubertal onset and seasonality in the female cat (Goodrowe et al., 1989; Tsutsui et al., 2004b). Maintaining queens in photoperiod-controlled conditions (12-h light:12-h dark daily) allows year-round cycling (Wildt et al., 1979a). Although historically classified as induced (reflex) ovulators, requiring cervical stimulation during estrus to elicit ovulation, a high incidence of spontaneous ovulation (either intermittent or constant) has been observed in both group-housed and single-caged queens in a laboratory setting (Lawler et al., 1993; Gudermuth et al., 1997; Pelican et al., 2005). Spontaneous ovulation also has been observed in non-domestic felids, including the fishing cat (Moreland et al., 2002), clouded leopard (Brown et al., 1995), lion (Graham et al., 1993), margay (Moreira et al., 2001) and leopard (Brown et al., 2001). The etiology and physiological mechanism regulating spontaneous ovulation in cats is poorly understood and warrants further investigation. Age, non-sexual physical interactions, self-stimulation, uterine pathology and pheromonal influences all have been implicated (Gudermuth et al., 1997). Additionally,

there may be an evolutionary component to this phenomenon (Lariviere and Ferguson, 2003).

Numerous studies have examined behavioral, endocrine and physiological traits across the feline estrous cycle. Proestrus is short (1-2 days) and characterized by the presence of flat, clear ovarian follicles, vaginal cornification and increasing serum estradiol (Shille et al., 1979; Wildt et al., 1999; Bristol-Gould and Woodruff, 2006). The female generally becomes more active during proestrus but refuses copulation attempts by the male (Goodrowe et al., 1989). During estrus (typically 6 to 7 days), serum estradiol increases to > 20 ng/ml as advanced follicular development occurs in several dominant follicles (> 2 mm in diameter) (Wildt et al., 1999). During estrus, the female becomes receptive to the male and exhibits a variety of behaviors that serve to attract potential mates and facilitate breeding, including tail deviation, lordosis, rolling, increased vocalizations and foot-treading (Tsutsui and Stabenfeldt, 1993). Queens will allow up to 30 matings in a 36 hour period and have been observed to breed for as long as six consecutive days (Concannon and Verstegen, 1999). After successful mating, the uterotubal junction and the uterine crypts serve as sperm reservoirs (Chatdarong et al., 2004). Sperm reservoirs also have been observed in other species of domestic and laboratory animals, but are usually located in the oviductal isthmus (Suarez, 1998; Suarez, 2002).

Following estrus, unmated queens that do not ovulate enter a period of anestrus (also termed interestrus; typically 7 to 21 days), characterized by cessation of estrous behaviors, follicular atresia and a decline in serum estradiol levels (Wildt et al., 1999; Bristol-Gould and Woodruff, 2006). In contrast, mated queens undergo complex neuroendocrine responses culminating in a coitus-induced luteinizing hormone (LH) surge, final follicular maturation and ovulation. Sharp increases in serum LH occur within five minutes of intromission (Goodrowe et al., 1989). Timing of ovulation post-

copulation varies greatly (24 to > 52 h), where delaying coitus until later in estrus typically results in a shorter interval to ovulation. The LH surge and ovulation rarely occur if mating is restricted to just one copulation (Wildt et al., 1980). Immediately following ovulation (metestrus), prominent corpora hemorrhagica (CH) are observed on the ovaries that give rise (via luteinization) to progesterone-producing CL. CL dominate during diestrus and high levels of serum progesterone are maintained.

Following ovulation, non-pregnant cats exhibit elevated serum progesterone for 36-38 days (Paape et al., 1975; Wildt et al., 1999), whereas pregnant cats display elevated serum progesterone for the duration of gestation (63-67 days). Luteal phase length and fecal progestin concentrations are similar following spontaneous versus coitus-induced ovulation (Graham et al., 2000). In non-pregnant cats, CL remain visible for 35-44 days (Wildt et al., 1981) until luteolysis and formation of luteal scars on the ovary. In pregnant cats, CL appear to be the primary source of progesterone throughout gestation, although the placenta has been implicated in progesterone production during late pregnancy (Verstegen et al., 1993). Indeed, maintenance of pregnancy is possible following ovariectomy at Day 55 (Concannon and Verstegen, 1999). Relaxin production by the fetoplacental unit begins around Day 20, peaks at Day 35 and gradually declines until parturition (Stewart and Stabenfeldt, 1985; Klonisch et al., 1999). Prolactin may be luteotrophic in the cat and is first detected around Day 35, sharply increasing just before parturition (Banks et al., 1983; Tsutsui and Stabenfeldt, 1993). After parturition (2-6 kittens), the CL remain intact throughout lactation and regress ~ 2 months postpartum (Goodrowe et al., 1989). Return to estrus following pregnancy is typically observed 4 weeks after normal weaning; if the kittens are weaned prematurely, estrus is reestablished within 6 to 8 days (Concannon and Verstegen, 1999).

Early embryonic development, implantation and placentation have been characterized in the cat. Each ovulated oocyte has an ~ 70% chance of undergoing



successful fertilization and surviving to implantation following natural breeding (Swanson et al., 1994). After fertilization in the mid to proximal oviduct (ampulla), embryos migrate through the uterotubal junction, entering the uterus as morulae or early blastocysts on Day 5 or 6 (Day 0 = first copulation) (Denker et al., 1978b; Swanson et al., 1994). Transuterine embryo migration has been observed, where the greater the disparity in CL number between ovaries, the higher the incidence of relocation within the uterus (Tsutsui et al., 1989; Swanson et al., 1994). The uterine epithelium begins to undergo decidualization in the immediate vicinity of each blastocyst by Day 12, indicating early pre-implantation interactions between the endometrium and conceptus (Denker et al., 1978a). Attachment and implantation of hatched blastocysts occurs by Day 14 (Denker et al., 1978a; Concannon, 1991) and, like human implantation, involves invasion of the uterine epithelium by the trophoblast (Boomsma et al., 1991; Carson et al., 2000).

Similar to most carnivores, cats possess a zonary, endotheliochorial placental structure that has been well characterized both developmentally and morphologically (Leiser, 1982; Leiser and Koob, 1993; Leiser and Kaufmann, 1994; Walter and Schonkypl, 2006). Maternal-fetal blood flow is established via a simple crosscurrent system (Leiser and Kaufmann, 1994). By Day 22, the fetal heart rate is detected and organogenesis has begun (Nelson and Cooper, 1975; Concannon, 1991; Concannon and Verstegen, 1999). Numerous studies have used ultrasonography to diagnose and evaluate pregnancy in queens from as early as Day 10 (Zambelli et al., 2002a; Zambelli and Prati, 2006), providing a database of fetal and placental morphological norms from early (Zambelli et al., 2002b) to late (Zambelli et al., 2004) gestation. The fetal skull and spine are radio-opaque by Day 38 and radiography remains a useful tool for confirming pregnancy and determining litter size in late gestation (Concannon and Verstegen, 1999).

*Male domestic cats.* Sperm can be recovered from the testes of male kittens as early as 5 months of age; however, most normal young males begin to show sexual maturation and sperm production by the age of 8 months (Tsutsui et al., 2004a). Free-ranging toms generally complete puberty by 12 months of age, indicated by the advent of complete spermatogenesis, mating behaviors and the emergence of 100-200 androgen-dependent spines on the penis (Goodrowe et al., 1989). Sperm production does not appear to be seasonally influenced (Spindler and Wildt, 1999; Wildt et al., 1999). Factors related to spermatogenesis have been elucidated in the domestic cat, including seminiferous epithelial cycle length, testis morphology, daily sperm production, DNA replication and germ cell apoptosis (Blanco-Rodriguez, 2002; Franca and Godinho, 2003). Eight stages of the seminiferous epithelium cycle have been characterized in the cat, with a cycle length of 10.4 days, indicating that the total duration of spermatogenesis, from A-spermatogonia to fully-differentiated spermatozoa, is approximately 7 weeks (Franca and Godinho, 2003).

Outbred domestic cat males produce a high proportion (> 70%) of structurally-normal sperm in a typical ejaculate (Howard, 1992; Wildt et al., 1999). However, teratospermia (production of > 60% pleiomorphs per ejaculate) has been observed in both domestic and non-domestic felids (Wildt et al., 1988; Howard, 1993). For example, Florida panthers, clouded leopards, and cheetahs typically have less than 20% normal spermatozoa (Pukazhenthil et al., 2006b). Commonly observed sperm abnormalities in cats include head and midpiece defects, cytoplasmic droplets, bent flagella, bent midpieces and tightly coiled flagella (Howard, 1992; Howard, 1993). In multiple felid species, malformed sperm have been linked to decreased genetic variability, which in turn leads to low fertility (Pukazhenthil et al., 2001). Structurally-malformed domestic cat spermatozoa exhibit abnormal sperm function and reduced ability to penetrate the zona pellucida and fertilize an oocyte (Howard et al., 1991; Howard et al., 1993a). Even

normal spermatozoa from a teratospermic donor are compromised, displaying decreased ability to undergo acrosome reaction, capacitation, penetration of the zona pellucida and fertilization of conspecific oocytes (Howard et al., 1991; Howard et al., 1993a; Long et al., 1996). There is evidence that teratospermic males may compensate with higher sperm output, as evidenced by increased sperm concentration, testes volume, more germ cells per Sertoli cell and reduced germ cell loss during spermatogenesis compared to normospermic individuals (Howard et al., 1990; Neubauer et al., 2004). Poor nutrition has been implicated as one cause for low sperm concentrations and morphological abnormalities (Swanson et al., 2003; Howard and Allen, 2007).

Mean serum testosterone, FSH and LH concentrations for both normospermic and teratospermic cats have been documented (Howard et al., 1990). However, limited information is available on the temporal patterns in circulating levels of these hormones. Plasma testosterone rapidly increases at 8 months of age, reaching a peak of ~ 2.6 ng/ml by 10 months of age in outbred cats (Tsutsui et al., 2004a). Teratospermic males have similar FSH and LH concentrations compared to normospermic males, but serum testosterone levels are lower (Howard et al., 1990). Species-specific differences in serum testosterone values also have been documented for several non-domestic species, including the cheetah, leopard, tiger, puma, ocelot, margay and tigrina (Wildt et al., 1988; Morais et al., 2002; Genaro et al., 2007).

*Gonadal control.* Manipulating felid reproduction has two important applications: (1) contraception; and (2) ovarian control for assisted reproduction. The latter will be explored in detail later in this chapter. Indeed, while this research project focuses on assisted reproduction, many cats are capable of producing offspring prolifically. Alternatives to euthanasia and surgical sterilization for control of rampant pet

overpopulation remain a focus of significant interest and research (Kutzler and Wood, 2006). For optimal genetic management of zoo animals, there is a need for contraception that is safe and effective, as well as reversible (Jewgenow et al., 2006). Methods for inhibiting fertility in felids can include same-sex housing, disruption of gamete production, fertilization or implantation, and direct termination of pregnancy (Burke, 1982; Munson, 2006).

Cats are most commonly contracepted through the administration of exogenous hormones that directly interfere with folliculogenesis or spermatogenesis. These hormones act primarily to disrupt the hypothalamic-pituitary-gonadal (HPG) axis, although additional modes of action at the level of the female reproductive tract (e.g. altering endometrial tone, influencing cervical mucous secretion, etc.) are known to occur in many species (Burriss, 1999). In females, the progestin implants levonorgestrel (Norplant®) (Baldwin et al., 1994; Looper et al., 2001) and melengesterol acetate (MGA) (Munson, 2006) and the oral progestins megestrol acetate (Ovaban®) (Øen, 1977; Romatowski, 1989) and medroxyprogesterone acetate (Provera®) (Munson, 2006) have been used for contraception in domestic and non-domestic felids. Duration of ovarian suppression and return to estrus following removal vary by drug and dosage.

While progestins are highly effective at contracepting domestic and non-domestic queens, long-term use has been associated with a host of health problems including infertility, mammary cancer, uterine cancer, endometrial hyperplasia, pyometra and diabetes (Hinton and Gaskell, 1977; Kollias et al., 1984; Munson and Mason, 1993; Munson et al., 2002; Munson, 2006). To combat these problems, alternative means for contraception are currently under investigation. Exogenous androgens have been studied for their ability to suppress follicular phase in domestic cats (Gardner et al., 1985), but adverse effects including increased aggression and masculinization have prevented widespread use. GnRH agonists have shown promise in both male and

female felids. Leuprolide acetate (Lupron®) and deslorelin effectively suppress ovarian activity in females (Munson et al., 2001; Munson, 2006). Deslorelin also has been used to down-regulate spermatogenesis in cheetahs (Bertschinger et al., 2006). Finally, immunocontraception with anti-GnRH vaccines has shown promise but requires further investigation (Levy et al., 2004; Robbins et al., 2004).

Disruption of pregnancy after gamete production can be achieved through the prevention of sperm-egg interactions in some species. Several vaccines have been developed that target the zona pellucida antigens required for sperm binding to the oocyte. One such vaccine, a porcine zona pellucida (PZP) derivative (SpayVac®), has been studied in the domestic cat with unimpressive results (Gorman et al., 2002; Levy et al., 2005). While cats display high anti-PZP titers following vaccination, they do not display a significant reduction in fertility. Safety of these PZP vaccines, which have been associated with adjuvant-related sarcomas, also make their use undesirable in felids (Munson, 2006). Development of a feline-specific ZP vaccine currently is ongoing (Ringleb et al., 2004).

Termination of pregnancy has been used in some cases as a means for contraception, albeit very rarely in zoo felids. In domestic cats, pregnancy termination can be achieved through the use of drugs including prostaglandins (Nachreiner and Marple, 1974), a combination of cabergoline (a dopamine agonist) and cloprostenol (a prostaglandin) (Onclin and Verstegen, 1997) and the progesterone antagonist aglepristone (Georgiev and Wehrend, 2006).

## **Tools for Assessing Reproductive Potential**

*Female fertility assessments.* Reproductive potential can be assessed in females using several minimally-invasive techniques. Documenting presence or

absence of estrous behavior as an index of fertility is of limited use because some felids do not readily display outward signs of estrus (Shille et al., 1979; Wielebnowski and Brown, 1998). Conversely, females with significant uterine pathology or reproductive abnormalities can continue to display cyclic estrous behaviors. Generally, a more detailed, direct examination of the reproductive tract is necessary. Laparoscopic examination of the ovaries, oviducts and uterus is a common approach (Wildt et al., 1977). This surgical procedure is performed under general anesthesia using a 5 to 10 mm laparoscope inserted through a small incision cranial to the umbilicus to visualize the abdominal cavity. A two mm Verres probe, which is used to insufflate the abdominal cavity with air before the laparoscopic exam, serves as a reference point for obtaining dimensions of reproductive organs and ovarian structures.

A less invasive technique, which provides reduced information compared to laparoscopy, is ultrasonography. Ultrasound imaging can diagnose pregnancy, provide reproductive tract dimensions and uncover certain pathologies. However, ultrasound technology is not advanced enough, at least in the domestic cat, to assess ovarian activity (e.g. number of follicles vs. CL) or diagnose more subtle uterine pathologies such as mild cystic endometrial hyperplasia (Baker, 2007). Hystero-graphy, where a contrast medium is injected transcervically to subsequently visualize the reproductive tract via radiography, has shown promise as a nonsurgical alternative to laparoscopic examination (Chatdarong et al., 2005). Finally, more direct evaluation of oocyte quality and function is possible through laparoscopic oocyte retrieval and subsequent evaluation of *in vitro* developmental competence and fertilization (Goodrowe et al., 1988b).

*Male fertility assessments.* In males, evaluation of fertility potential is possible through semen collection and analysis. Semen can be recovered by electroejaculation or an artificial vagina, and also by flushing the reproductive tract post-castration or post-

mortem (Howard et al., 1986; Howard, 1992). Electroejaculation in the domestic cat typically yields a 100-200  $\mu$ l ejaculate containing 30-50 million sperm (Wildt et al., 1999). An artificial vagina yields a lower volume ejaculate (50-60  $\mu$ l) but higher concentration compared to electroejaculation (Tanaka et al., 2000a). In addition to performing conventional semen analyses (motility, forward progressive status, concentration, morphology, etc.), the ejaculate also can be assessed for functional competence using a variety of techniques (Wildt et al., 1992; Howard, 1993), including the zona-free hamster ovum and zona-intact cat oocyte penetration bioassays (Howard et al., 1991). While raw sperm in a fresh ejaculate rapidly deteriorates, dilution in culture medium can extend viability considerably (Howard et al., 1986). Furthermore, domestic and non-domestic felid sperm contain numerous microbes and require washing with culture medium to remove seminal plasma and bacteria prior to AI, IVF and sperm cryopreservation (Howard et al., 1993b). Sorting of X and Y- chromosome-bearing felid spermatozoa via high speed flow cytometry has been performed with relatively high accuracy (~ 85%) and may prove useful in assisted reproduction applications (Spinaci et al., 2007).

*Fecal hormone monitoring.* Traditional approaches to monitoring endocrine function rely on serial blood sampling (Wildt et al., 1981); however, many animals are difficult to restrain without anesthesia and easily stressed during blood collection (Brown et al., 1994). Because stress can perturb reproductive function (Graham and Brown, 1996; Breen et al., 2005), the development of non-invasive hormone monitoring techniques has been valuable for assessing reproductive and adrenal activity in a host of mammals including ungulates, primates and carnivores (Brown et al., 1997; Brown, 2006). This approach utilizes recently excreted products (typically urine, feces or saliva) that can be obtained with no deleterious effect on the animal. In addition to eliminating potentially stressful blood collections, fecal and urinary analyses provide a pooled

hormone value, rather than a peak or nadir which may not be representative of the overall physiological response (Brown et al., 2001).

In cats, steroid hormones are excreted almost exclusively (> 95%) in the feces (Shille et al., 1990; Brown et al., 1994). Using high performance liquid chromatography and gas chromatography/mass spectrometry, it has been demonstrated that estradiol is excreted in nearly equal amounts as unconjugated estrogen metabolites (estradiol 17 $\beta$  and estrone) and non-enzyme-hydrolyzable conjugates (primarily estrogen sulfates) (Brown et al., 1994). In contrast, and unlike most other species for which information is available, progesterone is excreted primarily as unknown conjugated metabolites in cat feces. Unconjugated progestins, which account for ~ 20% of fecal progesterone metabolites, include several pregnenolone epimers.

Hormone extraction techniques have been developed and validated for felids to concentrate and isolate steroids from individual fecal samples. In cats, fecal samples are first frozen and lyophilized (freeze-dried). The most prominent extraction method involves boiling the dried feces in a 90% ethanol solution (Brown et al., 1994). Enzyme immunoassays (EIA) or radioimmunoassays (RIA) then are used to quantify fecal estrogens and progestins in the resulting extract. These extraction methods also have proven successful for monitoring androgens (Brown et al., 1996a) and corticoids (Graham and Brown, 1996) in several felid species. Fecal steroid hormone monitoring has been employed to characterize baseline reproductive function (Brown et al., 2001), identify mode of ovulation (Moreira et al., 2001; Moreland et al., 2002), assess response to assisted reproduction hormone treatments (Brown et al., 1995; Pelican et al., 2005), determine correlations between adrenal activity and perceived stress (Terio et al., 1999; Wielebnowski et al., 2002) and evaluate incidence of reproductive seasonality (Brown et al., 2002; Morais et al., 2002; Morato et al., 2004) in domestic and wild felids.

Pregnancy detection using fecal hormone monitoring remains difficult due to the inherent



“lag time” associated with sample processing and the fact that a non-pregnant luteal phase can last one half to two thirds the duration of pregnancy in felids. Furthermore, fecal progesterin concentrations during pregnancy versus a non-pregnant luteal phase can not be distinguished. Thus, a positive pregnancy diagnosis can't be made until very close to parturition using fecal hormone analysis alone. A urinary canine relaxin radioimmunoassay has been successful in diagnosing pregnancy within 3-4 weeks after mating in the domestic cat and leopard (de Haas van Dorsser et al., 2006).

### **Assisted Reproduction Techniques in Felids**

*Ovulation induction.* Ovarian stimulation using exogenous hormones generally requires that two consecutive physiological events occur: (1) stimulation of follicular recruitment, selection and dominance (folliculogenesis); and (2) stimulation of final follicular maturation and ovulation. These exogenous hormone regimens must mimic the endogenous actions of FSH and LH, respectively. In multiple felid species, the most common exogenous hormone used to induce folliculogenesis is equine chorionic gonadotropin (eCG; also known as pregnant mare's serum gonadotropin or PMSG), which initiates the follicular phase with a single intramuscular injection (Pelican et al., 2006b). Exogenous porcine FSH also has been used for this purpose in the domestic cat (Goodrowe et al., 1988a) and several wild felids (Phillips et al., 1982; Pope et al., 1993; Crichton et al., 2003). However, FSH treatment requires multiple injections over a course of several days and can result in high numbers of cystic-appearing follicles that fail to ovulate (Goodrowe and Wildt, 1987). Efforts to characterize the amino acid sequence of tiger FSH eventually may lead to a felid-specific follicle-stimulating gonadotropin, yet the need for frequent daily injections remains a significant roadblock, particular in applications with wild felids (Crichton et al., 2003). After follicular activity

has been induced, various hormones can be used to complete follicular maturation and induce ovulation, including human chorionic gonadotropin (hCG), gonadotropin-releasing hormone (GnRH) or exogenous porcine LH (Goodrowe and Wildt, 1987; Howard, 1999; Crichton et al., 2003).

A single injection of eCG and hCG has become the regimen of choice in both domestic cats and their non-domestic counterparts (Roth et al., 1997b; Howard, 1999). These drugs are preferred over regimens involving multi-day injections, particularly in stress-sensitive wild felids where each treatment becomes progressively more challenging to administer as the cat anticipates the next injection. Interestingly, eCG and hCG dosages are largely species-specific. For example, the ~ 10 kg ocelot requires 500 IU eCG to stimulate adequate follicular development, whereas the ~ 20 kg clouded leopard requires only 100 IU eCG (Swanson et al., 1996b; Howard et al., 1997). Furthermore, a trend has been observed in several South American felid species, where decreased sensitivity to gonadotropins is observed (Swanson and Brown, 2004). The optimal interval between eCG and hCG continues to be studied; however, an interval of 80-84 hours is currently used (Donoghue et al., 1992).

*Artificial insemination.* Vaginal insemination is moderately effective in domestic cats; however, high concentrations of spermatozoa are necessary to achieve acceptable pregnancy rates (> 50%) and results can be highly variable (Tanaka et al., 2000b; Tsutsui, 2006). An additional consideration is that anesthesia is required to perform AI in most felids. Yet, anesthesia is known to inhibit ovulation in the cat, making vaginal insemination of little use in zoological applications (Howard et al., 1992a). Transcervical insemination, which is currently under development in the cat, poses similar challenges but could show promise in the future (Zambelli and Cunto, 2005b; Zambelli and Cunto, 2005a).

A laparoscopic AI technique was developed in felids to combat the limitations of vaginal and transcervical insemination (Howard et al., 1992a). The laparoscope allows direct visualization of ovarian response and enables intrauterine sperm deposition using a catheter inserted through the body wall and directly into the uterine lumen. This procedure must be timed carefully to ensure that anesthesia induction and insemination occurs after ovulation, yet within the fertilization lifespan of the ova (~ 14 h post-ovulation). The eCG/hCG regimen in combination with post-ovulatory laparoscopic AI using fresh or frozen-thawed spermatozoa has been used to successfully produce offspring in domestic (Howard et al., 1992a) and non-domestic felids including the cheetah (Howard et al., 1992b), clouded leopard (Howard et al., 1996), tiger (Donoghue et al., 1993), puma (Barone et al., 1994b), leopard cat (Howard, 1991), snow leopard (Roth et al., 1997a), tigrina (Swanson and Brown, 2004) and ocelot (Swanson et al., 1996b).

*In vitro fertilization and embryo transfer.* Multiple studies have been conducted to develop techniques and elucidate culture media requirements for successful oocyte *in vitro* maturation (IVM), IVF and ET in the domestic cat. These studies have served as the foundation for successful IVF/ET in the tiger (Donoghue et al., 1990), African wild cat (Pope et al., 2000), ocelot (Swanson and Brown, 2004), caracal (Pope et al., 2001), and fishing cat (Pope et al., 2006a). Oocytes are harvested either from freshly excised ovaries (post-ovariohysterectomy or post-mortem) or via ovarian stimulation followed by pre-ovulatory laparoscopic follicular aspiration (Goodrowe et al., 1988b). Optimal temperature and culture conditions for IVM have been studied extensively, and most immature cat oocytes complete nuclear maturation *in vitro* within 32 h of culture (Johnston et al., 1989; Pope et al., 1993; Wood et al., 1995). Circannual declines in

oocyte quality and maturation stage at the time of harvest can be mitigated by altering culture conditions (Comizzoli et al., 2003).

Following IVM and IVF with high quality sperm, a 60 to 80% fertilization success has been achieved (Johnston et al., 1989). Intracytoplasmic sperm injection (ICSI), a modification to traditional IVF in which a single sperm is injected directly into an ovum, also has been successful in the cat (Gomez et al., 2000; Comizzoli et al., 2006). One of the greatest challenges following IVF in cats is the apparent morula to blastocyst developmental block that is observed (Roth et al., 1994). This block does not appear to be concurrent with the transition from maternal to zygotic control of development, which occurs by the 5-8 cell stage (Hoffert et al., 1997). Studies have shown that co-culturing cat embryos with conspecific companions can enhance *in vitro* development and increase the likelihood of survival to the blastocyst stage (Spindler and Wildt, 2002; Spindler et al., 2006). Furthermore, a feline-optimized culture medium was developed to elucidate the specific metabolic requirements for successful embryo culture, with the ultimate goal of increasing incidence of blastocyst formation following IVF (Herrick et al., 2007). Despite the continued challenge to improve felid embryo culture, transfer of both early stage embryos to the oviduct (Goodrowe et al., 1988b) and morulae/blastocysts to the uterus (Pope et al., 1993) of synchronized embryo recipients has resulted in pregnancies in the domestic cat.

*Cryopreservation of gametes and embryos.* Significant strides have been made in cats to develop and refine techniques for long-term cryopreservation of biological materials including gametes and embryos (Wildt, 1997; Wildt and Wemmer, 1999). The development of 'genome resource banks' is an important tool for enhancing management of populations with low heterozygosity, as well as maintaining an insurance policy against extinction should a catastrophic event occur in the animal's native range

(Wildt, 2000). There also is growing support for the use of cryopreserved materials to maintain lines of rare biomedical models (Critser and Russell, 2000).

In cats, sperm cryopreservation techniques have been developed and refined to reduce membrane damage and minimize rapid osmotic changes during cooling, freezing and thawing. This is achieved through the carefully-timed addition of cryoprotective agents to fresh ejaculates, typically glycerol in an egg yolk buffer, before storage in liquid nitrogen (Luvoni, 2006). Rates of cooling, thawing and cryoprotectant removal have been refined to improve sperm motility and minimize membrane damage post-thaw (Pukazhenthii et al., 2002). However, studies also have demonstrated that teratospermic ejaculates are more vulnerable to cold-induced damage compared to normospermic ejaculates (Pukazhenthii et al., 1999; Pukazhenthii et al., 2000), and techniques aimed at optimizing sperm cryopreservation in teratospermic individuals continue to be refined. The use of conspecific, frozen-thawed spermatozoa for AI has resulted in the successful birth of offspring in the domestic cat (Platz et al., 1978), ocelot (Swanson et al., 1996b), cheetah (Howard et al., 2002) and leopard cat (Howard, 1991). Cryopreservation of epididymal sperm also is feasible post-castration or post-mortem; however fertility of the resultant sperm is variable and some studies indicate increased susceptibility to freezing damage (Hay and Goodrowe, 1993; Tebet et al., 2006).

Oocyte cryopreservation remains a significant challenge in cats, largely due to a high surface area to volume ratio in the ovum which makes them vulnerable to osmotic stress and membrane damage (Luvoni, 2006). An alternative approach is cryopreservation of whole ovarian follicles or ovarian cortex samples, which has shown promise in preliminary studies (Jewgenow et al., 1998; Jewgenow and Paris, 2006). Techniques for freezing immature oocytes using novel cryoprotectant protocols also are under development (Comizzoli et al., 2004). Embryo cryopreservation has been more successful than oocyte freezing in felids (Pope et al., 1993; Pope, 2000). Using slow,

controlled-rate cooling methods, transfer of *in vitro* derived frozen-thawed embryos has resulted in live births in the domestic cat (Pope et al., 1994), ocelot (Swanson and Brown, 2004), African wild cat (Pope et al., 2000) and caracal (Pope et al., 2006a).

### **Challenges to Assisted Reproduction Success**

*Ovarian response and the endocrine environment.* Despite significant efforts to develop and refine assisted reproduction techniques in domestic and wild felids, the incidence of pregnancy following AI remains variable across species (0% to ~ 50%). There are multiple factors implicated in AI failure, from poor ovarian response at the time of AI (Day 2 post-hCG) to failure of the embryo(s) to successfully attach during the critical peri-implantation period (~ Day 12). Variable ovarian response to exogenous gonadotropins is a common observation in cats (Roth et al., 1997b; Graham et al., 2000). Some of this variability is attributed to administration of gonadotropins during the luteal phase, where presence of functional CL at the time of the AI can attenuate ovarian response and lead to ovulation failure. This is common in species that spontaneously ovulate (Howard et al., 1997; Bauer et al., 2004). Even if ovulation does occur, elevated progesterone levels at the time of ovarian stimulation have been associated with lower pregnancy rates following IVF/ET in other species (Kolibianakis et al., 2004). Alternatively, when exogenous gonadotropins are given during follicular phase, several follicular cohorts of inconsistent age and maturation phase can result; ovulation of these follicles in response to exogenous gonadotropins is, therefore, variable. Whether oocytes exhibit decreased ability to undergo fertilization as a direct result of ovarian stimulation is not well characterized in the cat (Roth et al., 1994). In several species, exposure to exogenous gonadotropins has been associated with poor oocyte quality (Moor et al., 1985; Hyttel et al., 1986; Yun et al., 1987).

Following AI, disruptions in the maternal environment are common and can be linked, in part, to residual effects of eCG and/or hCG in the cat (Swanson et al., 1997). Pharmacokinetic data demonstrate that eCG and hCG persist in domestic cat circulation for at least 120 hours and 96 hours, respectively. This persistence in circulation supports ancillary follicular growth after the initial folliculogenic surge (Donoghue et al., 1992; Swanson et al., 1996a). Secondary (and even tertiary) cohorts of follicles can ovulate, leading to ancillary CL formation and further disruption of endocrine dynamics. Attempts to neutralize residual eCG and hCG have been ineffective at preventing secondary follicular and CL development (Swanson et al., 1996a). Oral melatonin pre-treatment only marginally reduced the incidence of secondary follicular development (Graham et al., 2004). Additionally, repeated eCG/hCG treatment is associated with immunologically-mediated refractoriness to ovarian stimulation (Swanson et al., 1995a). Alternating gonadotropin regimens between eCG/hCG and porcine FSH/LH has successfully mitigated immunological complications in ocelots and tigrinas (da Paz et al., 2006); however, avoidance of gonadotropin overuse is likely to be a more optimal long-term strategy (Swanson et al., 1995a).

Specific endocrine perturbations known to occur following gonadotropin stimulation in cats include sustained high fecal estrogens and abnormally-elevated fecal progestins (Graham et al., 2000). The implications of these altered endocrine patterns are not fully understood. Protracted serum estradiol has been associated with diminished embryo development in the cat (Goodrowe et al., 1988a; Gonzalez-Bulnes et al., 2003). Hyper-elevated levels of serum progesterone are associated with poor embryo quality (Swanson et al., 1995b), but have not been directly associated with decreased fertility (Roth et al., 1997b) in the cat. Yet, fecal hormone analyses indicate that abnormal ovarian steroid patterns are not associated with changes in embryo quality or developmental stage (Graham et al., 2000). Thus, further study is needed to clarify

this relationship. In cows, abnormally high progesterone following IVF/ET is linked to decreased conception rates (Nogueira et al., 2004). One hypothesis to explain this phenomenon is that early exposure to high progesterone can accelerate down-regulation of progesterone receptors in the endometrium and endogenous prostaglandin release (a luteolysin in cows) (Mann et al., 1998). Whether a related mechanism may occur in cats remains to be determined.

*Early embryonic development and oviductal transport.* The influence of exogenous gonadotropins on early embryonic mortality has been characterized in the cat (Roth et al., 1995). While eCG/hCG administration does result in more unfertilized oocytes and fewer high-quality blastocysts compared to naturally-bred controls, good quality embryos can be obtained from gonadotropin-stimulated cats. It is likely that residual hCG, more so than eCG, plays a role in early embryo mortality. This is supported by observations that embryo quality is improved in naturally-bred cats treated with eCG, compared to cats treated with both eCG and hCG and then artificially inseminated (Roth et al., 1997b). The specific mechanisms by which residual gonadotropin effects may influence embryo quality demand further investigation in felids. In mice, superovulation is associated with delayed embryo development and abnormal blastocyst formation both *in vivo* and *in vitro* (Ertzeid et al., 1993; Ertzeid and Storeng, 2001; Van der Auwera and D'Hooghe, 2001). Similar observations have been made in the rabbit (Molina et al., 1991) and hamster (McKiernan and Bavister, 1998).

The amount of time the domestic cat embryo remains in the oviduct before transversing the uterotubal junction is long (144-168 h) compared to most species, including the rabbit (56-62 h), mouse (72 h) and human (60-70 h) (Croxatto and Ortiz, 1975). Duration of oviductal transport is influenced by exogenous hormone treatment in the cat, where embryos produced by naturally-mated queens reach the uterus sooner



than embryos produced following gonadotropin stimulation and AI (Graham et al., 2000). This may be related to the finding that estradiol is a key regulator in oviductal transport (Croxatto and Ortiz, 1975; Roblero and Garavagno, 1979; Bigsby et al., 1986; Zenteno et al., 1989). More specifically, endogenous ovarian steroids (both estradiol and progesterone) are implicated in smooth muscle contraction of the oviduct (Nozaki and Ito, 1987). Accordingly, prolonged elevations in fecal estrogens in the cat are negatively correlated with the proportion of embryos recovered in the uterus, versus the oviduct, 5 days after AI (Graham et al., 2000). This is further supported by the observation that administering exogenous estradiol retards embryo transport in the cat (Herron and Sis, 1974). Interestingly, this regulatory mechanism is largely species-specific. Exogenous estrogen accelerates embryo transport in the rat, whereas it has no influence on transport in the hamster (Croxatto, 2002). Exogenous progesterone does not appear to influence rat ovum transport (Fuatealba et al., 1987) and also is capable of antagonizing the effects of exogenous estrogen on the oviduct (Fuatealba et al., 1988b).

*Implantation.* Implantation involves a carefully orchestrated series of events within the uterine lumen that culminate in the establishment of one or more maternal-fetal units. Following hatching of the blastocyst from its zona pellucida in the uterus, the embryonic trophoblast must establish contact with the endometrium (Carson, 1999; Carson et al., 2000). This process involves embryonic attachment (apposition and adhesion) followed by trophoblastic invasion of the uterine epithelium (Carson, 1999). Successful implantation depends on precise coordination of embryonic development and uterine morphology, as well as steroid, cytokine and growth factor release.

In cats, little is known about the influence of exogenous hormone administration on implantation success. Beyond anecdotal evidence of low litter size following

gonadotropin stimulation and AI in felids, no distinct associations have been made between ovarian stimulation protocols and implantation failure. In the mouse, ovarian hyperstimulation inhibits embryo implantation (Fossum et al., 1989). Specifically, gonadotropin stimulation has been associated with delayed implantation, fetal death, prolonged gestation, and low birth weight (Ertzeid et al., 1993; Ertzeid and Storeng, 2001). Gonadotropin administration also is associated with decreased post-implantation viability in the hamster (McKiernan and Bavister, 1998). In humans, a similar relationship between ovarian stimulation and poor endometrial receptivity has been demonstrated (Gidley-Baird et al., 1986; Forman et al., 1988; Devroey et al., 2004). Remarkably, very little research has been conducted to elucidate the specific causes for implantation failure in humans following IVF and other assisted reproduction procedures (Devroey et al., 2004) or in females with endometriosis (Giudice et al., 2002).

### **Improving Assisted Reproduction with Estrous Cycle Control**

*Fundamentals of estrous cycle control.* Ovarian suppression prior to ovulation induction and AI or IVF is a common strategy in mammals, including humans, to provide a more synchronized ovarian response at the time of assisted reproduction. Control of the estrous cycle typically is performed by: (1) disruption of CL function; or (2) direct suppression of follicular activity (Foxcroft, 1999).

CL function can be disrupted in many species using exogenous prostaglandins, which lyse active CL and return the animal to follicular activity at a predictable time interval (Lofstedt, 1988; Schiewe et al., 1991; Fralix et al., 1996; Xu and Burton, 1999; Whitley and Jackson, 2004). Prostaglandins can be used alone or in conjunction with progestins, and may or may not be followed by exogenous ovarian stimulation. In cats, however, CL disruption using prostaglandins is not feasible for assisted reproduction

applications, because cats are refractory to prostaglandins up to day 40 post-ovulation (Shille and Stabenfeldt, 1979; Wildt et al., 1979b). Indeed, the mechanisms and hormonal control of luteolysis are poorly understood in the cat, making disruption of CL function an impractical approach.

Alternatively, follicular activity can be directly suppressed by administering GnRH analogs, which act directly at the level of the pituitary gland, or progestins, which act via negative feedback on the hypothalamic-pituitary-gonadal axis. These approaches have proven successful in felids (Pelican et al., 2006b). After temporary inhibition, a new cohort of synchronized, early antral follicles develops. These young follicles are under limited endogenous gonadotropin control and are believed to be highly susceptible to ovarian stimulation by exogenous gonadotropins (McGee and Hsueh, 2000). Understanding the changes in ovarian sensitivity to exogenous gonadotropins following down-regulation is poorly documented and could benefit from further investigation.

*Ovarian inhibition using GnRH analogs.* GnRH analogs act primarily at the level of the pituitary to inhibit ovarian activity; however, direct gonadal actions are observed in some species (Conn et al., 1999). GnRH agonists elicit a biphasic endocrine response. Initially, GnRH agonists stimulate ovarian activity by inducing an increase in FSH and/or LH secretion via liberation of stored gonadotropins (Shalev and Leung, 2003). This activity often culminates in ovulation. After the initial surge, continued administration of the agonist leads to down-regulation of gonadotropin production and gonadotropin receptor expression, ultimately resulting in suppression of ovarian activity. The pituitary remains refractory to GnRH action until the agonist is discontinued or voided from circulation. In contrast, GnRH antagonists provide more immediate ovarian suppression through competitive binding of the GnRH receptor (Janssens et al., 2000). This, in turn, inhibits FSH and/or LH secretion and down-regulates GnRH receptors on the pituitary.

The resultant suppression of ovarian activity is rapid and reversible (Shalev and Leung, 2003). This approach has been used routinely in conjunction with AI or IVF and ET in humans (Albano et al., 1999; Barbieri and Hornstein, 1999).

GnRH analogs have been used in felids for short-term ovarian suppression before assisted reproduction with some success. The GnRH antagonist antide provides reversible inhibition of ovarian activity in the domestic cat (Pelican et al., 2005). Furthermore, antide-treated cats display similar oocyte quality and *in vitro* fertilization rates following gonadotropin stimulation, compared to untreated controls (Pelican, 2002). Use of the GnRH agonist leuprolide (Lupron®) in clouded leopards has been far less promising. While leuprolide does suppress ovarian activity, its use before gonadotropin stimulation leads to a high incidence of ovulation failure (Pelican et al., 2006a).

*Ovarian inhibition using progestins.* Progestins are widely used in multiple species to synchronize estrous activity before assisted reproduction, including humans (Gonen et al., 1990; Burry et al., 1991), cetaceans (Robeck et al., 2004; Robeck et al., 2005), non-domestic ungulates (Monfort et al., 1993; Thompson and Monfort, 1999; Morrow et al., 2000), the cow (Stegner et al., 2004), horse (Webel and Squires, 1982), goat (Fonseca et al., 2005) and pig (Wood et al., 1992). These compounds act via a mechanism similar to endogenous progesterone, providing constant negative feedback at the level of the hypothalamus (primarily) and pituitary gland (Romagnoli and Concannon, 2003). This results in attenuated gonadotropin release, disruption of the hypothalamic-pituitary-gonadal axis and suppression of ovarian activity until removal of the progestin.

In cats, the progestin implant levonorgestrel (Norplant®) has been investigated for its ability to improve response to exogenous gonadotropins before IVF (Pelican et al., 2002). Following removal of levonorgestrel, cats display increased embryo yield with no

negative effect on oocyte quality or *in vitro* fertilization success, when compared to untreated females. Similar observations have been seen in humans, where oral contraceptives are used before ovarian stimulation and IVF to prevent spontaneous LH surges with no observed deleterious effects on subsequent ovarian response (Gonen et al., 1990; Burry et al., 1991). Studies using levonorgestrel in clouded leopards and fishing cats confirm the efficacy of progestin implants for improving consistency of ovarian response to exogenous gonadotropins, but AI attempts with this regimen have not resulted in pregnancies (Pelican and Howard, 2003; Bauer et al., 2004). The two potentially-stressful surgical events required for insertion and removal of the implant may be one cause for reduced fertility following AI using this approach.

*Altrenogest.* The oral progestin altrenogest (ALT; Regu-Mate®) has been used in horses (Webel and Squires, 1982; Lofstedt and Patel, 1989), livestock (Kraeling et al., 1981; Wood et al., 1992), and marine mammals (Robeck et al., 2004; Robeck et al., 2005) to temporarily suppress ovarian activity and synchronize return to follicular activity. Additionally, ALT can provide pregnancy support in mares (Hinrichs et al., 1999) and bitches (Root Kustritz, 2001). In horses, ALT typically is administered for 14-15 days, which coincides with the mean duration of the equine luteal phase (Lofstedt and Patel, 1989; Bollwein et al., 2004). This strategy is employed because any functional CL already present on the ovary should regress within 2 weeks, thereby ensuring that the animal is fully down-regulated by the time of ALT removal. After ALT treatment concludes in the horse, ovulation typically is observed within 9-11 days (Lofstedt and Patel, 1989). Studies on long-term use of ALT in mares have detected no changes in body composition or behavior (Hodgson et al., 2005). In pigs, the drug is used in a manner similar to horses to synchronize follicular phase in sows and gilts with no deleterious effects on subsequent ovarian function (Diehl et al., 1986; Guthrie et al.,

1997; Estienne et al., 2001). ALT also has been effective for providing estrous cycle synchronization in killer whales and dolphins before trans-cervical AI, resulting in the birth of live calves in both species (Robeck et al., 2004; Robeck et al., 2005). Indeed, the extensive use of ALT in a variety of mammal species, including the dog, lends support to its study in cats for estrous cycle control.

### **Assessing Reproductive Potential Following Assisted Reproduction**

*Ovarian morphology and function.* Ovarian morphology is an important indicator of reproductive potential following assisted reproduction. For example, presence of fresh CL (e.g. CH) near the time of insemination is a prerequisite to pregnancy. Conversely, older CL at the time of AI almost certainly hinders establishment of pregnancy because of the significant disruption to the endocrine milieu. What is not as well understood is how the proportion of CL versus follicles at the time of insemination influences pregnancy success in felids. Furthermore, the influence of exogenous gonadotropins used in assisted reproduction on ovarian ultrastructure has yet to be correlated with subsequent fertility. Changes in ovarian histology throughout early pregnancy have been characterized in the naturally-bred cat, serving as a useful database of information for future comparative analyses (Roth et al., 1995).

Ovarian function following assisted reproduction can be assessed indirectly by monitoring fecal steroid hormone fluctuations in felids. It also can be evaluated more directly by measuring progesterone levels in CL tissue following ovariectomy. Queens with poor fertility have consistently higher CL progesterone levels following ovulation compared to high fertility females; however, these differences are not seen after Day 4 of gestation (Swanson et al., 1995b). Furthermore, gonadotropin treatment has not been associated with changes in CL progesterone production in the cat (Roth et

al., 1997b). In contrast, ovarian stimulation is correlated with abnormal luteal function in sheep. Specifically, lower CL weight, decreased progesterone content and reduced ability of the CL to secrete progesterone *in vitro* are observed (McNeilly et al., 1981). Pre-treating sheep with progestins before ovarian stimulation appears to mitigate these luteal deficiencies (Hunter et al., 1986). LH receptor expression also has been measured in the cat CL across early pregnancy, but does not appear to be correlated with fertility (Swanson et al., 1995b). Characterizing feline ovarian steroid receptor expression during early pregnancy, which has been done in multiple species including the baboon (Hild-Petito and Fazleabas, 1997), llama (Powell et al., 2007) and cow (Berisha et al., 2002), may also serve as an interesting indicator of pregnancy potential.

*Oviductal morphology and function.* Ultrastructure of feline oviductal tissue is not well characterized in the cat. In the rat, changes in the proportion of secretory versus ciliated cells lining the ampullae are observed across the estrous cycle (Shirley and Reeder, 1996). Similar cyclic patterns of cell expression are observed in the dog (Steinhauer et al., 2004), goat (Abe et al., 1999), sheep (Murray, 1996) and rabbit (Anzaldúa et al., 2002). These changes in cell proportions appear to be regulated by alterations in ovarian steroid levels (Bareither and Verhage, 1981). Oviductal histology also has been studied in the rabbit following hCG treatment, indicating that changes in non-ciliated secretory cell expression occur following gonadotropin treatment (Bondi et al., 1997).

Techniques for assessing oviductal function have been developed in multiple mammal species. One factor of particular interest is the expression of steroid receptors during early pregnancy. While estrogen receptor (ER) and progesterone receptor (PR) expression has not been characterized in the cat oviduct, it has been assessed across the estrous cycle and during pregnancy in human and non-human primates (Brenner

and Slayden, 1994), the rat (Okada et al., 2003), cow (Ulbrich et al., 2003) and sheep (Garcia-Palencia et al., 2007). In these species, changes in ER and PR expression patterns are correlated with alterations in oviductal function (Jansen, 1984) and regulation of egg transport (Fuentelba et al., 1988a). Furthermore, in sheep, treatment with exogenous progestins leads to a reduction in ER alpha and PR expression in oviductal and uterine cells (Garcia-Palencia et al., 2007). Exogenous estradiol also appears to differentially influence ER and PR expression patterns in sheep, leading to an initial reduction in receptor concentrations, subsequently followed by receptor up-regulation (Rodriguez-Pinon et al., 2005).

*Uterine morphology and function.* Uterine histology can serve as an important indicator of fertility in multiple species (Psychoyos and Martel, 1985). However, there are no strong correlations between pregnancy success and histological characteristics in naturally-bred cats. Females with significant abnormal uterine pathology are capable of normal ovarian function and can produce high-quality embryos (Roth et al., 1995). Exogenous hormone treatment (gonadotropins or steroid hormones) influences uterine and endometrial histological characteristics in a variety of species including the rat (Stein and Kramer, 1989), human (Kolb et al., 1997), dog (Dhaliwal et al., 1999) and rabbit (McCarthy et al., 1977). In rats, for example, exogenous hormones alter cell types throughout the endometrium, including the surface epithelium, glandular epithelium and underlying stromal cells. These alterations have been linked to implantation failure and fetal loss (Stein and Kramer, 1989).

Several markers of uterine function have been studied in the cat. Uterine ER and PR expression has been characterized following steroid treatment and across pregnancy (Li et al., 1992a). Early embryonic development in naturally-bred cats (presumably concurrent with the shift to progesterone dominance) results in down-regulation of ER



and PR in the uterus without alterations in receptor distribution (Li et al., 1992a). This is similar to observations in the dog, where high serum estradiol leads to an up-regulation of both receptor types, and high serum progesterone leads to down-regulation (Vermeirsch et al., 1999; Galabova-Kovacs et al., 2004). Thus, changes in the hormonal milieu brought about by exogenous hormone therapy could, in theory, result in altered expression of ER and PR not conducive to establishment of pregnancy. Administering exogenous estrogens and progestins does appear to influence receptor expression in cat uterine tissue, but the specific regulatory mechanisms remain to be tested (Li et al., 1992a).

Cytokines, growth factors and other proteins have been implicated in uterine function and the implantation process. In cats, these proteins include a progesterone-dependent protein (PDP) that is high in pregnant cats only until Day 16 of gestation (Boomsma and Verhage, 1987) but remains elevated in pseudopregnant females for up to 5 weeks post-coitus (Boomsma et al., 1991). Further characterization of this PDP in cat uterine flushings (Li et al., 1991) and the pregnant cat uterus (Li et al., 1992b) determined that the protein is cathepsin L, which also has been implicated in mouse implantation (Reese et al., 2001). A feline estrogen-dependent protein (CUPED) is produced in response to estradiol fluctuations (Murray et al., 1986), and a similar protein has been found in the uterine flushings of the ferret, dog and baboon (Scalzo et al., 1990). Other proteins expressed in the cat uterus include transforming growth factor alpha, epidermal growth factor (EGF), EGF receptor and insulin-like growth factor binding protein-1 (Boomsma et al., 1994; Boomsma et al., 1997). Finally, one growth factor yet to be characterized in the cat uterus but of particular interest in the implantation process is vascular endothelial growth factor (VEGF) (Das et al., 1997). VEGF induces vasculogenesis and angiogenesis, both key requirements for successful placentation and embryo survival. In primates, endometrial VEGF is regulated by ER

and PR ligands (Greb et al., 1997). VEGF also is an important regulator of angiogenesis in the ovary (Kaczmarek et al., 2005).

Clearly, although several implantation-associated proteins have been identified in the cat, there is a need to characterize gene expression more comprehensively to understand how these various factors act alone or in concert to influence the peri-implantation environment. Overall, determining the presence or absence of these factors during critical time points in the implantation process could serve as useful markers for pregnancy and placental health (Paria et al., 2001). Techniques for characterizing factors produced by the feline conceptus itself are under development (Thatcher et al., 1991). Microarray analyses have been used to comprehensively characterize uterine gene expression across the estrous cycle and during pregnancy in the mouse (Reese et al., 2001; Bethin et al., 2003; Tan et al., 2003) and human (Kao et al., 2002; Bethin et al., 2003). Microarray technologies now are being developed using domestic cat reproductive tissues, and ultimately may serve to better identify specific molecular markers of uterine receptivity and embryo implantation for which further analyses can be conducted in the future.

### **Summary of Objectives**

The primary objective of this dissertation research was to understand how oral progestin priming before exogenous gonadotropin administration and AI influences endocrine dynamics, ovarian responsiveness, fertilization success, early embryonic development and peri-implantation morphology and function in the cat. The studies were designed to assess whether oral progestin priming can mitigate adverse fertility effects known to occur in cats following exposure to exogenous gonadotropins. Specific objectives were to: (1) assess the influence of different ALT dosages on endocrine

function and ovarian suppression; (2) evaluate the influence of progestin priming on ovarian sensitivity to exogenous gonadotropins; and (3) compare fertilization rates, early embryonic development and ovarian morphology and function in progestin-primed versus unprimed queens following AI. Overall, these studies provide a foundation for developing refined hormone regimens that may be used to enhance AI efficiency in a host of wild cats, including endangered species. There also are potential applications in contraception research for both feral cats and zoo populations. Finally, because the domestic cat is an emerging model for infertility research, these data may be useful for studying the influence of exogenous hormones on reproductive function in humans.

## CHAPTER 2

### ORAL PROGESTIN INDUCES RAPID, REVERSIBLE SUPPRESSION OF OVARIAN ACTIVITY IN THE CAT

#### Abstract

The influence of oral progestin (altrenogest; ALT) on ovarian activity was characterized in the domestic cat using non-invasive fecal steroid analyses. Queens were assigned to one of four treatments administered for 38 consecutive days: (1) 0 mg/kg ALT (control;  $n = 5$  cats); (2) 0.044 mg/kg (LOW;  $n = 5$ ); (3) 0.088 mg/kg (MID;  $n = 6$ ); and (4) 0.352 mg/kg (HIGH;  $n = 6$ ). Fecal estrogens and progestins were quantified for 60 days before, 38 days during and 60 days after ALT treatment. The initiation of follicular activity was suppressed in all cats receiving ALT, whereas ovarian activity was not suppressed in the control group. Females ( $n = 11$ ) exhibiting baseline fecal estrogens at ALT initiation remained at baseline, whereas females ( $n = 6$ ) with elevated fecal estrogens completed a normal estrogen surge before returning to baseline by Day 6 of treatment and remaining suppressed. All cats receiving ALT entered a follicular phase following withdrawal of the drug; however, MID cats displayed a more synchronized ( $P < 0.05$ ) return to follicular activity compared to HIGH cats. LOW queens displayed a return to activity that was similar ( $P > 0.05$ ) to both MID and HIGH cats. Females ( $n = 2$ ) that did not demonstrate follicular activity before ALT exhibited at least two estrogen surges in the 60 days following treatment. Mean baseline fecal estrogens and progestins were higher ( $P < 0.05$ ) after ALT in HIGH but not LOW or MID cats when compared to pre-treatment values. Before treatment with ALT, fecal progestin profiles revealed a lower incidence of spontaneous ovulation (2 of 13; 15.4%) in the study

population, compared to previous studies in laboratory-housed queens (~ 50%). Results demonstrate that: (1) ALT therapy induces rapid suppression of ovarian activity in the cat; (2) ALT does not influence the characteristics of a follicular phase existing at treatment initiation; and (3) ovarian suppression is reversible upon cessation of ALT therapy. This study provides the foundation for future research aimed at using progestin priming to improve exogenous gonadotropin regimens for assisted reproduction in felids.

## **Introduction**

Endangered felids have benefited from the development of assisted reproduction technologies designed to increase capacity for genetic management in *ex situ* populations (Wildt and Roth, 1997). These techniques, which include artificial insemination (AI) and *in vitro* fertilization (IVF) followed by embryo transfer (ET), have emerged as tools for increasing reproductive efficiency in wild felid species (Howard, 1999; Swanson, 2003). A laparoscopic intrauterine AI technique has been successful for producing live offspring in the cheetah (Howard et al., 1992b), clouded leopard (Howard et al., 1996), tiger (Donoghue et al., 1993), puma (Barone et al., 1994b), leopard cat (Howard, 1991), snow leopard (Roth et al., 1997a), tigrina (Swanson and Brown, 2004) and ocelot (Swanson et al., 1996b). While AI efficiency is ~ 50% in the cheetah using fresh sperm (Howard et al., 1997), success remains low (< 5%) in species including the clouded leopard (Howard et al., 1997) and tiger (Graham et al., 2006). Attempts have not been successful to date in the fishing cat (Bauer et al., 2004) or Pallas' cat (Brown et al., 2002).

The etiology of pregnancy failure following assisted reproduction is poorly understood; however, some contributing factors have been documented. It is well established that the exogenous gonadotropins used to stimulate the ovary before AI can

perturb the maternal environment in the cat (Swanson et al., 1996a; Graham et al., 2000). Most gonadotropin regimens in felids involve the administration of species-specific dosages of equine chorionic gonadotropin (eCG) to initiate folliculogenesis followed by human chorionic gonadotropin (hCG) to induce ovulation. However, studies have demonstrated that eCG and hCG share dual roles in the cat ovary, promoting both folliculogenesis and ovulation (Wildt et al., 1978; Goodrowe and Wildt, 1987; Swanson et al., 1997). Thus, when eCG and hCG are administered in combination, ancillary ovarian structures and abnormal estrogen elevations can result (Brown et al., 1995; Swanson et al., 1996a; Roth et al., 1997b; Graham et al., 2000). These physiological disruptions parallel similar observations in the cow (Alcivar et al., 1992), sheep (Gonzalez-Bulnes et al., 2003) and mouse (Fossum et al., 1989) following ovarian stimulation.

The unpredictable feline estrous cycle makes timing assisted reproduction a significant challenge. Historically considered to be induced ovulators (Wildt et al., 1980), investigations have determined that both laboratory-housed domestic cats and a wide range of wild felid species, including the fishing cat and clouded leopard, exhibit spontaneous ovulation with no discernable pattern or consistency (Lawler et al., 1993; Gudermuth et al., 1997; Graham et al., 2000; Brown et al., 2001; Moreira et al., 2001; Moreland et al., 2002; Pelican et al., 2005; Brown, 2006). Spontaneous ovulation and the existence of mature corpora lutea (CL) at the time of ovulation induction generally lead to unpredictable and often poor ovarian responses to exogenous gonadotropins (Pelican et al., 2006b). Heightened follicular activity and the presence of preovulatory follicles at the time of exogenous gonadotropin administration can have similar implications.

A quiescent ovary at the time of ovulation induction is advantageous, increasing the likelihood of a consistent and uniform ovarian response and diminishing the risk of an altered endocrine milieu during the critical peri-implantation period. This is evident in

the cheetah, an induced ovulator that also is known to display periods of anestrus. Cheetahs demonstrate a ~ 50% success rate following exogenous gonadotropin therapy and AI (Howard et al., 1992b; Howard et al., 1997). In the ocelot, also an induced ovulator, incidence of pregnancy following AI is ~ 25% (Swanson et al., 1996b). For species exhibiting spontaneous ovulation, such as the clouded leopard and fishing cat, AI success is significantly compromised (Pelican et al., 2006b).

Inconsistent ovarian response can be mitigated through temporary ovarian suppression before ovulation induction. This approach synchronizes early follicle cohorts that are subsequently highly susceptible to ovarian stimulation with exogenous gonadotropins (McGee and Hsueh, 2000). Short-term ovarian suppression also has been linked to decreased incidence of ovarian hyperstimulation (Kol, 2004). Temporary ovarian inhibition has been used successfully in humans (Albano et al., 1999; Barbieri and Hornstein, 1999), domestic livestock (Lofstedt, 1988; Wood et al., 1992; Deligiannis et al., 2005) and wild ungulates (Monfort et al., 1993; Morrow et al., 2000) to synchronize follicular activity and improve ovarian response for AI.

Previous investigations confirmed the efficacy of a progestin implant (levonorgestrel, Norplant®) for short-term suppression of follicular activity and spontaneous ovulation in the domestic cat (Pelican et al., 2005). However, return to follicular activity following levonorgestrel implant removal is highly variable in the cat, ranging from ~ 2 weeks to greater than 2 months. Additionally, use of levonorgestrel implants requires two anesthesia events (for insertion and removal), which is an impractical approach for applications in wild felids. The oral progestin altrenogest (ALT; Regu-Mate®) has been used in dogs (Root Kustritz, 2001) to maintain pregnancy, and in horses (Lofstedt and Patel, 1989), pigs (Wood et al., 1992), killer whales (Robeck et al., 2004) and bottlenose dolphins (Robeck et al., 2005) to synchronize follicular activity before assisted reproduction or natural breeding. This study evaluated the efficacy of

three dosages of ALT for short-term inhibition of ovarian activity in the domestic cat using non-invasive fecal steroid monitoring. The hypothesis was that oral progestin would provide rapid, reversible inhibition of ovarian activity in a dose-dependent manner, with a consistent return to cyclicity following removal.

## **Materials and Methods**

### ***Animals***

Thirteen adult (1-5 year old) female domestic cats were housed at the Smithsonian's National Zoological Park's Conservation and Research Center (CRC). Queens were maintained individually in stainless steel cages (0.5 m<sup>3</sup>) under artificial fluorescent illumination (12L:12D) during the ~ 14 month study. All cats were provided a dry commercial diet (Purina ONE®, Nestlé Purina PetCare Co., St. Louis, MO) and had continual access to water, toys, perches and bedding. Cats were housed in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996), and all research activities were approved by the CRC's Institutional Animal Care and Use Committee (IACUC; # 05-25) and the University of Maryland IACUC (R-06-06).

### ***Altrenogest administration***

Duration of ovarian cycle inhibition was based on the domestic cat luteal phase length (36-38 d) (Brown et al., 1994; Pelican et al., 2005). This strategy was employed to ensure lysis of functional corpora lutea (if present) and promote complete ovarian quiescence by the time of treatment removal. The study was conducted in two time periods (Trial 1 and Trial 2) separated by a 4 month interval where resumption of



follicular activity was confirmed by fecal hormone analyses. Each cat was randomly assigned to two different treatments, and treatment combinations were assigned to balance potential carry-over effects between Trials 1 and 2.

ALT oral suspension (2.2 mg/ml; Intervet Inc., Millsboro, DE) was stored at room temperature in an opaque container. In a preliminary study, the colorless, odorless suspension was determined to be palatable to domestic cats. During each trial, ALT was administered daily in 5 g wet food (Friskies®; Nestlé Purina PetCare Co.) for 38 consecutive days. In Trial 1, cats were assigned to one of four daily ALT treatments: (1) 0 mg/kg ( $n = 3$  cats); (2) 0.088 mg/kg ( $n = 3$ ); (3) 0.176 mg/kg ( $n = 3$ ); and (4) 0.352 mg/kg ( $n = 4$ ). Based on results from Trial 1, the 0.176 mg/kg treatment was removed and replaced with a lower dosage (0.044 mg/kg). In Trial 2, cats were assigned to one of four daily ALT treatments: (1) 0 mg/kg ( $n = 2$ ); (2) 0.044 mg/kg ( $n = 5$ ); (3) 0.088 mg/kg ( $n = 3$ ); and (4) 0.352 mg/kg ( $n = 2$ ). For both trials, each cat was weighed (range, 2.3 to 4.9 kg) the day before treatment initiation to calculate dosages (range, 0.05 to 0.78 ml ALT daily).

### ***Fecal hormone extraction***

Over the course of the study, fecal samples were collected daily (if present), sealed in plastic bags labeled with the individual's name and the date, and stored at -20°C. Fecal samples from 60 days before, 38 days during and 60 days after each ALT treatment period were extracted to isolate and concentrate estrogens and progestins using a validated protocol for domestic cats (Brown et al., 1994). Briefly, individual fecal samples were lyophilized, pulverized and 0.18 to 0.2 g of dry fecal powder was boiled in 5 ml of 90% ethanol for 20 minutes. During the boiling process, 100% ethanol was added as needed to maintain approximate pre-boil volumes. After centrifugation (500g,

20 min), the supernatant was recovered and the pellet resuspended in 5 ml of 90% ethanol, vortexed for 30 seconds and re-centrifuged at 500g for 15 minutes. The first and second supernatants were combined, dried under air, and reconstituted in 1 ml methanol. Methanol extracts were briefly vortexed and placed in a sonicator for 15 minutes to free particles adhering to the glass tube. Each extract was diluted 1:10 in steroid dilution buffer (0.2 M NaH<sub>2</sub>PO<sub>4</sub>, 0.2 M Na<sub>2</sub>HPO<sub>4</sub>, 0.15 M NaCl; pH 7.0) and stored in polypropylene tubes at -20°C until enzyme immunoassay (EIA) analyses.

### ***Estrone sulfate EIA***

A single antibody estrone sulfate (E1S) EIA was used to quantify estrogen metabolites in all fecal extracts (Stabenfeldt et al., 1991). This assay cross-reacts with a broad range of estrogen metabolites previously identified in domestic cat feces by high performance liquid chromatography (Brown et al., 1994). Specifically, the assay employed a polyclonal antibody (R583; 1:1,500; C. Munro, University of California, Davis, CA) produced against estrone-3-glucuronide dissolved in coating buffer (0.015 M Na<sub>2</sub>CO<sub>3</sub>, 0.035 M NaHCO<sub>3</sub>, pH 9.6), added to 96-well, flat-bottom microtiter plates (Nunc-Immuno, Fisher Scientific Inc., Pittsburgh, PA) and incubated overnight at 4°C. Plates were washed (0.05% Tween 20 in 0.15 M NaCl solution) to remove un-adsorbed antibody and 0.025 ml steroid assay buffer (0.2 M NaH<sub>2</sub>PO<sub>4</sub>, 0.2 M Na<sub>2</sub>HPO<sub>4</sub>, 0.15 M NaCl, 2.0 g/L BSA, pH 7.0) was added to each well and maintained at room temperature for 2 to 5 hours. Next, 0.05 ml diluted sample (range, 1:100 to 1:500) or E<sub>1</sub>SO<sub>4</sub> standard (range, 1.95-500 pg; Sigma-Aldrich Chemical Co., St. Louis, MO) was added to wells in duplicate immediately followed by 0.05 ml estrone sulfate horseradish peroxidase (1:20,000; C. Munro). Following a 2 hour incubation at room temperature, plates were washed and 0.1 ml substrate (0.04 M ABTS, 0.5 M H<sub>2</sub>O<sub>2</sub> in a 0.05 M citric acid solution) was added to each well. Optical densities (OD) were read using a microplate reader

(MRX, Dynex Technologies, Chantilly, VA) at 405nm when 0 pg standard wells reached an OD of 0.9 to 1. Serial dilutions of domestic cat feces yielded a displacement curve that was parallel to the standard curve ( $R^2 = 0.99$ ). Recovery of known amounts of  $E_1SO_4$  standard added to a pool of domestic cat fecal extracts (1:400) was  $70.4\% \pm 4.1\%$  ( $y = 0.82x - 3.3$ ;  $R^2 = 0.99$ ). Intra-assay variation was  $< 10\%$  and inter-assay variation was 10.3% and 13.4% at 30% and 70% binding, respectively ( $n = 124$  plates). Sensitivity for this assay was 2 pg/well.

### ***Pregnane EIA***

A single antibody pregnane (Pg) EIA was utilized to quantify progesterone metabolites in every other fecal extract (Schwarzenberger et al., 1991; Graham et al., 2001). This assay cross-reacts with a broad range of progesterone metabolites previously identified in domestic cat feces by high performance liquid chromatography (Brown et al., 1994). The procedures and assay reagents were the same as described previously for the E1S assay unless otherwise noted. The EIA relied upon a monoclonal antibody (CL425; 1:10,000; C. Munro) in coating buffer that was added to 96-well, flat-bottom microtiter plates (Nunc-Immuno) and incubated overnight. Plates were washed and 0.05 ml diluted sample (range, 1:2000 to 1:6000) or progesterone standard (range, 0.78 to 200 pg; Sigma-Aldrich) was added to wells in duplicate immediately followed by 0.05 ml enzyme conjugate (progesterone-3CMO horseradish peroxidase; 1:40,000; C. Munro). Following a 2 hour incubation, plates were washed, 0.1 ml substrate was added to each well and OD were read. Serial dilutions of domestic cat feces yielded a displacement curve that was parallel to the standard curve ( $R^2 = 0.99$ ). Recovery of known amounts of progesterone standard added to a pool of domestic cat fecal extracts (1:1600) was  $65.9\% \pm 11.9\%$  ( $y = 1.03x - 5.4$ ;  $R^2 = 0.99$ ). Intra-assay variation was  $<$

10% and inter-assay variation was 12.8% and 16.8% at 30% and 70% binding, respectively ( $n = 68$  plates). Sensitivity for this assay was 1 pg/well.

### ***Statistical analyses***

For each individual, baseline fecal estrogen concentrations were determined using an iterative process in which all values exceeding the mean plus two standard deviations (SD) were removed from the data set. The average was then recalculated and the elimination process repeated until no values exceeded the mean plus two SD (Brown et al., 1994; Pelican et al., 2005). The final mean obtained through this process was considered the baseline mean for that animal, and all values removed from the data set during the iterative process were classified as elevated. Duration of the follicular phase was defined as the number of consecutive days where estrogens were elevated (minimum 3 days), and the highest fecal estrogen value within an array of elevations was the peak for that follicular phase. Estrous cycle length was calculated as the number of days between fecal estrogen peaks with no subsequent elevation in fecal progestins. Baseline progestin concentrations were determined using a similar iterative process, except the mean plus 1.5 SD was used. Values greater than twice the progestin baseline were considered elevated for that individual. 1.5 SD was chosen for progestins because 2 SD was too sensitive to differentiate baseline from elevations. A luteal phase was defined when progestin levels rose above baseline and remained elevated for at least 3 consecutive weeks. Luteal phase length was the total number of days progestins remained above baseline. Following treatment, return to follicular activity was calculated as the number of days from ALT removal until the first day fecal estrogens were above baseline for that individual.

Data from Trials 1 and 2 were combined for data analyses to compare: (1) 0 mg/kg ALT daily ( $n = 5$  cats, control); (2) 0.044 mg/kg ( $n = 5$ , LOW); (3) 0.088 mg/kg ( $n =$

6, MID); and (4) 0.352 mg/kg ( $n = 6$ , HIGH). Treatment differences were evaluated for the 60 days before (PRE), 38 days during and 60 days after (POST) ALT treatment. Estrous cycle traits (duration of follicular phase, mean estrogens/follicular phase, peak estrogens/follicular phase, estrous cycle length, baseline and mean estrogens, baseline and mean progestins) were calculated for each individual and then averaged within each treatment. To normalize the iterative process, only the 38 days before, during and after ALT treatment were used when determining baseline fecal steroid concentrations. Data within treatment across time were evaluated using a mixed model repeated measures ANOVA followed by least significant difference (LSD) mean comparisons. A toeplitz variance-covariance structure was chosen for repeated measure analyses based on: (1) low number of parameters; and (2) acceptable fit for the residuals. Data between treatments within a single time interval were analyzed using a mixed model one-way ANOVA followed by LSD mean comparisons. When necessary, data were corrected for non-normal distribution before ANOVA using log transformations (Sokal and Rohlf, 1994). Differences in the range of return to follicular activity among treatments were compared using a F-test for variance in Excel 2003 (Microsoft Corporation, Redmond, WA). All other analyses were performed using SAS 9.1.3 (SAS Institute Inc., Cary, NC). Data are presented as mean  $\pm$  SEM.

## **Results**

### ***Estrous cycle characteristics before treatment***

Estrous cycle characteristics for the 60 days before treatment showed that the duration of the follicular phase ranged from 3-17 days (Table 2.1). The interval between consecutive estrogen peaks (estrous cycle length) varied extensively (range, 7 to 43 days). Frequency of the follicular phase, estrous cycle length, mean estrogens/ follicular

phase, peak estrogens/ follicular phase and baseline progestins did not differ ( $P > 0.05$ ) among treatment groups before ALT or placebo administration. However, baseline estrogen metabolites were higher in LOW ( $P < 0.05$ ) compared to HIGH cats, and similar ( $P > 0.05$ ) to MID cats during the pre-treatment period. Additionally, follicular phase duration was longer ( $P < 0.05$ ) in LOW and HIGH compared to control cats before ALT treatment. Incidence of spontaneous ovulation before and after treatment was 15.4% (two of 13 cats). Luteal phase length could not be characterized because in all cases elevated progestins extended beyond the time period subject to fecal hormone analyses.

### ***Influence of altrenogest dosage on estrous cycle characteristics***

A comparison of fecal estrogen profiles during ALT or placebo treatment demonstrated that no female receiving ALT initiated follicular activity during treatment, whereas all control cats exhibited follicular activity at least two times during placebo administration (Fig. 2.1). Consequently, number of estrogen peaks was similar ( $P > 0.05$ ) in control cats across time periods, but lower ( $P < 0.05$ ) during ALT treatment at all dosages assessed (Fig. 2.2). Additionally, HIGH cats displayed fewer ( $P < 0.05$ ) estrogen peaks before ( $1.5 \pm 0.4$  peaks/60 days) versus after ( $2.7 \pm 0.2$  peaks/60 days) treatment. Baseline estrogens were elevated ( $P < 0.05$ ) in HIGH cats after ALT ( $195.7 \pm 15.4$  ng/g dry feces) compared to before treatment ( $164.1 \pm 6.9$  ng/g) (Fig. 2.3A). Baseline progestins also were elevated ( $P < 0.05$ ) in HIGH cats after ALT ( $3.2 \pm 0.3$   $\mu$ g/g dry feces) compared to before treatment ( $2.6 \pm 0.2$   $\mu$ g/g) (Fig. 2.3B). In contrast, baseline estrogens and progestins were similar ( $P > 0.05$ ) across time in LOW and MID cats. An increase ( $P < 0.05$ ) in baseline estrogens was observed in control cats during placebo treatment (Fig. 2.3A).

Two females did not display follicular activity before ALT treatment; however, both exhibited at least two follicular phases in the 60 days following treatment (Fig.

2.4A). Females ( $n = 11$ ) demonstrating follicular activity before ALT treatment but exhibiting baseline estrogen levels at ALT initiation remained at baseline (Fig. 2.4B), whereas females exhibiting follicular activity on Day 1 of treatment ( $n = 6$ ) returned to baseline by Day 6 and remained inhibited (Fig. 2.4C). In the six females displaying follicular activity at treatment initiation, duration of the follicular phase ( $7.7 \pm 2.0$  days), mean fecal estrogens ( $315.0 \pm 21.9$  ng/g feces) and peak fecal estrogens ( $395.2 \pm 47.2$  ng/g feces) were similar ( $P > 0.05$ ) to estrous cycle characteristics preceding treatment initiation. Following ALT treatment, all cats demonstrated a return to follicular activity in the LOW ( $8.2 \pm 1.8$  d post-ALT withdrawal), MID ( $12.5 \pm 0.9$  d) and HIGH ( $16.0 \pm 4.0$  d) treatments (Fig. 2.5). However, a more synchronized ( $P < 0.05$ ) return to follicular activity was observed in MID (range, 10 to 16 d) compared to HIGH (range, 9 to 35 d) cats, with intermediate variation ( $P > 0.05$ ) in the LOW cats (range, 2 to 12 d; Fig.2.6).

## Discussion

Exogenous progestin therapy has proven effective for contraception and inhibition of follicular activity in felids previously (Baldwin et al., 1994; Looper et al., 2001; Pelican et al., 2005). To our knowledge this is the first study to evaluate the effect of varied oral progestin dosages on ovarian cycle characteristics in the cat using non-invasive fecal hormone analyses. These results demonstrate the efficacy of oral progestin for short-term, reversible inhibition of ovarian activity with no observed side effects. Consistent with previous studies using progestin implants for ovarian suppression, oral progestin did not alter follicular activity already in progress. The use of fecal samples proved advantageous over blood sampling for assessing differences in the dose-response relationship among treatments, eliminating stressful collections that may have disrupted reproductive function and confounded results (Graham and Brown,

1996). Furthermore, fecal hormone monitoring revealed a lower incidence of spontaneous ovulation (15.4%) in this laboratory-housed domestic cat population compared to historical data (~ 50%) (Pelican et al., 2005).

Given the large body of literature available on the use of progestins for ovarian cycle control, we correctly hypothesized that oral ALT would inhibit follicular activity in the domestic cat. The mechanism for this inhibition is consistent with previous research on progestin implants in the cat (Pelican et al., 2005), where oral progestin prevented initiation of follicular activity, but had no influence on follicular activity that was in progress at the time of treatment onset. These results indicate that ALT acts only to prevent initial recruitment of small antral follicles but is unable to override selection and dominance of mature follicles when that process is already underway. Because follicular recruitment is primarily under the influence of high FSH and low LH, whereas follicular selection is regulated by low FSH and increasing LH, it is possible that ALT is acting directly at the level of the pituitary and/or ovary to differentially influence FSH and LH release and/or follicular dynamics. However, previous studies in the mare indicate that ALT has little or no effect on LH production (Squires et al., 1983). Potential actions on inhibin and/or activin also must be considered. Additionally, oral progestin appears to provide a priming effect on the ovary of anestrus cats, as indicated by the return to consistent follicular activity following treatment in previously acyclic females. This could lead to interesting applications in wild felids that demonstrate ovarian 'shut down' for unknown reasons, where a return to estrous cyclicity is required to facilitate natural breeding.

We hypothesized that ovarian suppression with ALT would increase in a dose-dependent manner, yet results indicated abolishment of follicular activity at even the lowest dosage assessed. It is probable that the threshold dosage of ALT required for ovarian suppression in the cat falls below the range of dosages assessed. This



threshold is not believed to be size-dependent, since a lower ALT dosage is required in the horse (0.044 mg/kg) versus the dog (0.088 mg/kg) (Lofstedt and Patel, 1989; Root Kustritz, 2001). Fecal hormone profiles in the cats administered the lowest dosage did appear to demonstrate some indications of breakthrough follicular activity; however, this could not be statistically differentiated from baseline values. These results indicate that a dosage of 0.044 mg/kg is likely close to the minimum dosage necessary to suppress follicular activity in the cat. The highest dosage assessed, while effective, did result in an increase in baseline estrogens and progestins following removal of the drug. This shift in baseline estrogens is consistent with similar observations in the cat following treatment with the progestin implant levonorgestrel (Pelican et al., 2005). While the implications for this baseline shift remain unknown, it could possibly influence implantation success if used in conjunction with assisted reproduction. Thus, the currently-utilized dosage for dogs (0.088 mg/kg) was the optimal dosage in domestic cats, with no indications of breakthrough cycling or alterations in baseline hormone levels following treatment.

This study demonstrated two associations between ALT dosage and return to follicular activity following removal of the drug. First, a positive relationship between dosage and mean duration of suppression was observed, where the interval from end of treatment to the follicular phase numerically increased as dosage increased. A similar correlation has been observed in pigs (Kraeling et al., 1981). While the pharmacokinetics of ALT have not been examined in the cat, this finding could be explained by dose-dependent differences in drug persistence in circulation. Second, we hypothesized that oral progestin generally would provide a more consistent return to cyclicity when compared to implant formulations. Instead, results indicated that dosage played a large role in determining the variability of estrous cycle return. For example, the high dosage yielded far greater variability in return to cyclicity (9-35 days) than the

mid-range dosage (10-16 days). Synchronization with ALT proved superior to levonorgestrel in the cat, particularly when comparing return to follicular activity in the mid-range ALT dosage (10-16 days) to six levonorgestrel implants (11-79 days) (Pelican et al., 2005). The use of ALT in cats supports similar observations in livestock species, where return to follicular activity can be timed within days following ALT cessation. This is evident in the horse, where ovulation typically is observed nine to 11 days after stopping ALT (Lofstedt and Patel, 1989).

Through daily fecal collection, a more precise assessment of the timing of ALT effects on ovarian activity was possible. In a laboratory-housed population of animals, daily blood sampling is feasible, however invasive procedures are known to perturb reproductive function (Graham and Brown, 1996). In addition to reducing stress, fecal hormone analyses provide a pooled steroid value excreted over several hours, rather than a peak or nadir that may not be representative of the overall physiological response (Brown et al., 2001). Fecal steroid hormone monitoring has previously been employed in cats to assess chorionic gonadotropin dosages (Brown et al., 1995; Graham et al., 2000), and to evaluate the efficacy of oral melatonin administration for prevention of ovarian hyperstimulation (Graham et al., 2004). This study further supports this application, indicating that fecal hormone analysis alone can provide adequate data to make informed decisions on optimal dosage of an exogenous hormone.

This population of domestic cats had a low prevalence of spontaneous ovulation (15.4%) compared to laboratory-housed queens assessed in earlier studies. Spontaneous ovulation rates ranging from 35-87% have been reported (Lawler et al., 1993; Gudermuth et al., 1997; Pelican et al., 2005). Domestic cats previously housed in the CRC colony have demonstrated a spontaneous ovulation rate of 56.3% (Pelican et al., 2005). Within that population, four cats were unpaired and two (50%) exhibited spontaneous ovulation. Incidence of spontaneous ovulation has been attributed, in part,

to stressful events, interactions with cage-mates and visual and olfactory cues from adjacent males (Concannon, 1991; Gudermuth et al., 1997). Cats in the current study were housed individually with olfactory and auditory contact with males. It also has been hypothesized that increased age may trigger ovulation (Lawler et al., 1993). The current study supports this finding, since both spontaneous ovulators were in the oldest age class assessed (6 years of age), however this should be considered conservatively given the low number of cats assessed. The low incidence of spontaneous ovulation does not appear to be attributed to previous reproductive history in this study, since one spontaneous ovulator had previously produced offspring and one had not.

The safety of exogenous progestins also is an important consideration, particularly if the ultimate goal is application in wild felids. Commonly used progestins in domestic and non-domestic cats have included melengestrol acetate (MGA), megestrol acetate (Ovaban®) and levonorgestrel (Norplant®) (Lofstedt and Patel, 1989; Romatowski, 1989; Baldwin et al., 1994; Romagnoli and Concannon, 2003; Pelican et al., 2005). However, administration of certain progestins for numerous years, most notably MGA, has been associated with increased incidence of pyometra and uterine neoplasia in cats (Munson and Mason, 1993). Newer progestin formulations have aimed at decreasing these side effects. For example, levonorgestrel implants inhibit follicular activity and spontaneous ovulation in the domestic cat with no observed adverse reactions (Baldwin et al., 1994; Looper et al., 2001). The present study was the first to demonstrate that short-term treatment with ALT can provide effective ovarian suppression in domestic cats with no known deleterious effects. Whether long-term or continual intermittent treatment with ALT is safe in domestic and non-domestic cats remains to be determined.

In conclusion, short-term treatment with three different dosages of ALT induced rapid and reversible inhibition of follicular activity in the cat. These findings provide the

necessary foundation for further investigations into the use of ALT prior to assisted reproduction in felids. Ongoing studies are evaluating whether an ALT-treated ovary exhibits altered sensitivity to exogenous gonadotropins, and whether ALT pre-treatment might mitigate adverse endocrine effects known to occur following ovarian stimulation in the cat.

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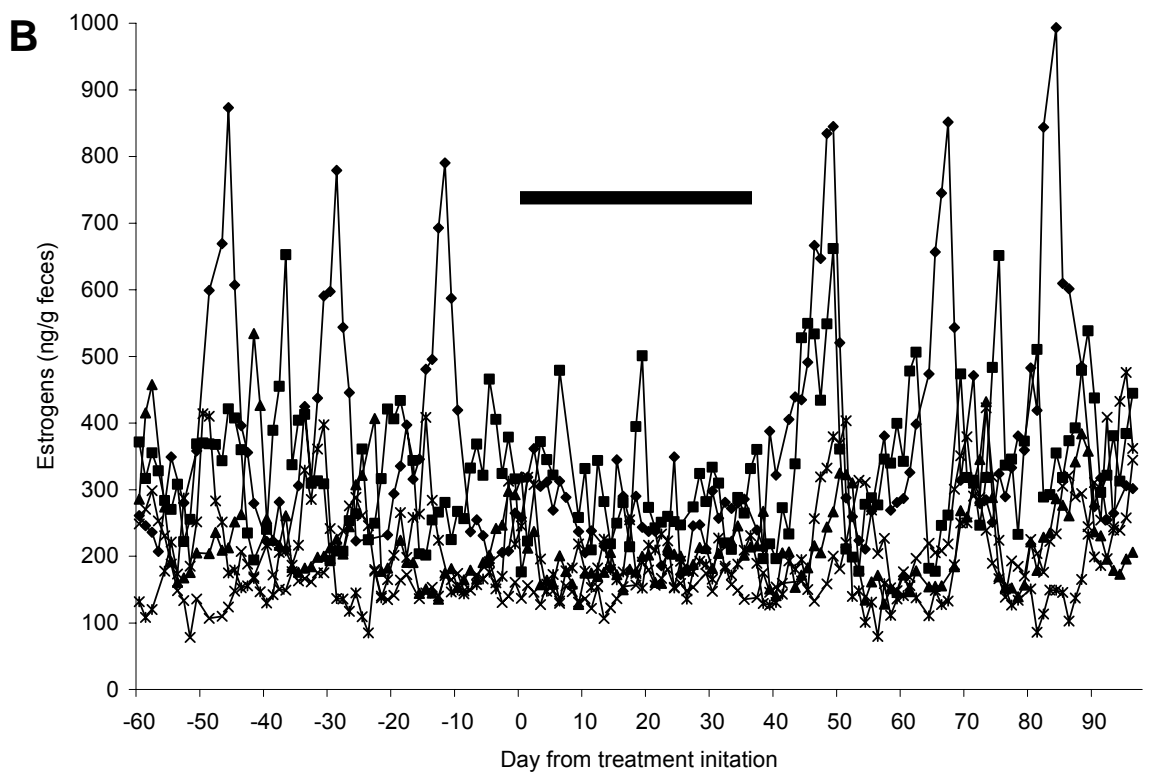
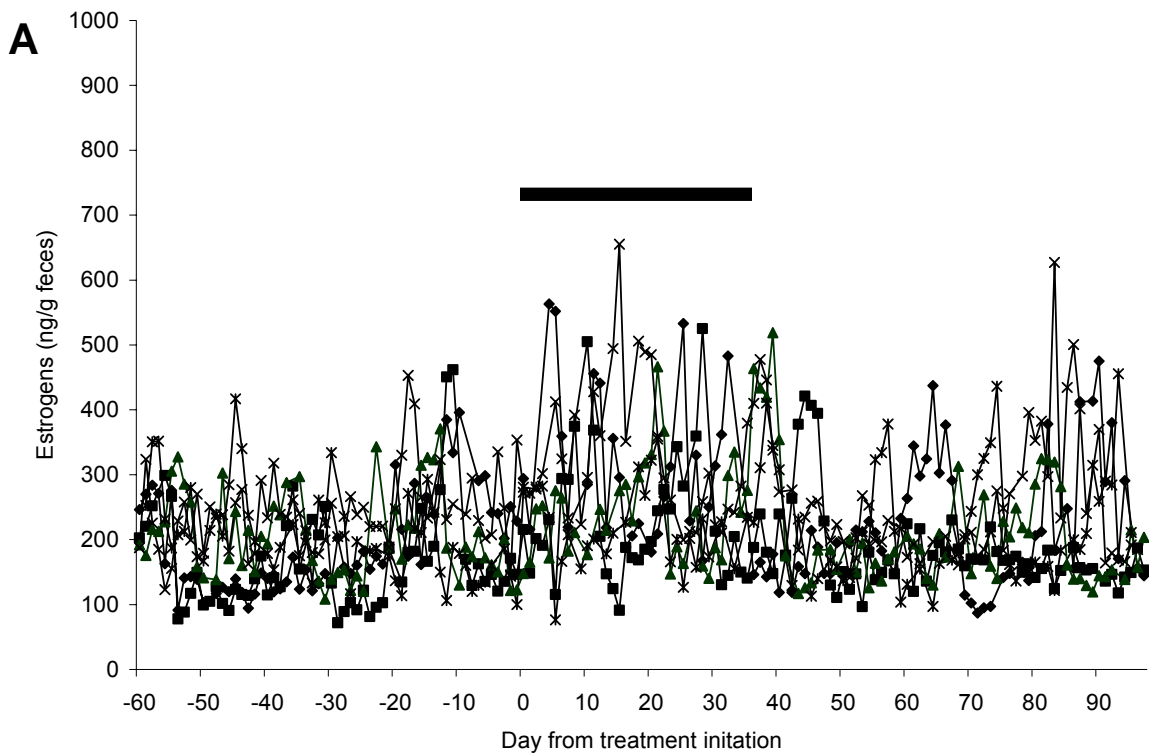
Table 2.1. Domestic cat reproductive traits before altrenogest treatment, assessed by longitudinal fecal steroid analyses ( $n = 13$  females).

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Duration of follicular phase (days)	5.3 $\pm$ 0.4
Estrous cycle length (days)	20.6 $\pm$ 1.8
Number of follicular phases/ 60 days	2.1 $\pm$ 0.3
Baseline estrogens (ng/g feces)	194.8 $\pm$ 12.5
Peak estrogens (ng/g feces)	401.8 $\pm$ 26.2
Mean estrogens/follicular phase (ng/g feces)	331.5 $\pm$ 16.5
Baseline progestins ( $\mu$ g/g feces)	2.8 $\pm$ 0.2

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Values are means  $\pm$  SEM.



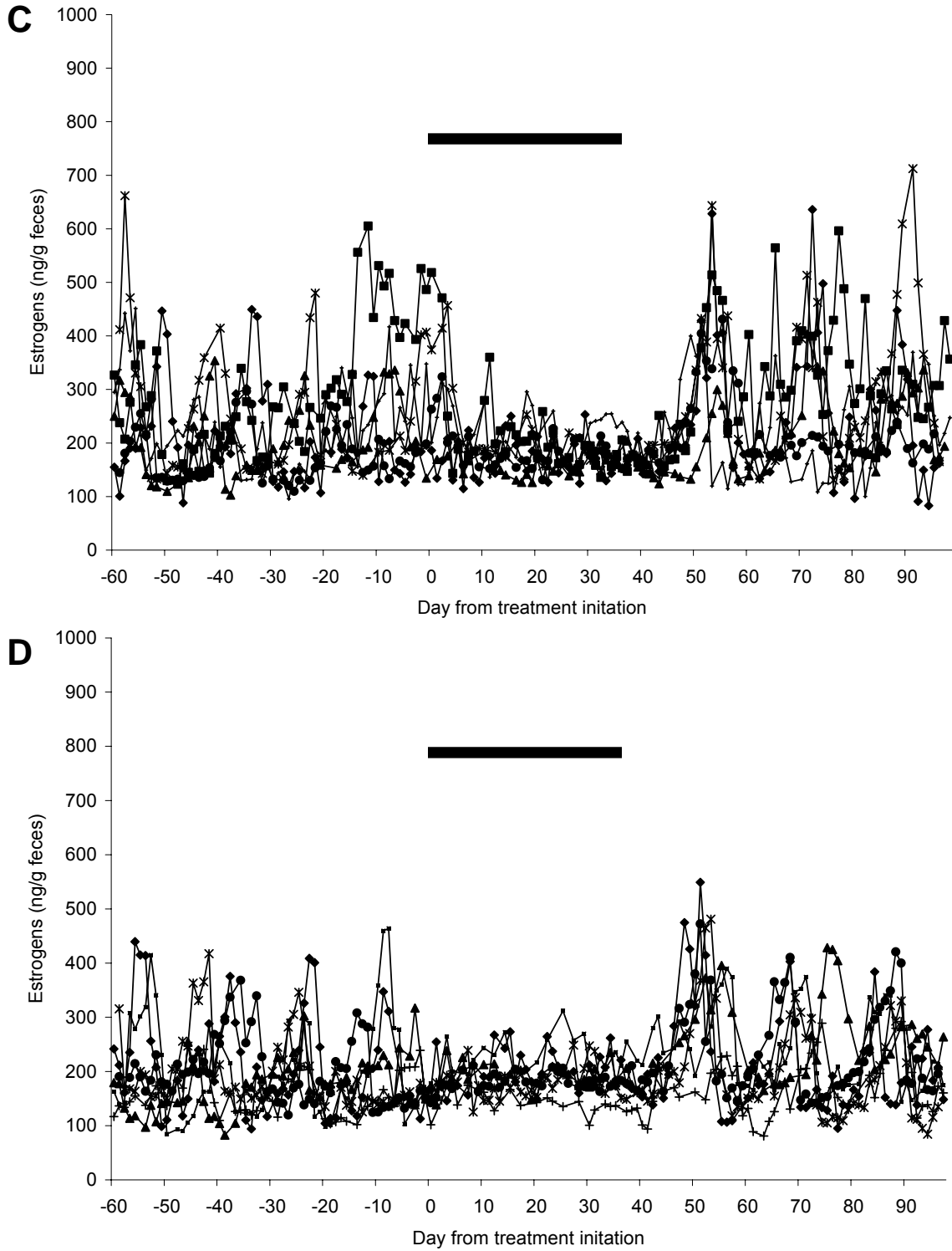


Fig. 2.1. Influence of altrenogest (ALT) on longitudinal fecal estrogens. Queens were assigned to: (A) 0 mg/kg ALT (control); (B) 0.044 mg/kg (LOW); (C) 0.088 mg/kg (MID); and (D) 0.352 mg/kg (HIGH). Black bars indicate the 38 day treatment period. Individual animals within each treatment are represented by different line markers.

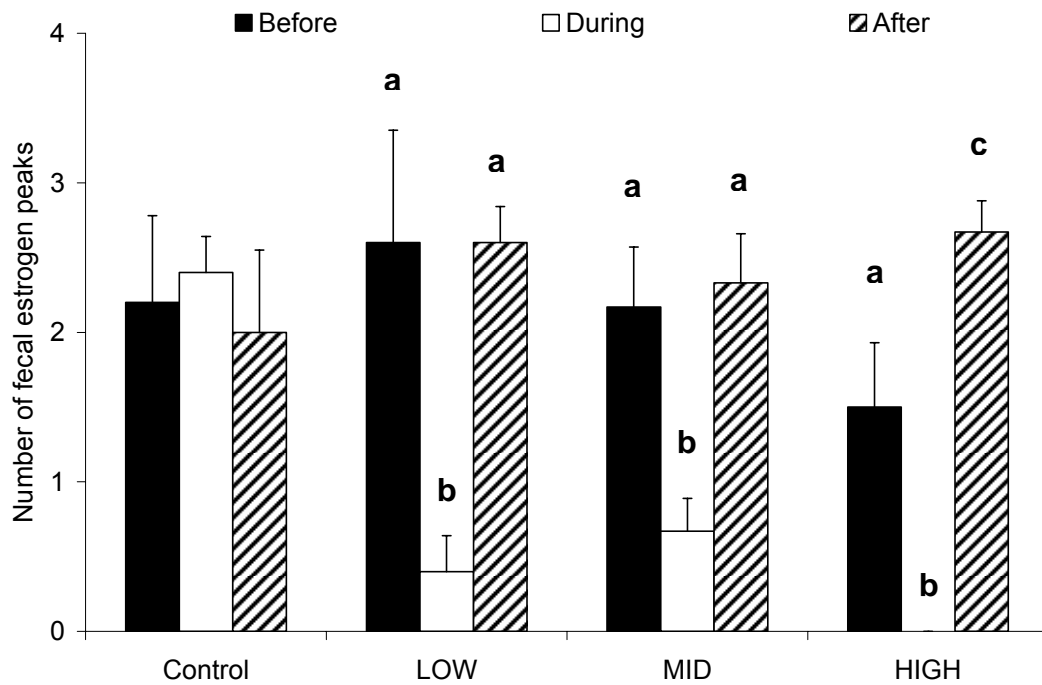


Fig. 2.2. Influence of altrenogest dosage on number of fecal estrogen peaks (mean  $\pm$  SEM) before (solid), during (open) and after (hatched) treatment. Within a treatment, means with different superscripts differ ( $P < 0.05$ ).



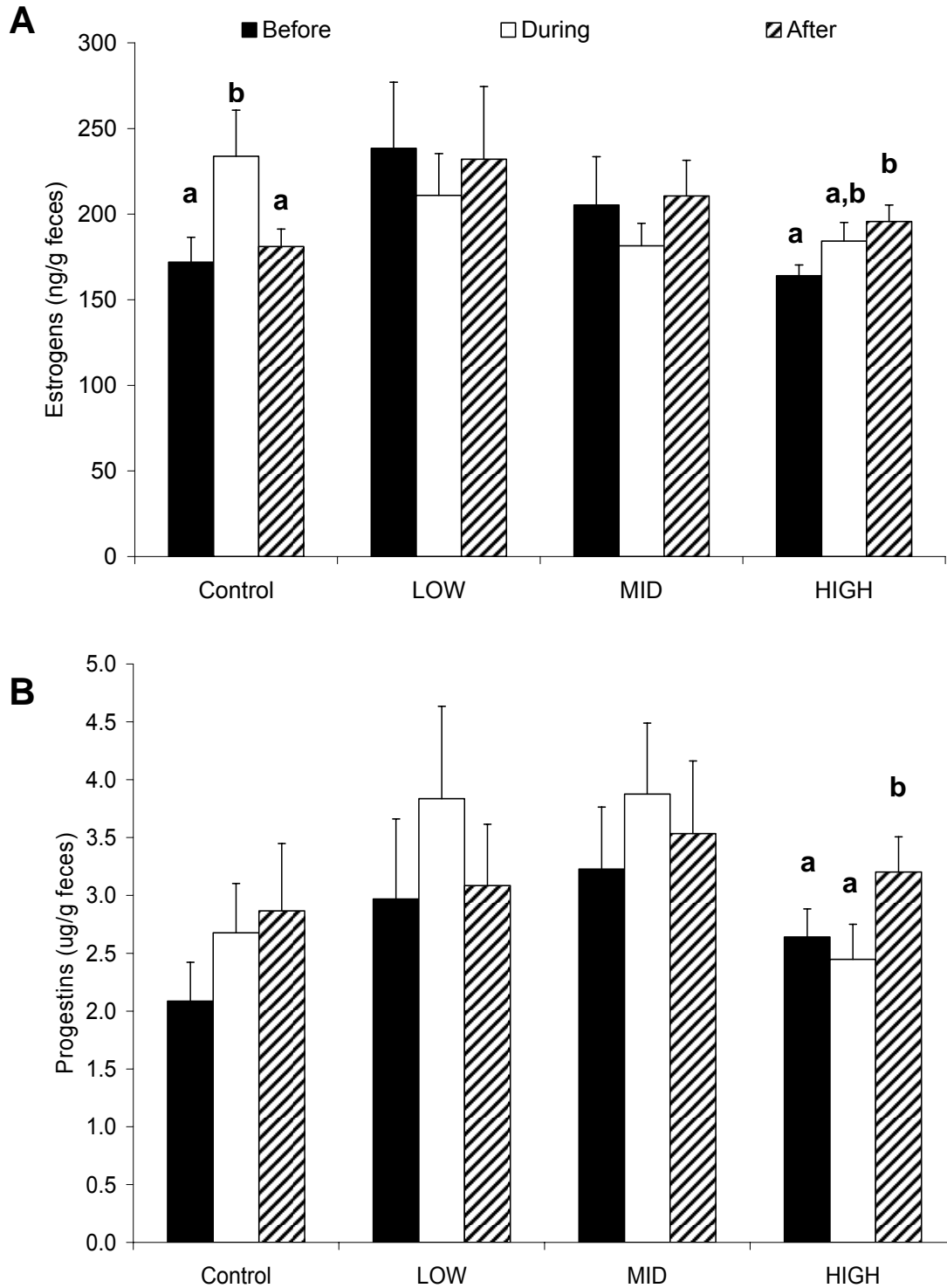
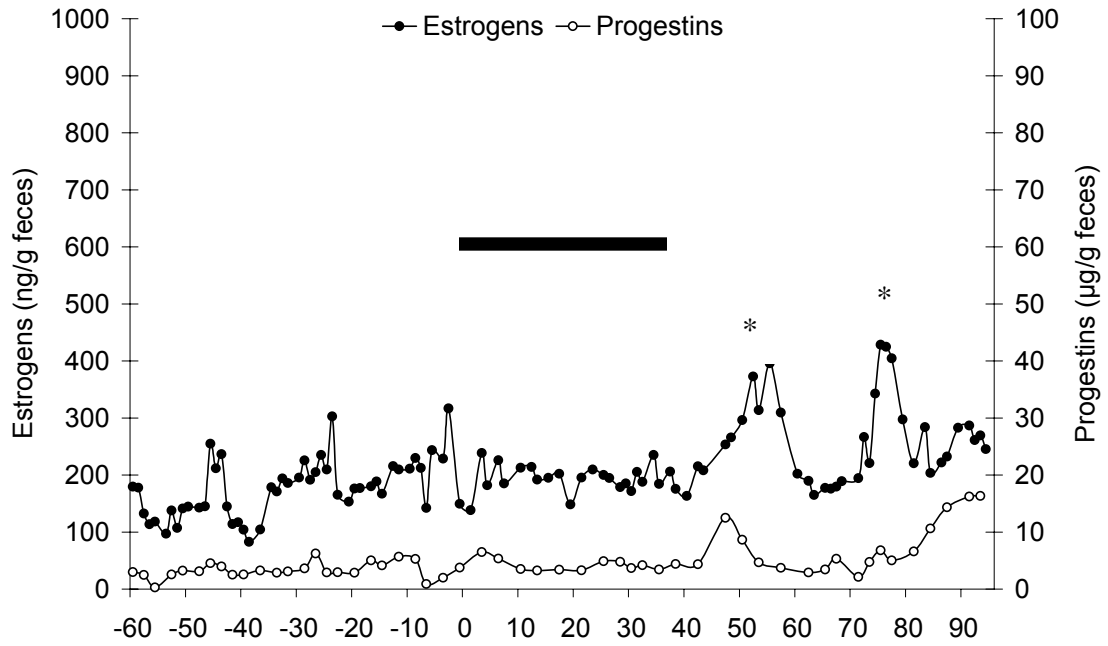
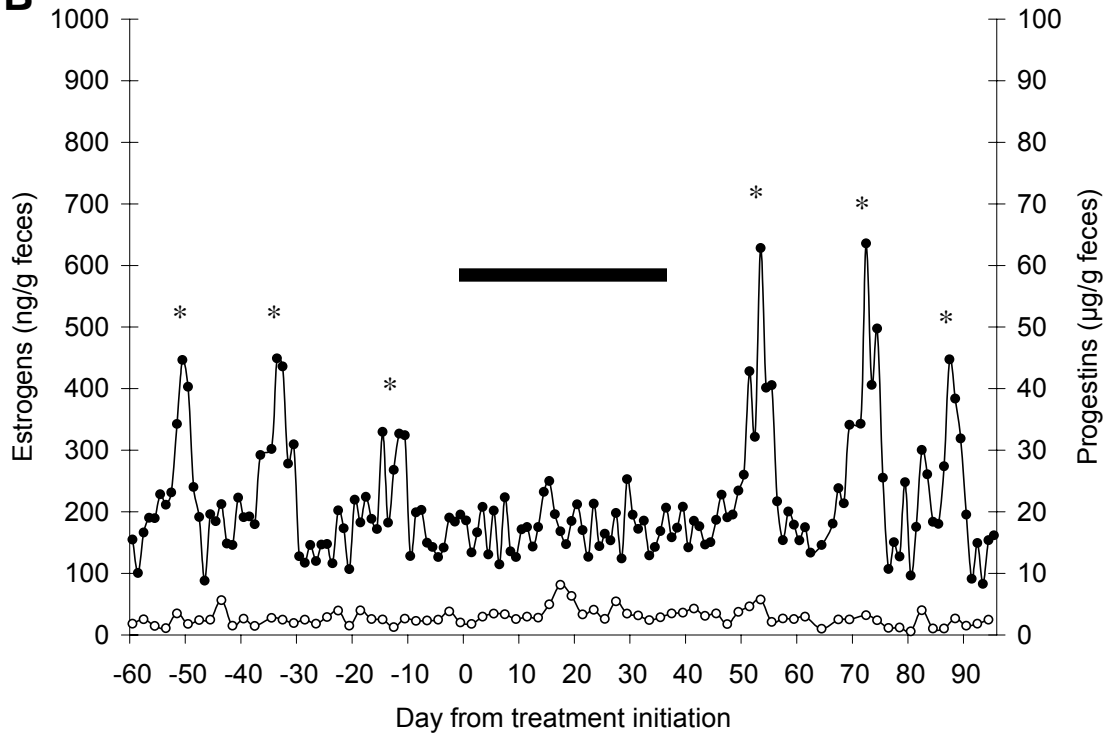


Fig. 2.3. Effect of altrenogest dosage on: (A) baseline fecal estrogens; and (B) baseline fecal progestins before (solid), during (open) and after (hatched) treatment (mean  $\pm$  SEM). Within a treatment, means with different superscripts differ ( $P < 0.05$ ).

**A****B**

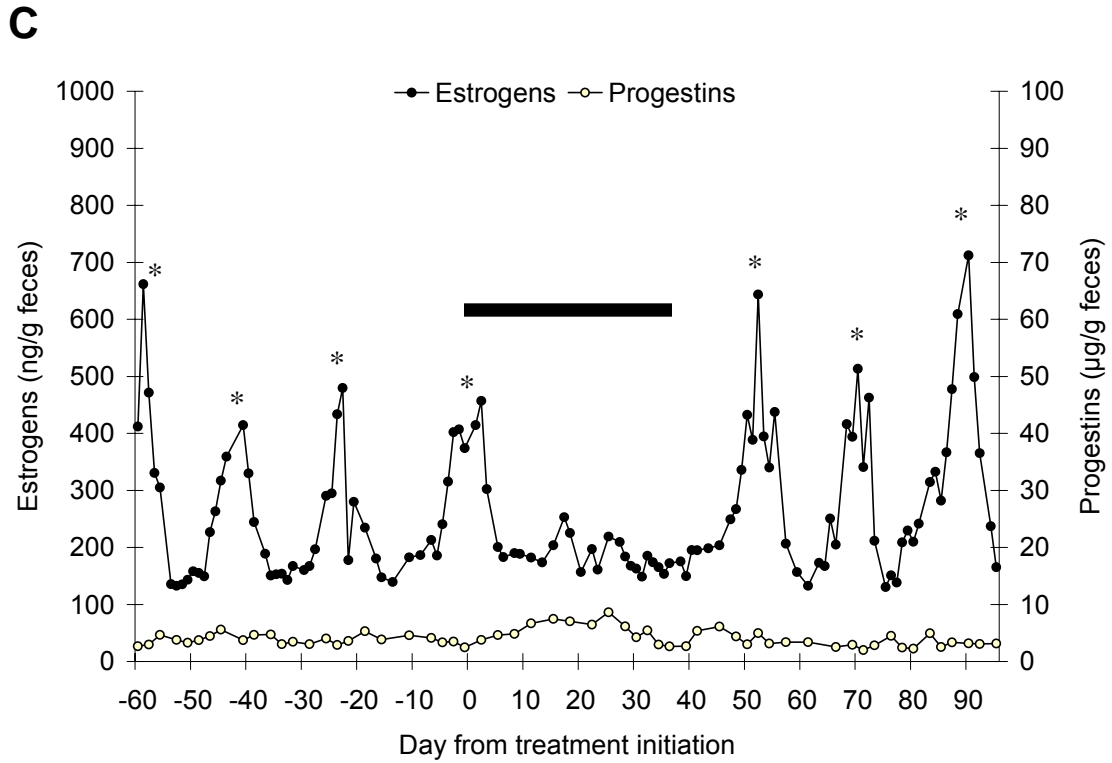


Fig. 2.4. Representative fecal steroid profiles before, during and after altrenogest (ALT) treatment. Profiles include: (A) a female demonstrating follicular activity only after ALT treatment; (B) a female exhibiting baseline fecal estrogens at the time of ALT treatment initiation; and (C) a female exhibiting follicular activity at the time of ALT treatment initiation. Asterisks indicate each follicular phase and the solid bar represents the 38 day treatment period.

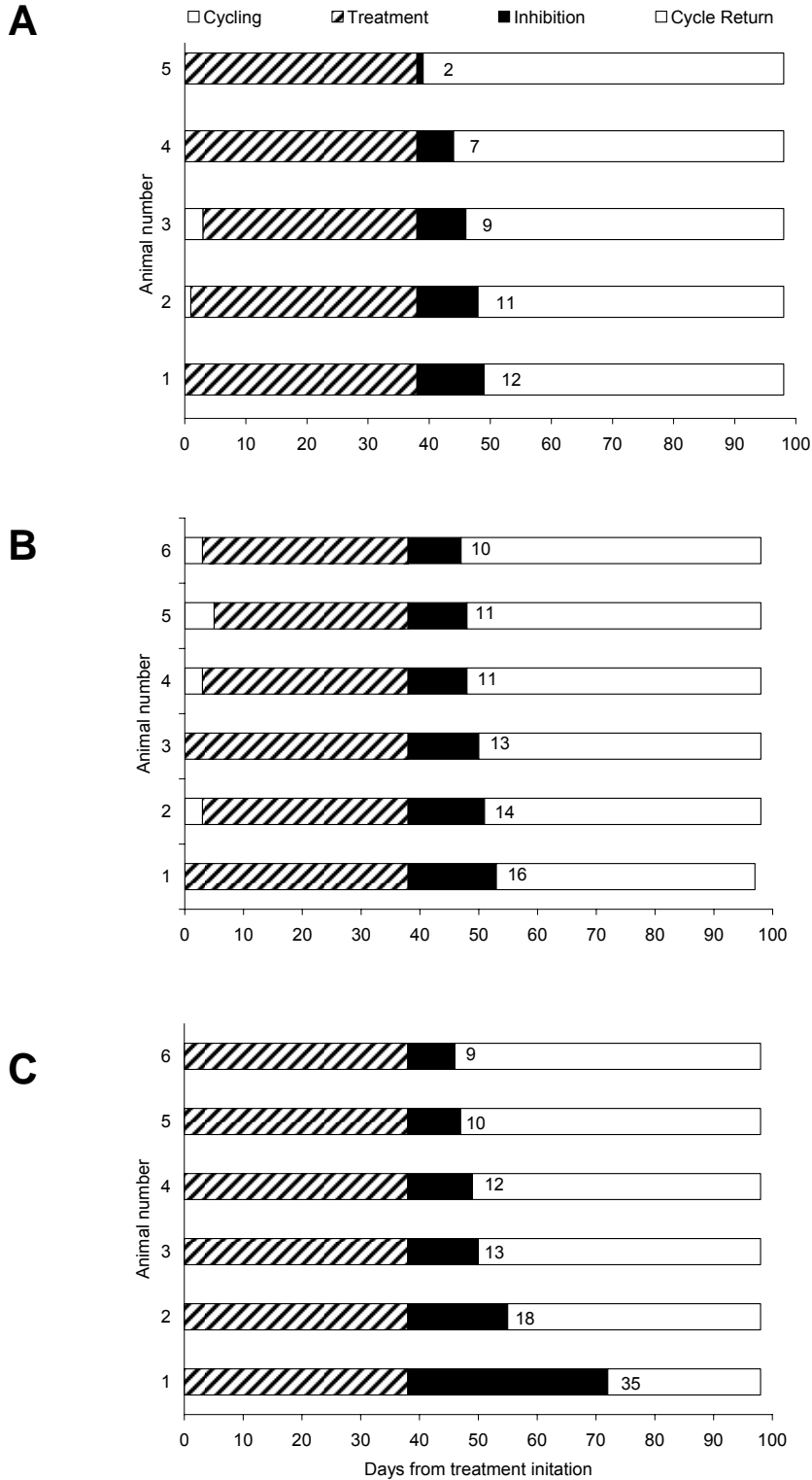


Fig. 2.5. Comparison of inhibition duration following altrenogest (ALT) treatment. Cats received: (A) 0.044 mg/kg ALT (LOW); (B) 0.088 mg/kg (MID); and (C) 0.352 mg/kg (HIGH). Bars represent inhibition during the treatment period (hatched), inhibition following removal of altrenogest (solid), and follicular activity (open). Numbers within bars represent interval (days) from ALT removal to the first follicular phase.

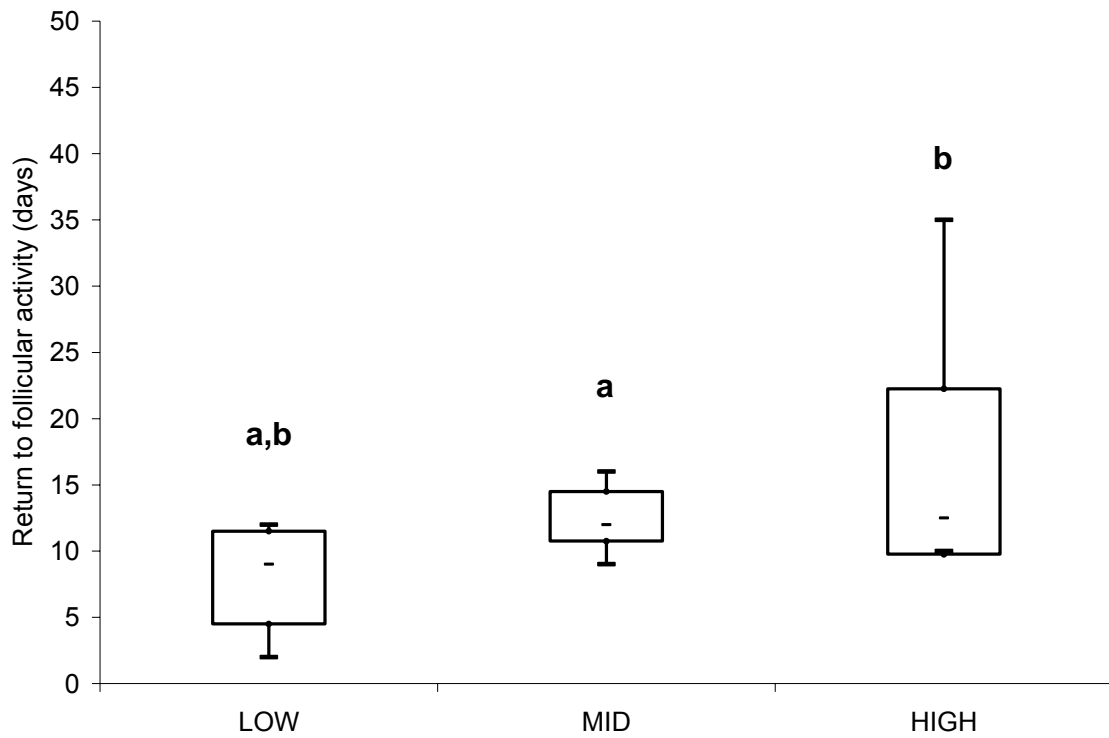


Fig. 2.6. Variation in return to follicular activity following treatment with altrenogest (ALT). Cats received 0.044 mg/kg ALT (LOW); 0.088 mg/kg (MID); and 0.352 mg/kg (HIGH). Variances with different superscripts differ among treatments ( $P < 0.01$ ).

## CHAPTER 3

### ORAL PROGESTIN PRIMING ELIMINATES SPONTANEOUS OVULATION AND INCREASES SENSITIVITY TO EXOGENOUS GONADOTROPINS IN THE CAT

#### Abstract

The impact of pre-treatment with oral progestin (altrenogest; ALT) before ovulation induction on ovarian morphology and function was examined in the domestic cat. A population of queens known to exhibit induced or spontaneous ovulation was assigned to two dosages of exogenous gonadotropins with and without ALT priming: (1) ALT + 100 IU equine chorionic gonadotropin (eCG) + 75 IU human chorionic gonadotropin (hCG;  $n = 7$  cats; ALT HIGH); (2) 100 IU eCG + 75 IU hCG ( $n = 7$ ; HIGH); (3) ALT + 50 IU eCG + 37.5 IU hCG ( $n = 5$ ; ALT LOW); or (4) 50 IU eCG + 37.5 IU hCG ( $n = 5$ ; LOW). Ovarian response and gonadotropin-induced ovulation were assessed by laparoscopy on Day 2 (Day 0 = day of hCG) and graded on a scale of one to four (1 = excellent; 2 = good; 3 = fair; 4 = ovulation failure). Spontaneous luteal activity was abolished in all 12 ALT-primed cats, whereas five of 12 (41.7%) unprimed cats had old corpora lutea (CL) on Day 2. All cats given ALT had a Grade 1 or 2 ovarian response, compared to only 50% of females given gonadotropins alone. Suboptimal ovarian response and ovulation failure were observed in LOW but not ALT LOW queens ( $P < 0.05$ ), demonstrating an increased sensitivity to eCG/hCG in the progestin-primed ovary. Longitudinal fecal hormone profiles from a subset of queens ( $n = 19$ ) demonstrated that the estrogen surge following gonadotropin-induced ovarian stimulation ( $7.3 \pm 0.9$  days) was similar ( $P > 0.05$ ) in all groups compared to pre-treatment values ( $6.1 \pm 0.6$  days). The interval from hCG administration to the first day of sustained elevated fecal

progestins also was similar ( $P > 0.05$ ) among treatments, averaging  $4.8 \pm 0.3$  days after hCG. On Day 17, laparoscopy was conducted to assess ovarian activity and corpora lutea (CL) morphology compared to Day 2. Then, ovariohysterectomy was performed and ovaries prepared for histology and luteal progesterone quantification. ALT did not prevent ancillary folliculogenesis and ovulation following the initial gonadotropin-induced ovarian response. Luteal progesterone measured in all treatments compared to a natural estrus/coitus-induced ovulation group spayed at a corresponding time point ( $n = 6$  cats) revealed that CL progesterone was abnormally low ( $P < 0.05$ ) in LOW, ALT HIGH and HIGH cats compared to ALT LOW and naturally-bred cats. Overall, this study demonstrated that oral progestin priming prevents spontaneous ovulation, provides a consistent ovarian response to ovulation induction, increases sensitivity to exogenous gonadotropin dosage and supports normal luteal progesterone production.

## **Introduction**

The domestic cat (*Felis catus*) is a valuable research model for investigating endocrine mechanisms important for optimizing assisted reproduction in rare wild felids. One technique developed in the cat that has been particularly successful is artificial insemination (AI), a critical tool for managing genetically-isolated populations (Wildt and Roth, 1997; Howard, 1999). AI circumvents breeding incompatibility, a common problem in zoo felids (Brown et al., 1995), while providing the potential for introducing new genes from wild populations into captive breeding programs via frozen sperm (Howard, 1992). Using a laparoscopic intrauterine AI technique, offspring have been produced in the cheetah (Howard et al., 1992b), clouded leopard (Howard et al., 1996), leopard cat (Howard, 1991), ocelot (Swanson et al., 1996b), puma (Barone et al., 1994b), snow leopard (Roth et al., 1997a), tiger (Donoghue et al., 1993) and tigrina (Swanson and

Brown, 2004). However, overall incidence of pregnancy following AI remains remarkably low in many wild felids (< 10%) (Pelican et al., 2006b).

Much of the etiology of AI failure can be traced back to the unique reproductive physiology of the female cat. Although classically considered induced ovulators (Wildt et al., 1980; Goodrowe et al., 1989), there is evidence of intermittent spontaneous ovulation in many wild felids (Brown, 2006) and in domestic cats (Lawler et al., 1993). Indeed, a strong correlate to poor AI success in felids is spontaneous ovulation and an accompanying inconsistent ovarian response to exogenous gonadotropins before AI (Howard et al., 1997). This variability is attributed to administration of gonadotropins during the luteal phase, where functional corpora lutea (CL) can attenuate ovarian response or lead to ovulation failure (Pelican et al., 2006b). Alternatively, if exogenous gonadotropins are given concurrent with a natural estradiol surge, follicular cohorts of inconsistent age and oocyte maturation stage can result. Ancillary folliculogenesis and ovulation (Swanson et al., 1996a), immunologically-mediated refractoriness to repeated exogenous gonadotropin exposure (Swanson et al., 1995a), an abnormal endocrine milieu (Brown et al., 1994), delayed oviductal transport (Graham et al., 2000) and poor embryo quality (Goodrowe et al., 1988a) also can mitigate reproductive success after gonadotropin stimulation.

Short-term ovarian suppression before AI or timed breeding routinely is used in spontaneous ovulators across multiple genera, including the human (Burry et al., 1991; Barbieri and Hornstein, 1999), cow (Patterson et al., 1997), pig (Wood et al., 1992), horse (Lofstedt, 1988), sheep (Deligiannis et al., 2005), sable antelope (Thompson and Monfort, 1999), scimitar-horned oryx (Morrow et al., 2000), killer whale (Robeck et al., 2004) and bottlenose dolphin (Robeck et al., 2005). This strategy temporarily down-regulates ovarian activity, thereby enabling a more uniform ovarian response at the time of insemination, oocyte retrieval or embryo transfer. Down-regulation before



gonadotropin stimulation also can reduce ovarian hyperstimulation in some species (Kol, 2004; Oshima et al., 2004). Indeed, one of the few wild felid species with a relatively high AI success rate (~45%), the cheetah, is an induced ovulator which frequently experiences periods of prolonged acyclicity (Brown et al., 1996b). These periods of anestrus provide an endogenous mechanism for ovarian suppression that results in a more consistent ovarian response to exogenous gonadotropins and higher pregnancy success following AI (Howard et al., 1992b; Howard et al., 1997).

Manipulating the ovarian cycle can be accomplished with various exogenous agents that differentially act on the hypothalamic-pituitary-gonadal (HPG) axis. In the cat, progestins are optimal for ovarian suppression compared to GnRH analogs or prostaglandins (Wildt et al., 1979b; Pelican et al., 2005). Progestins primarily act at the level of the hypothalamus and the pituitary, providing negative feedback that leads to attenuated gonadotropin release and suppression of ovarian activity (Burris, 1999; Romagnoli and Concannon, 2003). In cats, progestin implants have been used successfully to down-regulate ovarian activity without negatively affecting subsequent ovarian response to exogenous gonadotropins or oocyte quality (Pelican et al., 2001; Pelican et al., 2007). The oral progestin altrenogest (ALT) also has been used in cats for ovarian suppression (Chapter 2), as well as in other domestic and non-domestic animals prior to assisted reproduction or timed breeding including the horse (Lofstedt and Patel, 1989), pig (Wood et al., 1992), killer whale (Robeck et al., 2004) and bottlenose dolphin (Robeck et al., 2005).

This study evaluated the effect of short-term oral progestin priming on subsequent ovarian response to equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) in the domestic cat. Multiple endpoints associated with ovarian function and morphology were examined. We hypothesized that: (1) oral progestin priming would prevent spontaneous ovulation and result in a more

synchronized ovarian response to gonadotropins compared to unprimed females; (2) the down-regulated ovary would exhibit an altered dose-dependent sensitivity to gonadotropins; (3) progestin priming would reduce accessory CL formation; and (4) priming would mitigate adverse effects on ovarian morphology and function associated with exogenous gonadotropin treatment.

## **Materials and Methods**

### ***Animals***

Thirty adult (1-3 year old) female domestic cats were housed at the Smithsonian's National Zoological Park's Conservation and Research Center (CRC) during the ~ 14 month study. These cats were part of a research population known to exhibit intermittent spontaneous ovulation (Graham et al., 2000; Pelican et al., 2005). Queens were maintained alone ( $n = 11$ ) or in pairs ( $n = 19$ ) in stainless steel cages (minimum  $0.5 \text{ m}^3$  of space per female) under artificial fluorescent light (12 L: 12 D). Three proven breeder males (1-7 years old) were housed in an adjacent room in individual runs ( $> 1 \text{ m}^3$  of space per male). All cats were provided a dry commercial diet (Purina ONE®, Nestlé Purina PetCare Co., St. Louis, MO). Paired females were briefly separated daily and one cat from each pair received 5 g canned food (Friskies®; Nestlé Purina) with green food dye (Icing Colors, Wilton Industries, Woodbridge, IL) that served as a marker to differentiate fecal samples. All research activities were approved by the CRC's Institutional Animal Care and Use Committee (IACUC; #05-25) and the University of Maryland IACUC (R-06-06).

### ***Exogenous hormone administration and natural breeding***

Queens ( $n = 24$ ) receiving exogenous hormones were randomly assigned to four treatments: (1) ALT + 100 IU eCG + 75 IU hCG ( $n = 7$  cats; ALT HIGH); (2) 100 IU eCG + 75 IU hCG ( $n = 7$ ; HIGH); (3) ALT + 50 IU eCG + 37.5 IU hCG ( $n = 5$ ; ALT LOW); or (4) 50 IU eCG + 37.5 IU hCG ( $n = 5$ ; LOW). To account for potential variability across time, treatments were blocked by day with one alternating, matched pair (ALT HIGH versus HIGH; or ALT LOW versus LOW) beginning treatment on the same day.

Subjects were previously naïve to exogenous hormones, and procedures were aligned to the day of hCG, termed Day 0. The oral progestin ALT (Regu-Mate®; Intervet Inc., Millsboro, DE) was administered at a dosage of 0.088 mg/kg daily (Chapter 2) in 5 g canned food for 38 days, whereas unprimed females received wet food only.

Lyophilized eCG (Sigma-Aldrich Corporation, St. Louis, MO) and hCG (Sigma-Aldrich) were solubilized in sterile, preservative-free saline to a concentration of 250 IU/ml and stored in individual syringes at  $-20^{\circ}\text{C}$  until use. Three days after the last ALT dose or randomly in unprimed females, cats received a single intramuscular (i.m.) injection of eCG to induce follicular activity (Day -3). This was followed 80 hours later by a single i.m. injection of hCG to stimulate final follicular maturation and ovulation (Day 0).

A group of queens demonstrating natural estrus and coitus-induced ovulation was used to compare luteal progesterone concentrations with the exogenous gonadotropin groups. Females ( $n = 6$ ) were monitored daily for signs of behavioral estrus including lordosis, tail deviation, vocalization, rubbing and rolling. Beginning on Day 2-4 of behavioral estrus, a proven breeder male was introduced for natural breeding and allowed to mate three times a day (one intromission per encounter) in three hour intervals for two consecutive days (Wildt et al., 1981). The first day of breeding was denoted Day 0.

## ***Laparoscopic assessment of ovarian response on Day 2***

A laparoscopic ovarian exam was performed 36-40 hours after hCG (Day 2) in all gonadotropin-treated queens, which corresponds to the interval when post-ovulatory intrauterine artificial insemination is typically performed in the cat (Howard et al., 1992a). Females were anesthetized with a single i.m. injection of 10 mg/kg ketamine hydrochloride (Ketaved; Vedco Inc., St. Joseph, MO) combined with 1 mg/kg acepromazine maleate (Phoenix Pharmaceuticals, Inc., St. Joseph, MO) and anesthesia was maintained with isoflurane inhalant gas (Phoenix Pharmaceuticals). Following induction, cats were placed in dorsal recumbancy and examined laparoscopically (Wildt et al., 1977). Briefly, the surgical table was tilted to an angle of ~ 45 degrees with the animal's head down, and a two mm Verres probe was inserted through the abdominal wall to insufflate the abdomen with room air. Next, a five mm trocar-cannula was inserted midline cranial to the umbilicus. A five mm laparoscope containing an integrated camera system (Olympus Surgical and Industrial America, Orangeburg, NY) was inserted through the cannula to visualize the abdominal cavity, including the entire reproductive tract. Using the two mm Verres probe for reference, all ovarian structures were counted, described, measured and photographed. CL that were white and well-vascularized were termed 'old', and cats with old CL on Day 2 were classified as spontaneous ovulators. CL that were pink or red with developing vascularization were termed 'fresh', and presumed to be the result of gonadotropin stimulation. Follicles > 2 mm in diameter and demonstrating mild to moderate vascularization were classified as 'mature'. Overall ovarian response for gonadotropin-induced ovulation was graded on a scale of 1 to 4: (1) excellent; multiple fresh CL and no follicles > 2 mm (Grade 1); (2) good; mixed cohort of fresh CL and follicles > 2 mm (Grade 2); (3) fair; variable-aged CL including fresh and old (Grade 3); and (4) poor; ovulation failure (Grade 4; Fig. 3.1). The

Verres probe also was used to measure ovarian dimensions, oviductal diameter and uterine diameter, and ovarian volume was calculated using the formula for testes volume (length x width<sup>2</sup> x 0.524) (Howard et al., 1990).

### ***Laparoscopy, ovariohysterectomy and tissue processing on Day 17***

On Day 17, all naturally-bred females and a subset of gonadotropin-treated queens ( $n = 4$  to 5 cats per treatment) were subjected to a laparoscopic exam to assess ovarian activity and CL morphology compared to Day 2. Ovarian structures were counted, described, measured and photographed as described above. These queens then were immediately prepared for a routine ovariohysterectomy. Reproductive organs were moistened with saline-soaked sponges during the procedure and immediately processed upon removal. From one ovary, whole CL were excised and individually weighed. The ovary with more CL was chosen to maximize recovery of luteal tissue. Half of these CL were flash frozen in liquid nitrogen for progesterone quantification. The remaining ovary was bisected, and one hemi-ovary was fixed in 4% paraformaldehyde (Fisher Scientific Company, Pittsburgh, PA) for histology. Fixed hemi-ovaries were embedded in paraffin within 1 week by a commercial company (HistoServ, Rockville, MD), sectioned at 5 $\mu$ m and stained with hemotoxylin and eosin.

### ***Fecal collection and extraction***

Daily fecal samples were collected from ALT HIGH ( $n = 5$ ), HIGH ( $n = 5$ ), ALT LOW ( $n = 5$ ) and LOW ( $n = 4$ ) queens beginning 60 days prior to treatment initiation and ending on the day of ovariohysterectomy. Samples were placed in individual plastic bags and stored at -20°C until processing. Using a protocol previously validated for the

domestic cat (Brown et al., 1994), lyophilized fecal samples were crushed to a fine powder and 0.18 to 0.2 g of dry fecal matter was boiled in 5 ml of 90% ethanol for 20 minutes. Following centrifugation (500g, 20 min), the supernatant was recovered and the pellet resuspended in 90% ethanol and vortexed for 30 seconds. Following a second centrifugation (500g, 15 min), supernatants were combined, dried under air, and reconstituted in 1 ml methanol. Methanol extracts were vortexed briefly and placed in a sonicator for 15 minutes to bring any particles affixed to the vessel wall into solution. Extracts were immediately diluted in steroid dilution buffer (1:10; 0.2 M NaH<sub>2</sub>PO<sub>4</sub>, 0.2 M Na<sub>2</sub>HPO<sub>4</sub>, 0.15 M NaCl, pH 7.0) and stored in polypropylene tubes at -20°C until enzyme immunoassay (EIA) for estrogens and progestins.

### ***Estrogen conjugate EIA***

A single-antibody estrogen conjugate (EC) EIA was used to quantify estrogens in fecal extracts (Robeck et al., 2004). The polyclonal antibody (anti-EC R522-2; 1:20,000; C. Munro, UC Davis, CA) cross-reacts with a broad range of estrogen metabolites present in domestic cat feces (Brown et al., 1994). The antibody was dissolved in coating buffer (0.015 M Na<sub>2</sub>CO<sub>3</sub>, 0.035 M NaHCO<sub>3</sub>, pH 9.6), added to 96-well, flat-bottom microtiter plates (Nunc-Immuno, Fisher Scientific) and incubated overnight at 4°C. Plates were washed (0.05% Tween 20, 0.15 M NaCl) to remove un-adsorbed antibody, and 0.025 ml steroid assay buffer (0.2 M NaH<sub>2</sub>PO<sub>4</sub>, 0.2 M Na<sub>2</sub>HPO<sub>4</sub>, 0.15 M NaCl, 2.0 g/L BSA, pH 7.0) was added to each well and maintained at room temperature for 30 minutes to 2 hours. Next, 0.05 ml sample (range, 1:100 to 1:1,000) or estrone-B-glucuronide standard (range, 0.78 to 200 pg; Sigma-Aldrich) was added to wells in duplicate immediately followed by 0.05 ml enzyme conjugate (E1G horseradish peroxidase; 1:15,000; C. Munro). After a 2 hour incubation period at room temperature,

plates were washed and 0.1 ml substrate (0.04 M ABTS, 0.5 M H<sub>2</sub>O<sub>2</sub> in a 0.05 M citric acid solution) was added to each well. Optical densities (OD) were read using a microplate reader (MRX, Dynex Technologies, Chantilly, VA) at 405nm when 0 pg standard wells reached an OD of 0.9 to 1. Serial dilutions of domestic cat feces yielded displacement curves that were parallel to the standard curve ( $r = 0.99$ ). Recovery of known amounts of estrone-B-glucuronide standard added to a pool of domestic cat fecal extracts (1:500) was 51.2 %  $\pm$  15.0% ( $y = 0.89x - 3.9$ ;  $R^2 = 0.99$ ). Intra-assay variation was < 10% and inter-assay variation was 8.0% and 9.8% at 30% and 70% binding, respectively ( $n = 82$  plates). Assay sensitivity was  $\sim$  1 pg/well.

### ***Pregnane EIA***

A single monoclonal antibody (CL425; 1:10,000; C. Munro) pregnane (Pg) EIA was employed to quantify progestin metabolites (Chapter 2). Similar to the EC EIA, the assay was conducted in 96-well, flat-bottom microtiter plates (Nunc-Immuno). After overnight incubation with the antibody at 4°C, plates were washed to remove unadsorbed antibody, and 0.05 ml sample (range, 1:2,000 to 1:50,000) or progesterone standard (range, 0.78 to 200 pg; Sigma-Aldrich) was added to wells in duplicate immediately followed by 0.05 ml enzyme conjugate (progesterone-3CMO horseradish peroxidase ; 1:40,000; C. Munro). Following a 2 hour incubation, plates were washed and 0.1 ml substrate added to each well. Optical densities (OD) were read at 405nm when 0 pg standard wells reached an OD of 0.9 to 1. Intra-assay variation was < 10% and inter-assay variation was 7.8% and 14.8% at 30% and 70% binding, respectively ( $n = 95$  plates).

### ***CL progesterone RIA***

Luteal progesterone concentration was measured in individual CL using a radioimmunoassay (RIA) previously validated for the domestic cat (Swanson et al., 1995b). Briefly, flash-frozen CL were thawed, individually homogenized in 2 ml PBS (0.02 M NaH<sub>2</sub>PO<sub>4</sub>, 0.03 M Na<sub>2</sub>HPO<sub>4</sub>, 0.15 M NaCl, pH 7.0) using a ground-glass homogenizer and decanted into a glass tube. Homogenates were diluted in 3 ml 100% ethanol, vortexed for 1 min and boiled for 20 min. During the boiling process, 100% ethanol was added to maintain approximate pre-boil volumes. Following centrifugation (500g, 20 min), the supernatant was recovered and the pellet resuspended in 2 ml ethanol. Tubes were placed in a sonicator for 15 min to free residual luteal pellet adhered to the glass tube, vortexed for 1 min and re-centrifuged (500g, 15 min). The first and second supernatants were combined, dried under air and resuspended in 1 ml methanol. Methanol extracts were diluted 1:10 in PBS and stored at -20°C. Thawed extracts were diluted (range, 1:200 to 1:1,000) and analyzed using a solid-phase <sup>125</sup>I progesterone RIA kit (Coat-a-Count; Diagnostic Products Corporation, Los Angeles, CA). Intra- and inter-assay variation was < 10%.

### ***Statistical analyses***

For fecal hormone data, baseline estrogen concentrations were calculated for each individual using an iterative process in which all values greater than the mean plus two standard deviations (SD) were eliminated. The mean of the remaining values was recalculated and the process repeated until no values exceeded the mean plus two SD (Brown et al., 1994; Pelican et al., 2005). All values removed during the iterative process were considered elevated, and the final mean obtained through this process was denoted the baseline for that animal. Baseline progestin concentrations were



determined using a similar iterative process, except the mean plus 1.5 SD was used. Values greater than twice the progestin baseline were considered elevated from baseline. 1.5 SD was chosen for progestins because 2 SD was too sensitive to differentiate baseline from elevations.

For each individual, duration of the follicular phase was calculated as the number of consecutive days when fecal estrogens remained elevated, and the highest estrogen value within an array of elevations was the peak for that follicular phase. The end of a follicular phase was determined when estrogens returned to baseline for more than 2 days. The luteal phase was defined when progestin levels rose above baseline and remained elevated for at least 3 consecutive weeks. Luteal phase length was the total number of days progestins remained above baseline. The end of a luteal phase was determined when progestins returned to baseline for at least 4 days. Anovulatory estrous cycle length during the pre-treatment period was calculated as the number of days between fecal estrogen peaks with no elevation in fecal progestins following the first peak.

Estrous cycle traits (duration of follicular phase, mean fecal estrogens/follicular phase, peak estrogens/follicular phase, estrous cycle length, baseline and mean estrogens, baseline and mean progestins) were summarized by treatment and also between induced versus spontaneous ovulators. Fecal data among treatments at a single time interval (pre-treatment versus gonadotropin-stimulated) were analyzed using a two-way factorial ANOVA followed by a Tukey-Kramer honestly significant difference (HSD) multiple comparison test, whereas data summarized for induced versus spontaneous ovulators at a single time interval were compared using a one-way ANOVA. Within treatment or ovulation mechanism (induced versus spontaneous), follicular data was compared before and after gonadotropin stimulation using paired *t*-tests.

Laparoscopic ovarian data were summarized within each treatment and compared using a two-way factorial ANOVA followed by HSD mean comparisons. Main effects (ALT and gonadotropin dosage) were interpreted only when interactions were not present. CL progesterone concentrations were analyzed using a 2x3 ANOVA followed by HSD mean comparisons. Numbers of CL on Day 2 vs. Day 17 were compared using paired *t*- tests. When necessary, data were corrected for non-normal distribution before ANOVA. All analyses were performed using JMP IN 5.1 (SAS Institute Inc., Cary, NC) and differences were significant at  $P < 0.05$ . Trends were noted when the  $P$  value ranged from 0.05 to 0.1. Data are presented as means  $\pm$  SEM.

## **Results**

### ***Estrous cycle characteristics before treatment***

Fecal hormone monitoring demonstrated that eight of 19 (42.1%) females exhibited at least one spontaneous luteal phase during the 60 day pre-treatment period (Table 3.1). Of these females, six were subsequently randomly assigned to receive ALT, and two were not. Follicular phase duration ranged from 2-19 days and was similar ( $P > 0.05$ ) in spontaneous vs. induced ovulators, with an overall mean of  $6.1 \pm 0.6$  days. Ovulation mechanism did not influence ( $P > 0.05$ ) baseline estrogens ( $144.5 \pm 8.4$  ng/g feces), mean estrogens during the follicular phase ( $246.2 \pm 15.2$  ng/g) or peak estrogens during the follicular phase ( $290.9 \pm 14.4$  ng/g). Mean anovulatory estrous cycle length was  $18.3 \pm 1.3$  days (range, 5 to 28 d). In those females demonstrating luteal activity, the luteal phase spanned 25-44 days. Baseline fecal progestins were higher ( $P < 0.05$ ) in spontaneous versus induced ovulators (Table 3.1).

### ***Influence of altrenogest on ovarian response to gonadotropins***

On Day 2, five of 12 (41.7%) unprimed cats exhibited old CL indicative of spontaneous ovulation before ovarian stimulation. None of the 12 ALT-primed females had old CL on Day 2 or evidence of spontaneous ovulation (Table 3.2). All cats given ALT had a good-to-excellent ovarian response on Day 2 (Grade 1 or 2), compared to six of twelve (50%) females given gonadotropins alone. Ovarian grade was similar on Day 2 among ALT HIGH, HIGH and ALT LOW, and improved ( $P < 0.05$ ) compared to LOW queens (Table 3.2). Fewer ( $P < 0.05$ ) fresh CL were observed on Day 2 in LOW compared to ALT HIGH cats (Table 3.2). Fresh CL were smaller ( $P < 0.05$ ) on Day 2 in cats receiving high dosages of gonadotropins (Fig. 3.2A). On Day 2, more ( $P < 0.05$ ) mature follicles were observed in cats treated with low gonadotropin dosages (Fig. 3.2B). Accessory CL development (Fig. 3.3) was observed in a high proportion of females in all treatment groups, with 75-100% of females exhibiting more CL on Day 17 compared to Day 2. However, there was no increase ( $P > 0.05$ ) in the mean number of CL from Day 2 to Day 17 within or among treatment groups.

### ***Fecal hormone dynamics during exogenous hormone treatment***

At the time of ALT initiation, four females had elevated fecal progestins (Fig. 3.4A). Luteal phase duration and mean progestins during these ALT-associated luteal phases fell within the range of normal values observed in untreated females. While ALT did not influence an existing luteal phase, all females had returned to baseline by cessation of ALT treatment. Similarly, two females had elevated fecal estrogens at the time of ALT initiation. Those females exhibited a follicular phase of normal duration and magnitude before returning to baseline and remaining suppressed for the remainder of treatment (Fig. 3.4B). On the day of eCG administration, three of nine (33.3%) unprimed cats had elevated fecal estrogens, and two of nine (22.2%) unprimed cats had elevated

fecal progestins (Fig. 3.5 A-B). Overall, 100% of ALT-primed cats exhibited baseline fecal estrogens and progestins at the time of eCG compared to only 44.4% of unprimed individuals. These elevations observed in unprimed females at the time of eCG led to inconsistent ovarian responses on the day of laparoscopy, including ovulation failure (Fig. 3.5 C-D).

Estrous cycle traits following exogenous gonadotropin treatment were compared to pre-treatment values (Table 3.3). No significant differences were observed across time, between treatments or between ALT-primed and unprimed individuals. In unprimed females, peak estrogens during the follicular phase ( $P = 0.09$ ) showed a trend towards increasing following gonadotropin treatment, compared to unstimulated cycles (Fig. 3.6). This relationship was not observed in cats primed with ALT. There also were trends associated with gonadotropin dosage, where cats receiving HIGH gonadotropins (regardless of priming status) showed higher mean estrogen values during the follicular phase ( $P = 0.10$ ) and peak estrogens during the follicular phase ( $P = 0.07$ ). Ovulation mechanism (spontaneous versus induced) during the pre-treatment period did not influence ( $P > 0.05$ ) fecal estrogen dynamics following exogenous gonadotropin treatment, compared to pre-treatment values.

### ***Ovarian histology and luteal progesterone***

On Day 17, histological sections revealed diverse ovarian dynamics in all treatment groups. CL were large with smooth edges, primarily polygonal cells and no central cavities (Fig. 3.7A). CL with centrally-located vacuoles were observed in two females (Fig. 3.7B). These vacuoles were distinct from the irregular central cavities observed immediately post-ovulation in felid corpora hemorrhagica (CH) (Roth et al., 1995). Regressing CL, the product of spontaneous ovulation before ovarian stimulation, were smaller in size with an irregular shape and multiple lipid vacuoles (Fig. 3.7C).

Primordial, primary, secondary and tertiary follicles also were observed in ovarian sections across treatment groups (Fig. 3.8).

Differences were observed in CL progesterone production among the gonadotropin-treated groups and the natural estrus/coitus-induced ovulation cats. Mean ( $\pm$  SEM) progesterone in individual CL was abnormally low ( $P < 0.05$ ) in LOW ( $86.4 \pm 11.7$  ng/mg), ALT HIGH ( $118.7 \pm 6.6$ ), and HIGH ( $118.5 \pm 6.3$ ) cats, compared to ALT LOW ( $158.5 \pm 12.9$ ) and naturally-bred cats ( $185.2 \pm 17.8$ ; Fig. 3.9).

## **Discussion**

This study was significant because it supports the use of oral progestins before exogenous gonadotropin treatment in the domestic cat. Oral progestin prevented spontaneous ovulation, and laparoscopic ovarian data confirmed that response to eCG and hCG was less variable when preceded by a regimen of ALT. Non-invasive fecal steroid monitoring further substantiated this assertion, demonstrating multiple adverse scenarios in unprimed females that were prevented by ovarian down-regulation with ALT prior to gonadotropin stimulation. This study also established the foundation for examining proposed mechanisms for ovarian sensitivity in the progestin-treated cat. These findings support the use of lower eCG and hCG dosages in future applications, thereby lessening adverse fertility effects associated with their persistence in circulation following ovulation induction. Perhaps the most remarkable finding was that CL progesterone production is impeded in unprimed, gonadotropin-treated individuals, but can be mitigated with oral progestin priming.

In conducting this study, we were able to increase our understanding of how oral progestins act on the feline hypothalamic-pituitary-gonadal axis to suppress reproductive steroid production, subsequently halting folliculogenesis. Our data lend support to

previous work which has shown that both oral progestins (Chapter 2) and progestin implants (Pelican et al., 2005) prevent novel follicular recruitment in the domestic cat. However, progestin priming is unable to override or even attenuate a follicular phase when it is already in progress. This is consistent with findings in other species, where it has been demonstrated that the role of endogenous progestins in folliculogenesis is minimal (Drummond, 2006). Instead, we suspect that ALT acts differentially on GnRH, FSH and LH production (as well as other yet-unknown regulators of reproductive steroid production) to influence these dissimilar responses (Chapter 2). In other mammalian species, a multitude of paracrine factors (Monniaux et al., 1997; Hillier, 2001), as well as actions by the oocyte itself (Eppig, 2001), have been implicated in regulation of follicular development.

The current study supported previous findings that progestin priming prevents spontaneous ovulation by eliminating novel folliculogenesis (Pelican et al., 2005; Pelican et al., 2007). However, it does not affect the duration or amplitude of an existing luteal phase. Understanding the underlying mechanism(s) for this action is impeded by a lack of comprehensive information on luteotrophic and luteolytic agents in the domestic cat (Goodrowe et al., 1989; Verstegen et al., 1993). LH is one factor implicated in luteal function in the cat, suggesting that progestins act more at the level of the hypothalamus, versus the pituitary, to mediate ovarian suppression (Pelican et al., 2007). This is observed in the mare, where ALT has little or no effect on LH production (Squires et al., 1983). Prolactin also has been named a luteotrophic factor in the cat (Verstegen et al., 1993), suggesting that lactotroph cells are not likely to be influenced by oral progestin priming. Regardless of the mechanism, the duration of progestin priming used in this study was adequate to allow a return and maintenance of baseline estrogens and progestins by the time of ovulation induction. Based on the results, a shorter duration for progestin priming would be insufficient and a longer duration unnecessary.

This research has provided a greater understanding of ovarian sensitivity to exogenous gonadotropins in the cat. Across wild felid species, variable sensitivity to gonadotropin dosages has long been a significant roadblock to developing successful ovulation induction and AI protocols. Effective gonadotropin dosages are not based on animal weight. For example, why does the ~10 kg ocelot require 500 IU eCG to stimulate adequate follicular development when only 200 IU eCG is sufficient for the ~35 kg cheetah? (Swanson et al., 1996b; Howard et al., 1997). Genetics and ovulation mechanism (spontaneous versus induced) both are suspected factors in this phenomenon. The most striking support for a genetic component is an interesting relationship that has been observed in neotropical felid species in South America, where decreased ovarian sensitivity to gonadotropins is observed across the *Leopardus* genus (Swanson and Brown, 2004). Data relating ovarian sensitivity to ovulation mechanism are equally convincing.

In domestic cats, differential sensitivity to gonadotropins can relate back to the ovulation mechanism of that particular individual, where cats primed with either endogenous progestins (via spontaneous ovulation) or exogenous progestins (via progestin implants) are subsequently more sensitive to exogenous gonadotropins (Pelican et al., 2007). Our current data support this finding, as does research in wild felids, where induced ovulators such as the tiger (Graham et al., 2006) and ocelot (Swanson et al., 1996b) generally require much higher dosages compared to spontaneous ovulators like the clouded leopard (Brown et al., 1995) and fishing cat (Bauer et al., 2004). Follow-up studies will be required to confirm these findings in other species and to investigate the molecular basis for this phenomenon. Of particular interest would be to characterize changes in endogenous steroid and gonadotropin receptor populations, if any, following varied exogenous hormone regimens (Pelican et al., 2007).

The interaction between oral progestin priming and exogenous gonadotropin treatment and its subsequent effect on luteal sufficiency was an important endpoint examined in this study. While previous studies in the cat demonstrated that luteal progesterone concentrations are similar following exogenous gonadotropin treatment compared to those reported in naturally-mated queens (Swanson et al., 1995b; Roth et al., 1997b), the current study does not support these findings. Instead, both gonadotropin dosage and progestin priming appear to influence subsequent luteal function.

It is not surprising that exogenous gonadotropins affected luteal function, given similar observations in humans following ovulation induction (Tavaniotou et al., 2001; Tavaniotou et al., 2002). However, data also showed that progestin priming mitigates luteal insufficiency caused by exogenous gonadotropins. This appears to be a species-specific finding, since progestin pre-treatment has been linked to reduced luteal steroidogenesis in other species (Hunter et al., 1986). While the control of luteal progesterone production is not well-characterized in the cat, key regulators in other species include LH, prolactin, estradiol, growth hormone, androgens and progesterone (Berisha et al., 2002; Niswender, 2002; Stocco et al., 2007). Future studies will be needed to characterize expression of these factors in cats following exogenous hormone treatment and also to examine correlations, if any, between ovarian morphology and subsequent luteal function. More direct effects on cholesterol biosynthesis and the steroidogenic pathway also should be examined (Drouineaud et al., 2007).

Finally, our finding that ALT did not to reduce ancillary folliculogenesis and ovulation observed on Day 17 was unexpected, since progestin implants mitigate this phenomenon in the cat (Pelican, 2002). However, despite observations of ancillary ovarian activity in all treatment groups, there were no subsequent effects on either the duration of the gonadotropin-associated follicular phase or the shift to progestin



dominance following ovulation. Some alterations were observed in cats receiving higher gonadotropin dosages, and these trends are consistent with a previous report in the cat that documented endocrine perturbations (sustained estrogen elevations, abnormally-elevated fecal progestins) following gonadotropin stimulation with comparable dosages (Graham et al., 2000). It should be noted that mean progestins during the gonadotropin-induced luteal phase could not be characterized in the current study because females underwent ovariohysterectomy ~ 2 weeks after the start of that luteal phase. While specific effects of accessory CL development on fertility require further investigation, ancillary follicles and CL have been loosely associated with disruptions to the maternal-fetal environment during early pregnancy (Graham et al., 2000). Whether progestin priming can eliminate these potential disruptions remains to be determined.

In conclusion, the ultimate goal of any ovulation induction protocol for AI is to mimic a natural follicular and luteal phase as closely as possible without affecting the delicate endocrine balance during early embryonic development, endometrial remodeling and implantation. This study demonstrated that oral progestin priming eliminates spontaneous ovulation and enables a consistent ovarian response to ovulation induction in the cat. Furthermore, an ovulation induction regimen has been determined that enables CL progesterone production that is comparable to naturally-bred cats. Taken together, these results support the use of oral progestin priming in combination with minimal exogenous gonadotropin dosages for ovulation induction in the domestic cat. Research is underway to examine the impact of this regimen on incidence of fertilization and implantation following AI. Concurrent with this, parallel studies are planned to characterize the relationship between progestin priming and exogenous gonadotropins in the context of molecular control of luteal function in the cat.

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Table 3.1. Domestic cat reproductive traits before exogenous hormone treatment in induced versus spontaneous ovulators, assessed by longitudinal fecal steroid analyses ( $n = 19$  queens).

	Induced ( $n = 11$ )	Spontaneous ( $n = 8$ )
Anovulatory estrous cycle length (days)	16.3 ± 1.1	20.5 ± 2.4
Baseline estrogens (ng/g feces)	148.2 ± 12.8	139.4 ± 10.1
Duration of follicular phase (days)	5.5 ± 0.9	6.9 ± 0.8
Mean estrogens/follicular phase (ng/g feces)	253.6 ± 21.1	237.0 ± 22.8
Peak estrogens/follicular phase (ng/g feces)	301.1 ± 21.2	278.2 ± 19.0
Baseline progestins (µg/g feces)	2.4 ± 0.2 <sup>a</sup>	5.5 ± 0.9 <sup>b</sup>
Duration of luteal phase (days)	-----	35.4 ± 2.9
Mean progestins/luteal phase (µg/g feces)	-----	13.8 ± 2.5
Peak progestins/luteal phase (µg/g feces)	-----	27.2 ± 7.1

Values are means ± SEM. Within a row, means with different superscripts differ ( $P < 0.05$ ).

Table 3.2. Ovarian response to low and high dosages of exogenous gonadotropins in altrenogest (ALT)-primed versus unprimed females, as assessed by laparoscopic examination on Day 2 (Day 0 = day of hCG).

	ALT LOW ( <i>n</i> = 5)	LOW ( <i>n</i> = 5)	ALT HIGH ( <i>n</i> = 7)	HIGH ( <i>n</i> = 7)
Ovarian grade	1.8 ± 0.2 <sup>a</sup>	3.2 ± 0.4 <sup>b</sup>	1.4 ± 0.2 <sup>a</sup>	1.9 ± 0.3 <sup>a</sup>
Total no. of fresh and old CL/cat	12.4 ± 4.3	6.4 ± 2.3	15.1 ± 2.9	15.9 ± 1.4
Proportion of cats w/ fresh CL	5/5 (100%) <sup>a</sup>	3/5 (60%) <sup>b</sup>	5/7 (100%) <sup>a</sup>	5/7 (100%) <sup>a</sup>
No. of fresh CL/cat	12.4 ± 4.3 <sup>a,b</sup>	2.8 ± 1.4 <sup>b</sup>	15.1 ± 2.9 <sup>a</sup>	14.1 ± 2.4 <sup>a,b</sup>
Diameter of fresh CL (mm)	3.6 ± 0.2	3.8 ± 0.1	2.8 ± 0.2	3.2 ± 0.2
Proportion of cats w/ old CL	0/5 (0%) <sup>a</sup>	3/5 (60%) <sup>b</sup>	0/7 (0%) <sup>a</sup>	2/7 (28.6%) <sup>a,b</sup>
No. of old CL/cat	0	3.6 ± 1.8	0	1.7 ± 1.1
Total no. of follicles/cat	4.8 ± 1.5	7.6 ± 1.7	3.7 ± 1.0	4.6 ± 1.6
Proportion of cats w/ mature follicles	4/5 (80%)	2/5 (40%)	2/7 (28.6%)	3/7 (42.9%)
No. of mature follicles/cat	3.4 ± 0.9	1.4 ± 1.2	0.9 ± 0.6	1.6 ± 0.9
Ovarian volume (mm <sup>3</sup> )	217.3 ± 23.4	200.5 ± 27.0	188.2 ± 13.6	217.5 ± 55.1
Oviduct diameter (mm)	3.1 ± 0.3	3.7 ± 0.5	2.9 ± 0.2	2.8 ± 0.3
Uterine horn diameter (mm)	7.0 ± 0.4	6.7 ± 0.5	6.6 ± 0.3	6.1 ± 0.4

Values are means ± SEM except for proportional data. Within rows, means with different superscripts differ (*P* < 0.05).

Table 3.3. Estrous cycle traits before and after exogenous gonadotropin treatment in altrenogest (ALT)-primed and unprimed females, as assessed by fecal steroid monitoring.

	Pre-treatment	Gonadotropin-induced
<u>ALT-primed females (n = 10)</u>		
Duration of follicular phase (days)	6.9 ± 0.8	6.2 ± 0.8
Mean estrogens/follicular phase (ng/g feces)	223.5 ± 17.8	242.5 ± 26.9
Peak estrogens/follicular phase (ng/g feces)	272.1 ± 15.5	321.0 ± 45.6
<u>Unprimed females (n = 8)</u>		
Duration of follicular phase (days)	5.1 ± 0.9	7.8 ± 1.5
Mean estrogens/follicular phase (ng/g feces)	274.7 ± 23.2	429.4 ± 96.1
Peak estrogens/follicular phase (ng/g feces)	314.3 ± 24.4	625.2 ± 170.4

Values are means ± SEM. Means were compared among treatments and across time ( $P > 0.05$ ). One female from the unprimed group was excluded because she had no ovarian activity prior to gonadotropin stimulation.

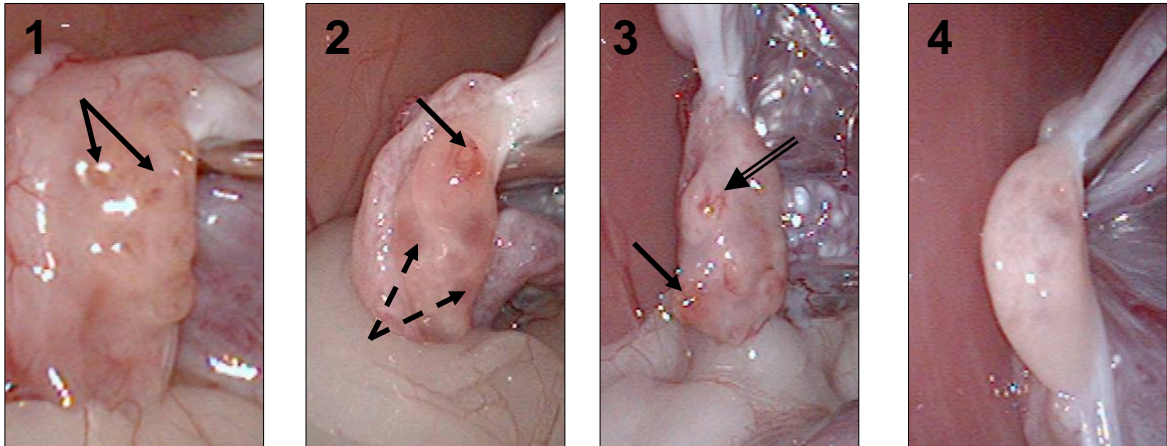


Fig. 3.1. Day 2 laparoscopic ovarian grading system. Ovarian response and gonadotropin-induced ovulation was categorized as (1) excellent, multiple fresh corpora lutea (CL) and no follicles > 2 mm (Grade 1); (2) good, mixed cohort of CL and follicles > 2 mm (Grade 2); (3) fair, variable-aged CL (Grade 3); or (4) poor, ovulation failure (Grade 4). Solid arrow denotes fresh CL, dashed arrow denotes mature follicles and double-lined arrow denotes old CL from a previous ovulation.

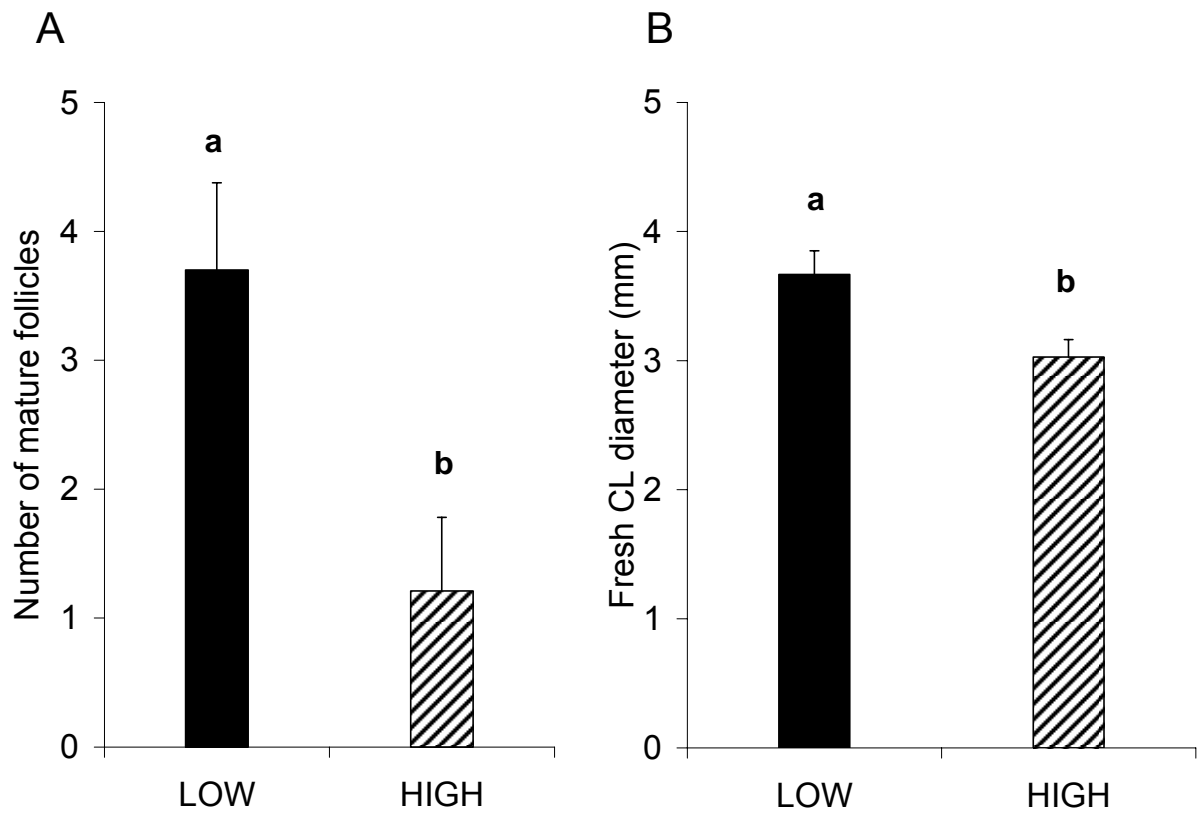


Fig. 3.2. Effect of low and high gonadotropin dosages on (A) diameter of fresh corpora lutea (CL); and (B) number of mature follicles on Day 2. Data were combined for ALT-primed and unprimed individuals. Means with different superscripts differ ( $P < 0.05$ ).

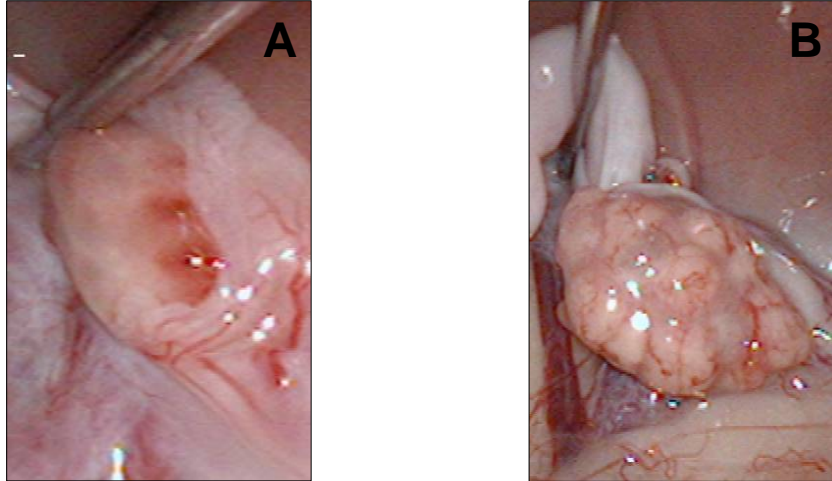


Fig. 3.3. Accessory corpora lutea (CL) development observed in representative laparoscopic photos of the right ovary from the same individual on (A) Day 2 and (B) Day 17.



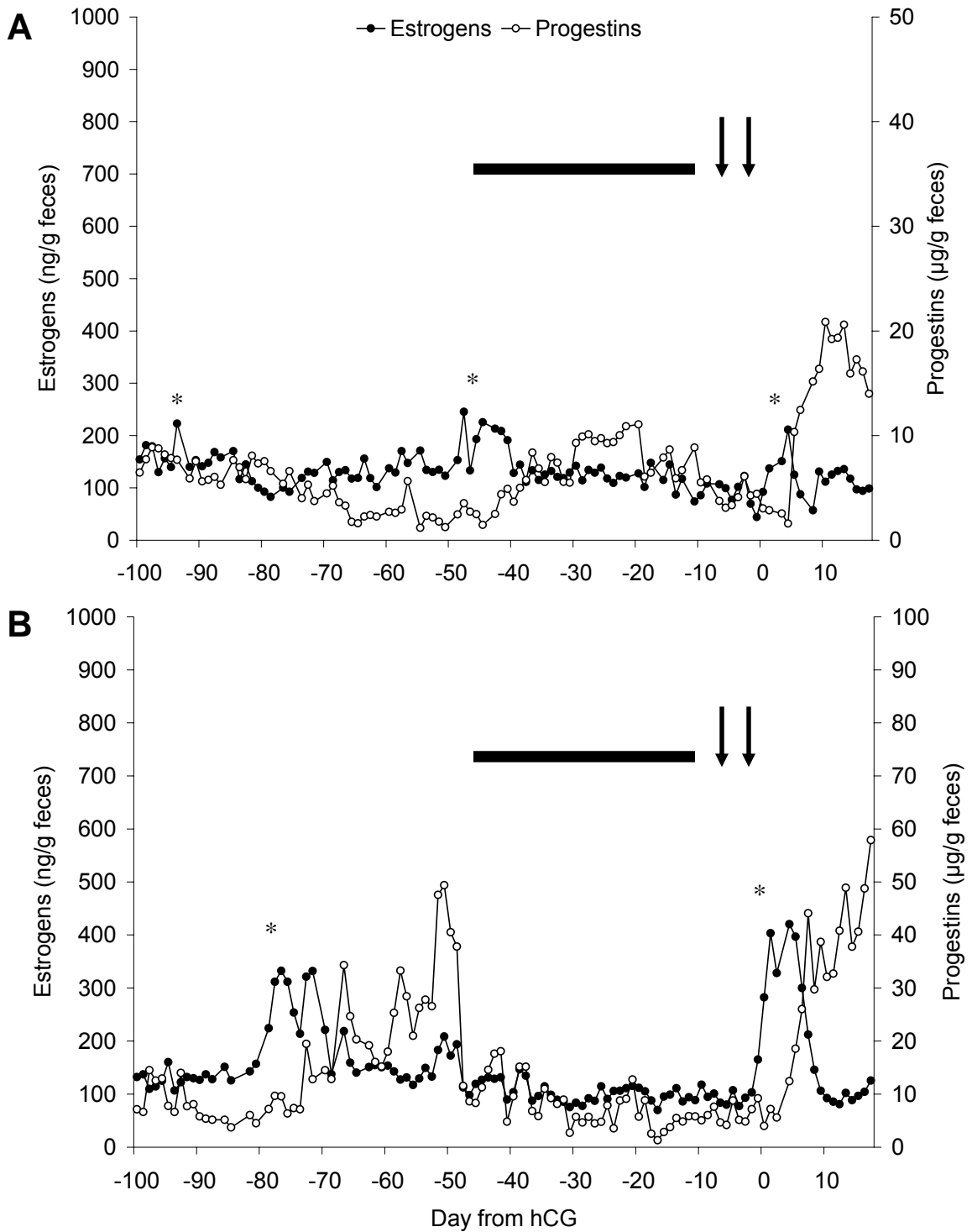
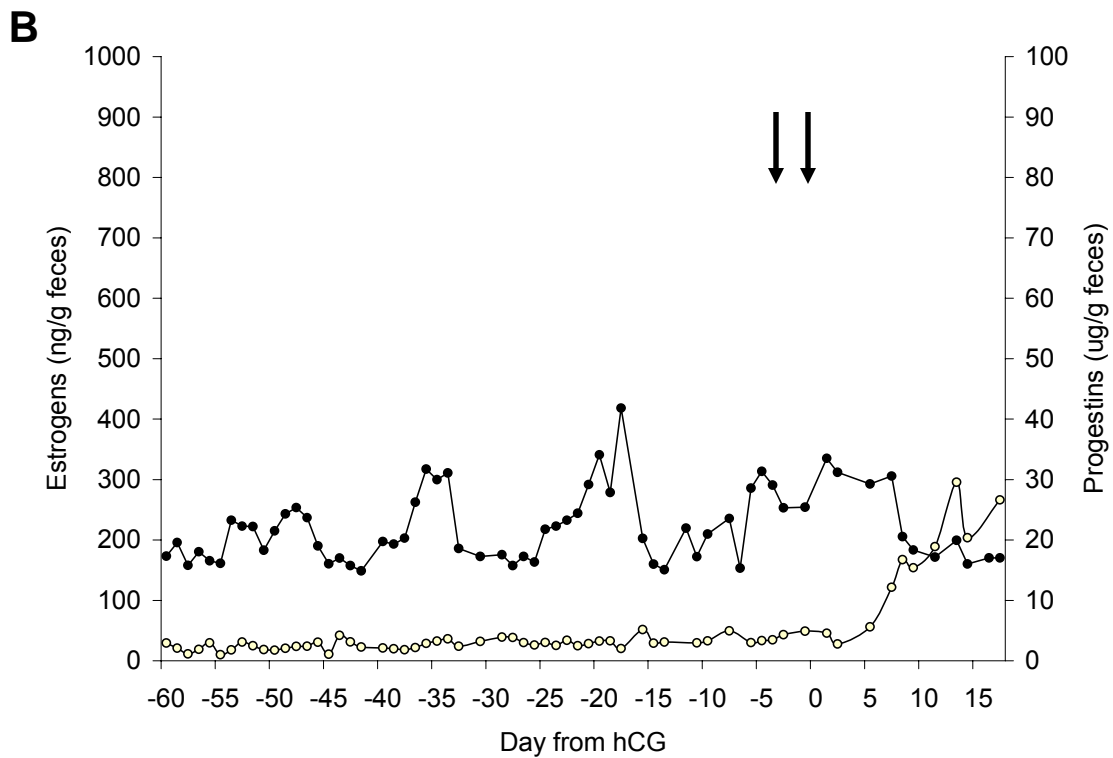
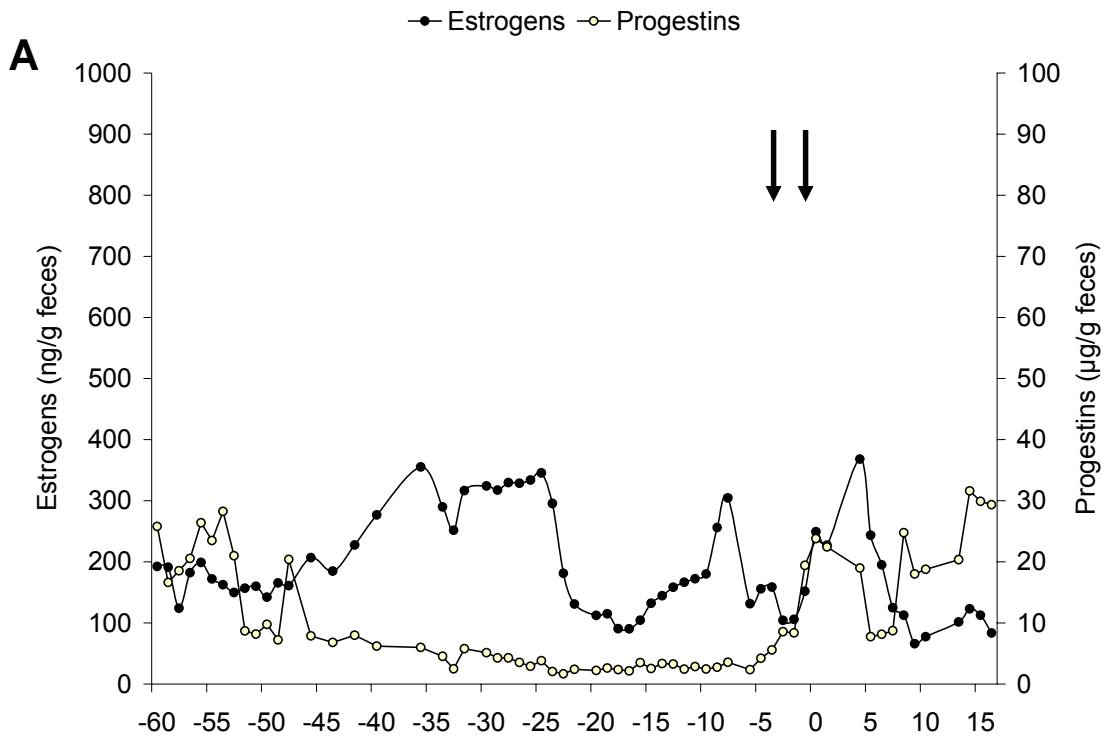


Fig. 3.4. Representative fecal steroid profiles in altrenogest (ALT)-primed females treated with LOW (50 IU eCG/37.5 IU hCG) gonadotropins. Queens were observed: (A) exhibiting elevated progestins at the time of ALT initiation; and (B) exhibiting elevated progestins before the time of ALT initiation. Asterisks indicate a follicular phase, solid bar represents ALT treatment and side-by-side arrows denote eCG/hCG injections. Axis was based on baseline hormone concentration for each individual.



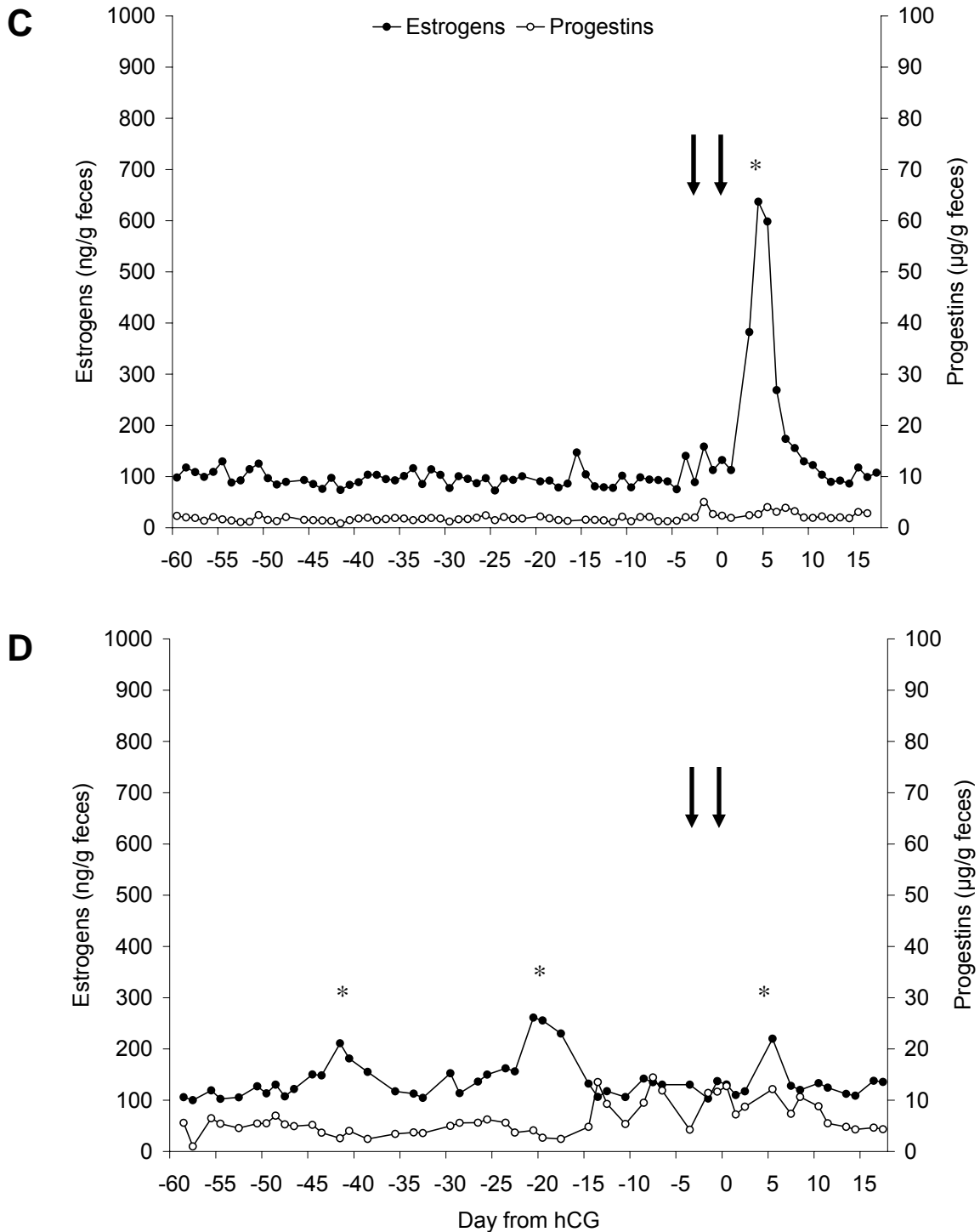


Fig. 3.5. Representative fecal steroid profiles in unprimed females treated with LOW (50 IU eCG/37.5 IU hCG) gonadotropins. Queens were observed (A) exhibiting elevated progestins at the time of eCG; (B) exhibiting elevated estrogens at the time of eCG; (C) exhibiting elevated estrogens at the time of eCG and subsequent ovulation failure; and (D) exhibiting elevated progestins at the time of eCG and subsequent ovulation failure. Asterisks indicate a follicular phase and side-by-side arrows denote eCG/hCG injections.

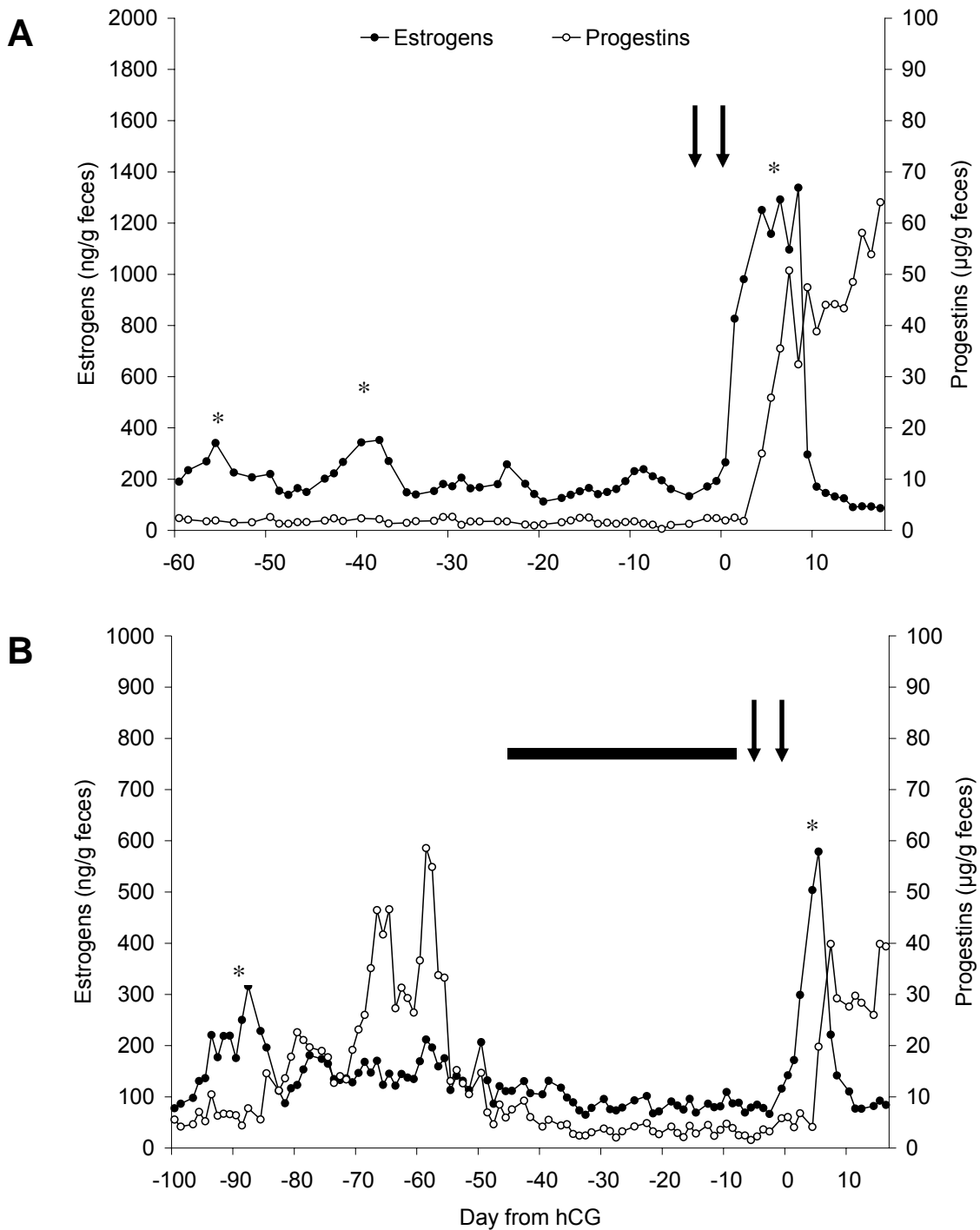


Fig. 3.6. Representative fecal steroid profiles in cats treated with HIGH (100 IU eCG/75 IU hCG) gonadotropins. (A) An unprimed HIGH female exhibiting elevated fecal estrogens following exogenous gonadotropins (note the scale on the y axis) and (B) an altrenogest (ALT) HIGH female exhibiting normal fecal estrogens following exogenous gonadotropins. Asterisks indicate a follicular phase, solid bar represents ALT treatment and side-by-side arrows denote eCG/hCG injections.

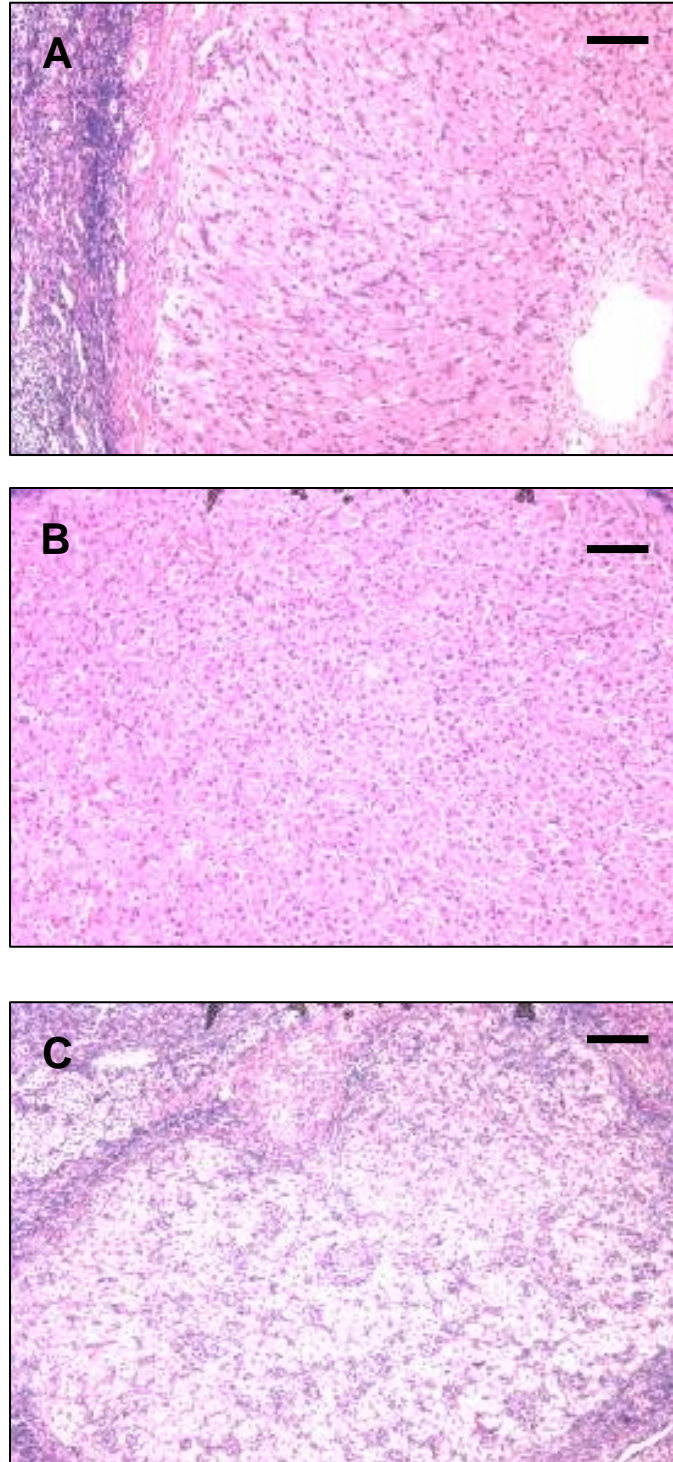


Fig. 3.7. Corpora lutea (CL) histomorphology on Day 17. (A) CL at this time point were large and expansile with no central cavity, smooth edges and a uniform population of polygonal cells. (B) In two females, CL with central vacuoles were noted. (C) Regressing CL were noted in several cats demonstrating spontaneous ovulation before ovulation induction. These CL were irregularly-shaped with prominent lipid vacuoles spanning the luteal tissue. Bar represents 100  $\mu$ m.

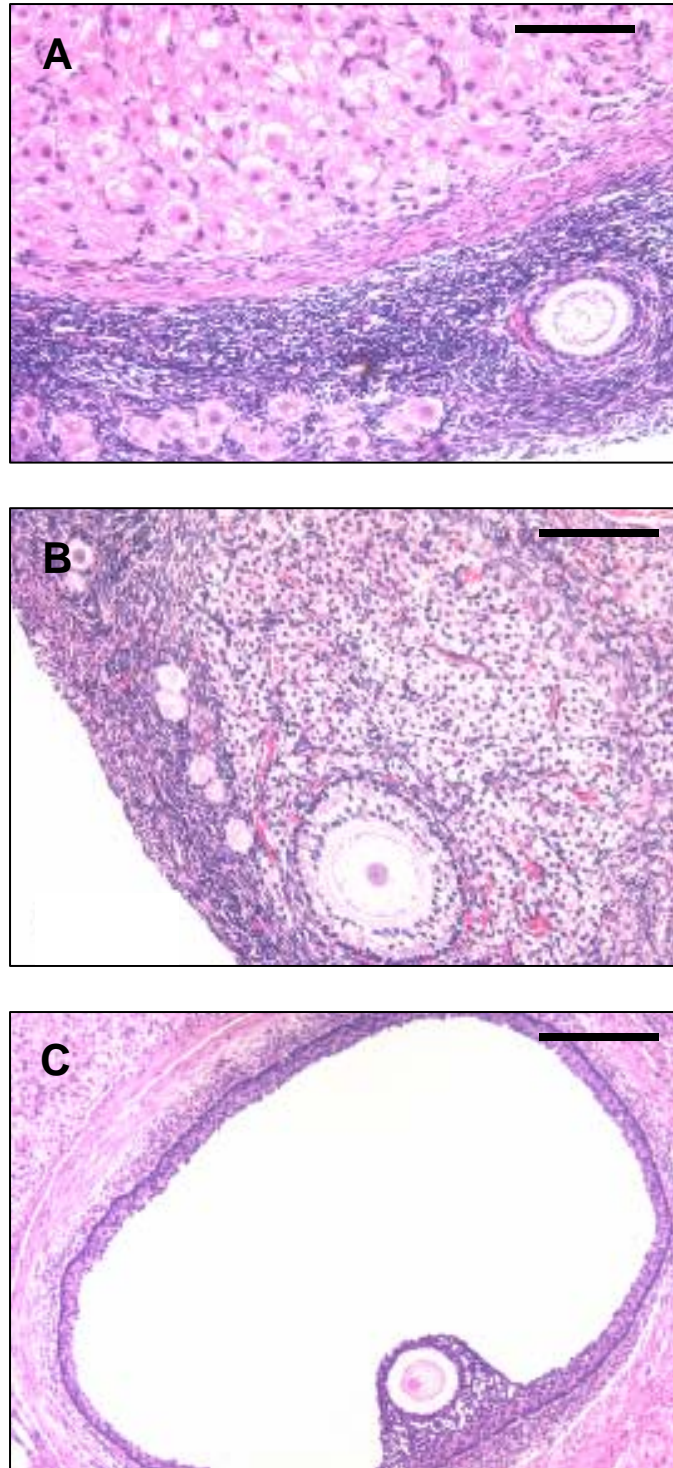


Fig. 3.8. Histomorphology of ovarian follicles observed on Day 17. (A) Mixed populations of primordial and primary follicles were frequently observed in the ovarian cortex. (B) A secondary follicle with several granulosa cell layers surrounding the oocyte. (C) A tertiary follicle with well-differentiated granulosa and theca cells and a large, distinct antrum. Bar represents 100  $\mu$ m.

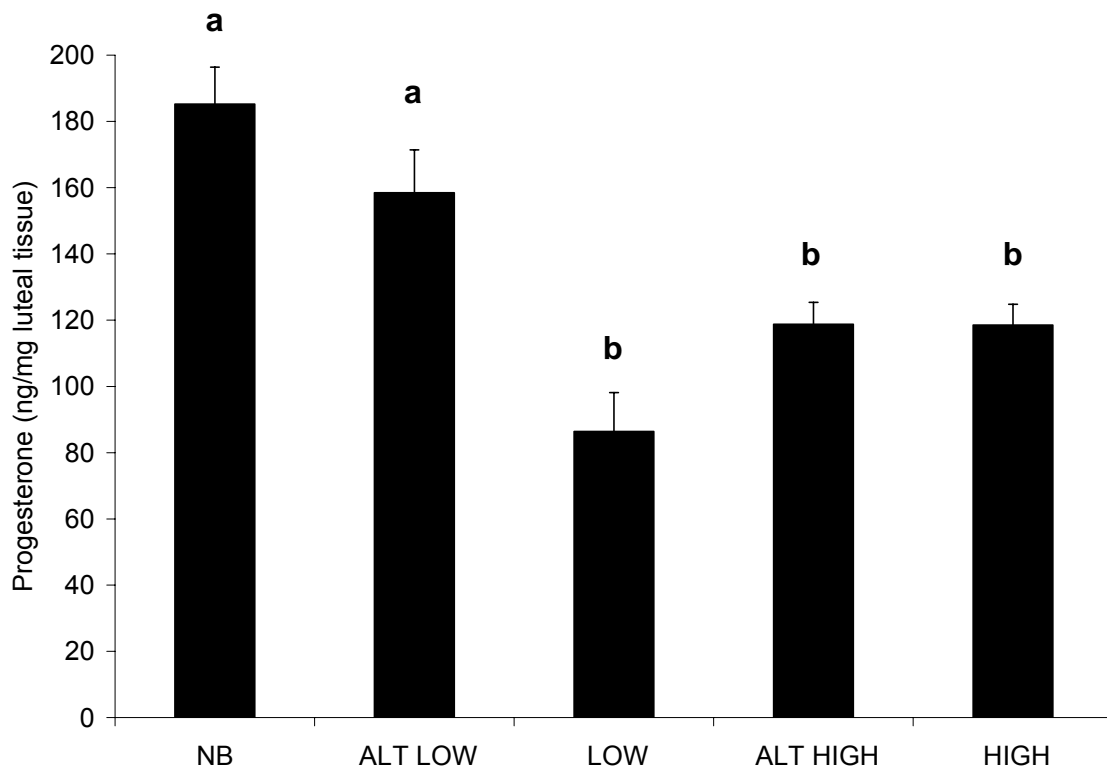


Fig. 3.9. Luteal progesterone concentration in individual corpora lutea recovered on the day of ovariectomy (Day 17 post-hCG or natural breeding; NB). Cats undergoing hormone treatment were administered low (50 IU eCG/37.5 IU hCG) or high (100 IU eCG/75 IU hCG) gonadotropins with or without altrenogest (ALT) treatment. Means with different superscripts differ ( $P < 0.05$ ).

## CHAPTER 4

### ORAL PROGESTIN PRIMING BEFORE EXOGENOUS GONADOTROPINS AND ARTIFICIAL INSEMINATION ENHANCES EARLY EMBRYONIC DEVELOPMENT AND LUTEAL FUNCTION IN THE CAT

#### Abstract

The effect of pre-treatment with oral progestin (altrenogest; ALT) before ovulation induction and artificial insemination (AI) was evaluated in the domestic cat to determine consequences on fertilization, early embryonic development and luteal progesterone production. Queens were randomly assigned to: (1) ALT + 50 IU equine chorionic gonadotropin (eCG) + 37.5 IU human chorionic gonadotropin (hCG;  $n = 8$  cats; ALT); or (2) 50 IU eCG + 37.5 IU hCG ( $n = 8$ ; control). Ovarian response was assessed 2 days after hCG; queens with fresh corpora lutea (CL) were inseminated *in utero* with fresh sperm. ALT priming enabled a consistent ovarian response to ovulation induction, whereas 25% of controls had variable-aged CL on Day 2. Ovariohysterectomy and oviduct flushing was performed on Day 5 to recover embryos and unfertilized oocytes (UFOs). Individual CL were enucleated from one ovary and assayed for progesterone concentration. Between treatments, no difference ( $P > 0.05$ ) in the number of UFOs recovered was observed. ALT embryos ranged in developmental stage from two to 16 cells, while control embryos ranged from two to eight cells. The distribution of embryos across developmental stages differed ( $P < 0.05$ ) between treatments, with more consistent development observed in ALT-primed females. Following *in vitro* culture, none of the 2-4 cell embryos progressed to morula. Of the remaining embryos at 5-16 cells, 86.7% of ALT and 62.5% of control embryos ( $P > 0.05$ ) developed to morulae or



blastocysts. Luteal progesterone was higher ( $P < 0.05$ ) in control ( $72.4 \pm 5.8$  ng/mg) versus ALT ( $52.2 \pm 5.5$  ng/mg) cats, and also higher than historical data from pregnant naturally-bred cats at a corresponding time interval ( $\sim 50$  ng/mg). In total, these data provide evidence for enhanced ovarian response and improved embryonic development following short-term ALT priming prior to ovulation induction and AI in the domestic cat.

## Introduction

Most of the 39 species in the Felidae family are threatened by extinction because of habitat loss and anthropogenic pressures (Seidensticker and Lumpkins, 1991; Wozencraft, 2005). Sustaining healthy *ex situ* populations of these endangered felids is a high priority of conservation specialists and the zoological community (Swanson, 2006). Equally important is the value in maintaining rare domestic cat biomedical models used to advance our understanding of diseases including HIV/AIDS, obesity and diabetes (O'Brien et al., 2002).

While free-ranging cats have a reputation for reproducing prolifically, this is not the case for domestic and wild felids in managed breeding programs. These cats often experience genetic or behavioral incompatibility (Wildt and Roth, 1997), and the disease status of certain domestic cats used in biomedical research can compromise reproductive performance (Magarey et al., 2006). Assisted reproduction techniques can be used to help maintain gene diversity in these populations. Accordingly, protocols for laparoscopic artificial insemination (AI) and *in vitro* fertilization (IVF)/ embryo transfer (ET) have been developed and refined for multiple cat species (Howard, 1999; Pelican et al., 2006b; Pope et al., 2006b). In theory, assisted reproduction combats reduced reproductive performance while allowing the introduction of under-represented genes into genetically-stagnant populations (Pukazhenthil and Wildt, 2004). Yet in practice,

incidence of pregnancy following assisted reproduction is too low in most felids (< 5%) to impact gene diversity, with the exception being the cheetah (Howard et al., 1997).

Low pregnancy success following assisted reproduction is largely attributed to variable reproductive dynamics observed across members of the Felidae family. Although historically considered induced ovulators, many felid species (including the domestic cat) exhibit spontaneous ovulation with no discernable pattern of predictability (Brown, 2006). Intermittent spontaneous ovulation has a considerable negative impact on ovulation induction and assisted reproduction success, since individuals within the same species displaying divergent ovulation strategies can respond very differently when receiving the same ovarian stimulation regimen (Pelican et al., 2007). Much of this inconsistency in ovarian response is associated with the administration of gonadotropins during the luteal phase, when either variable-aged corpora lutea (CL) or failure to ovulate in response to exogenous gonadotropins results (Pelican et al., 2006b). Conversely, individuals undergoing ovulation induction during a pre-existing state of heightened follicular development are at risk for ovarian hyperstimulation. Thus, it is not surprising that felids with the highest pregnancy success after AI (cheetah, ocelot) also are species that are strict induced ovulators. In cheetahs, intermittent periods of anestrus marked by prolonged ovarian quiescence are observed, which allows for a consistent response to exogenous gonadotropins (Howard et al., 1992b; Swanson et al., 1996b).

Even when ovulation induction coincides with a period of interestrus in cycling cats and results in a good ovarian response, numerous factors can lead to pregnancy failure following AI. Some of these factors are easily controlled for, such as poor sperm quality, while other factors including reduced oocyte quality, delayed oviductal transport, ancillary folliculogenesis/ovulation and abnormal endocrine dynamics are more difficult to manage (Pelican et al., 2006b). Prolonged actions of exogenous gonadotropins

(equine chorionic gonadotropin; eCG and human chorionic gonadotropin; hCG) after ovulation induction are known to influence these factors and impair fertility (Brown et al., 1995; Roth et al., 1997b; Swanson et al., 1997; Graham et al., 2000). Subsequently, it appears that limiting exposure to exogenous gonadotropins may be beneficial. Short-term treatment with the oral progestin altrenogest (ALT) suppresses folliculogenesis and prevents spontaneous ovulation in the cat (Chapter 2). Oral progestin priming also enables a consistent ovarian response to ovulation induction and increases ovarian sensitivity to exogenous gonadotropins, allowing for lower dosages of eCG/hCG for ovarian stimulation (Chapter 3). However, it is not clear if progestin priming affects pregnancy success, or if progestins are capable of mitigating adverse effects attributed to the prolonged action of exogenous gonadotropins.

The current study was designed to evaluate the effects of oral progestin priming and ovulation induction on AI success in a research population of domestic cats known to exhibit induced or spontaneous ovulation (Chapter 3). This population serves as an excellent model for wild felids that spontaneously-ovulate. Specific effects of oral progestin priming were evaluated relative to: (1) *in vivo* fertilization success and oviductal transport; (2) *in vitro* early embryonic development; and (3) luteal progesterone production following AI. Data also were compared to historical data from naturally estrual, mated queens at a corresponding time interval (Roth et al., 1994; Swanson et al., 1994; Swanson et al., 1995b). This study contributes to our overall objective of understanding feline ovarian function and embryonic competence following exogenous hormone stimulation, to ultimately improve assisted reproduction protocols in domestic and non-domestic felids.

## **Materials and Methods**

### ***Animals***

Sixteen adult (1-3 years) female domestic cats were housed under artificial fluorescent illumination (12L:12D) and provided dry food (Purina ONE®, Nestlé Purina PetCare Co., St. Louis, MO) with ad libitum water. Ten queens were nulliparous and six were proven breeders. Two proven males (4-5 years old) were housed individually in an adjacent room. Research activities were approved by the CRC's Institutional Animal Care and Use Committee (IACUC; # 05-25) and the University of Maryland IACUC (R-06-06). Housing was in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

### ***Exogenous hormone administration***

Queens were randomly assigned to: (1) ALT + 50 IU eCG + 37.5 IU hCG ( $n = 8$ ; ALT); or (2) 50 IU eCG + 37.5 IU hCG ( $n = 8$ ; control), and none had been previously exposed to exogenous progestins or gonadotropins. Treatment groups were blocked by parity and replicated over time with pairs (ALT versus control) undergoing gonadotropin stimulation, AI, spay and embryo culture on the same day. ALT oral suspension (0.088 mg/kg; Regu-Mate®; Intervet Inc., Millsboro, DE) was administered daily in 5 g wet food (Friskies®; Nestlé Purina) for 38 days; controls received wet food only (Chapter 2). Lyophilized eCG (Sigma-Aldrich Corporation, St. Louis, MO) and hCG (Sigma-Aldrich) were solubilized in sterile, preservative-free saline to a concentration of 125 IU/ml and stored frozen in individual syringes at -20°C until use. Three days after the last ALT treatment, cats received 50 IU eCG intra-muscularly (i.m.; Day -3) followed 80 hours later by 37.5 IU hCG i.m. (Day 0).

### ***Semen collection and analysis***

Semen was collected using a standard electroejaculation protocol from two proven breeder males on each day of AI (Howard, 1992). Briefly, males were anesthetized with a single i.m. injection of ketamine hydrochloride (5 mg/kg; Ketaved; Vedco Inc., St. Joseph, MO) combined with xylazine (1-2 mg/kg; AnaSed; BenVenue Laboratory, Bedford, OH). A one cm rectal probe delivered three series of low voltage electrical stimuli to the accessory sex glands (30 stimuli/series). Semen was collected into a warmed, sterile vial and immediately diluted in 25 mM HEPES-buffered Ham's F10 culture medium (Irvine Scientific, Santa Ana, CA) supplemented with 0.284 mg/ml glutamine, 0.026 mg/ml pyruvate, 130 IU/ml penicillin, 0.13 mg/ml streptomycin, 0.26 mg/ml neomycin (Sigma-Aldrich) and 5% (v:v) fetal calf serum (FCS; Irvine; HF10). An aliquot (3-5  $\mu$ l) of raw semen from Series 2 was removed before dilution in HF10 and fixed in 0.3% glutaraldehyde (pH 7.4, 340 mOsm) and 4.0% paraformaldehyde (Fisher Scientific Company, Pittsburgh, PA) for morphology and acrosome assessments, respectively. Following semen collection, males received subcutaneous fluids and 0.1 mg/kg yohimbine (Yobine; Lloyd, Shenandoah, IA) for reversal of anesthesia. A minimum 1 week interval was allowed between consecutive semen collections.

Diluted aliquots were immediately assessed under phase contrast microscopy (100x) for sperm motility (0 to 100%) and forward progressive status (scale; 0-5, 0 = no movement, 5 = rapid, forward progression). Aliquots displaying less than 70% motility or a status lower than three were discarded. Sperm concentration was determined by diluting 5  $\mu$ l sample in a red blood cell counting chamber (Unopette; Becton Dickinson and Co., Franklin Lakes, NJ) and then counting using a hemocytometer (Howard, 1993) (Table 4.1). Ejaculates from each male were pooled, centrifuged (300g, 8 min) and re-suspended in ~ 200  $\mu$ l fresh HF10. The supernatant was re-centrifuged (300g, 8 min),

and the second sperm pellet was combined with the re-suspended sperm. Additional HF10 was added as needed to obtain a final volume of 420  $\mu$ l. A 20  $\mu$ l aliquot was removed for final assessments of the sample for AI (sperm concentration, motility, status, morphology, acrosomal integrity; Table 4.1), and the remaining 400  $\mu$ l was divided into two equal aliquots for two AI, maintained at ambient temperature and shielded from light until insemination.

Sperm fixed in 0.3% glutaraldehyde were assessed for structural morphology using phase contrast microscopy at 1,000x (Howard, 1993). A total of 200 sperm per aliquot were classified either as normal or as having one of the following abnormalities: (1) head defects including macrocephaly, microcephaly, bicephaly, bent neck or damaged acrosome; (2) midpiece anomalies including bent midpiece, bent midpiece with cytoplasmic droplet, midpiece aplasia or damaged midpiece; or (3) flagellar deformities including tightly coil flagellum, bent flagellum without cytoplasmic droplet, bent flagellum with cytoplasmic droplet, proximal cytoplasmic droplet or distal cytoplasmic droplet.

Sperm fixed in 4% paraformaldehyde were stained with Coomassie blue to assess acrosomal integrity (Crosier et al., 2007). Briefly, fixed sperm were centrifuged for 8 min at 2000g, and the sperm pellet was gently resuspended in 500  $\mu$ l 0.1 M ammonium acetate. The suspension was re-centrifuged, washed two additional times in ammonium acetate (8 min, 2000g) and the supernatant was removed to leave a final volume of  $\sim$  50  $\mu$ l. The suspension was split and smeared onto two microscope slides, dried on a 37°C slide warmer, flooded with Coomassie blue stain (Fisher Scientific) in solution (Larson and Miller, 1999) for 90 s and rinsed thoroughly with deionized water. A coverslip was mounted on the dried slide (Permount; Fisher Scientific), and slides were maintained overnight at 20°C before long-term storage at 5°C. For each sample, 100 spermatozoal acrosomes were assessed under bright-field microscopy at 1,000x.

Acrosomes were categorized as either: (1) intact; uniform blue stain encompassing the entire acrosomal region; (2) damaged; patchy blue staining pattern; or (3) non-intact; no stain observed in the acrosomal region.

### ***Laparoscopic AI***

Thirty-six to 40 hours after hCG, queens were anesthetized with an i.m. injection of 10 mg/kg ketamine hydrochloride combined with 1 mg/kg acepromazine maleate (Phoenix Pharmaceuticals, Inc., St. Joseph, MO). A surgical plane of anesthesia was maintained with isoflurane gas (Phoenix Pharmaceuticals), and the cat was placed in dorsal recumbancy for laparoscopic AI (Wildt et al., 1977). In brief, the surgical table was tilted with the animal's head down, and a two mm Verres probe was inserted through the abdominal wall to insufflate the abdomen with room air. Next, a five mm trocar/cannula was introduced midline cranial to the umbilicus, and a five mm laparoscope with an integrated camera system (Olympus Surgical and Industrial America Inc., Orangeburg, NY) was inserted through the cannula to visualize the reproductive tract.

Using the two mm Verres probe for reference, all ovarian structures were counted, described, measured and photographed taking care not to disrupt the fimbriae and oviduct (possibly containing ovulated oocytes). CL that were white and well-vascularized were termed 'old', and cats with old CL on Day 2 were classified as spontaneous ovulators. CL that were pink or red with developing vascularization were termed 'fresh', and presumed to be the result of gonadotropin stimulation. Follicles > 2 mm in diameter and demonstrating mild to moderate vascularization were classified as 'mature'. Overall ovarian response for gonadotropin-induced ovulation was graded on a scale of 1 to 4: (1) excellent; multiple fresh CL and no follicles > 2 mm (Grade 1); (2)

good; mixed cohort of fresh CL and follicles > 2 mm (Grade 2); (3) fair; variable-aged CL including fresh and old (Grade 3); and (4) poor; ovulation failure (Grade 4; Fig. 4.1). The Verres probe also was used to measure ovarian dimensions, oviductal diameter and uterine diameter, and ovarian volume was calculated using the formula for testes volume (length x width<sup>2</sup> x 0.524) (Howard et al., 1990).

Following assessment of ovarian response, queens with fresh CL were inseminated *in utero* (Howard et al., 1992a). A second five mm cannula was inserted midline caudal to the umbilicus, and a five mm Babcock grasper with ratchet handle was inserted through the cannula to stabilize the uterine horn and bring it to the body wall for intrauterine AI. A 20-gauge feline indwelling catheter was inserted percutaneously directly into the uterine lumen for intrauterine sperm deposition. An aliquot (100 µl) of washed sperm in a 1 ml syringe was delivered through PE 10 silastic tubing inserted through the catheter. The procedure then was repeated in the contralateral uterine horn.

### ***Ovariohysterectomy and tissue processing***

On Day 5 post-hCG, females underwent routine ovariohysterectomy using the same anesthesia protocol described previously for laparoscopy. Reproductive tissues were covered in sterile saline-soaked sponges during the procedure and maintained at 37°C after removal. The ovary was removed at the base, the mesosalpinx tissue carefully dissected to elongate the oviduct, and a hemostat was placed at the utero-tubal junction. The oviduct was flushed retrograde from the isthmus to the infundibulum using 5 ml warmed (37°C) HEPES-buffered Ham's F-10 medium with bovine serum albumin (BSA; 4 mg/ml) via a latex-free syringe and 25 gauge needle (Roth et al., 1997b). Oviducts were flushed 4-6 times in alternating order into individual sterile Petri dishes. Transverse sections of oviduct and uterus were fixed in 4% paraformaldehyde. Whole



CL from one ovary were excised and individually weighed. The ovary with more CL was chosen to maximize recovery of luteal tissue. Half of these CL were flash frozen for progesterone quantification. The remaining ovary was bisected, and one hemi-ovary was fixed in paraformaldehyde. Fixed ovary, oviduct and uterus samples were embedded in paraffin by a commercial company (HistoServ, Rockville, MD), sectioned midway through the specimen at 5µm and stained with hemotoxylin and eosin.

### ***Embryo culture and staining***

The oviductal flush medium was thoroughly searched to locate unfertilized oocytes (UFOs) and embryos. Viable embryos (2 cell or greater) were immediately placed in culture (Roth et al., 1994). Embryos were assessed for developmental stage at the time of flush and categorized as: (1) 2 cell; (2) 3-4 cell; (3) 5-8 cell; or (4) 9-16 cell. These categories were chosen to encompass each cell division from 2 to 16 cells. Based on the total number of embryos recovered (maximum 5 embryos/drop; minimum 10 µl medium/embryo), 10-50 µl culture drops were prepared under mineral oil using equilibrated Ham's F10 culture medium (no HEPES; Irvine Scientific) supplemented with 0.284 mg/ml glutamine, 0.026 mg/ml pyruvate, 100 IU/ml penicillin, 100 IU/ml streptomycin and 5% (v:v) FCS. Embryos in the flush media were washed three times in culture medium, transferred to drops and placed in an incubator (38.5°C; 5% CO<sub>2</sub> in air). Developmental progression was noted 2 and 4 days post-flush. On Day 4 of culture (or Day 2, if the embryo showed no signs of progression during the first 2 day period), individual embryos were washed three times in culture medium to remove residual mineral oil, dried at room temperature on a microscopic slide and fixed overnight in ethanol. Embryos were stained directly with Hoechst 33342 (1 µg/ml in PBS; Sigma) (Comizzoli et al., 2004). Stained nuclei were examined under epifluorescence to

determine final cell number. UFOs were fixed in 2.5% paraformaldehyde at 37°C for 30 minutes on the day of ovariohysterectomy and stored at 4°C for subsequent staining. Fixed oocytes were rinsed in 5% FCS in phosphate buffered saline (PBS) for 30 minutes at 20°C and stained with Hoechst as described above to determine chromatin status.

### ***Statistical analyses***

All percentage data were arcsine transformed before analysis. Laparoscopic ovarian data and CL progesterone concentrations were analyzed by one-way ANOVA followed by Tukey-Kramer honestly significant difference (HSD) multiple comparison tests. A chi-square contingency test was performed to compare the distribution in developmental stages between treatments on the day of flush. Differences in initial (day of flush) and final (post-culture up to 4 days) stage of embryonic development were compared between treatments using non-parametric Wilcoxon rank-sum tests. All statistical analyses were performed using JMP IN 5.1 (SAS Institute Inc., Cary, NC), and data were expressed as mean  $\pm$  SEM.

## **Results**

### ***Ovarian response at AI***

On the day of AI, evidence of spontaneous ovulation was not observed in any ALT-primed cat, whereas two of eight controls (25%) had old CL indicating an ongoing luteal phase at the time of ovarian stimulation (Table 4.2). Both control females with old CL also had fresh CL, presumed to be the result of exogenous gonadotropin treatment. Mean ovarian grade, number of follicles and number of fresh CL did not differ ( $P > 0.05$ ) between treatments (Table 4.2). Fifty percent of cats in both treatment groups had at

least one mature, vascularized follicle on Day 2 at the time of post-ovulatory AI. Ovulation failure was observed in two of eight (25%) ALT and one of eight (12.5%) control females; thus, a total of 6 ALT females and 7 controls were inseminated. Although sperm output varied by day, there was no correlation with fertilization success, with all females receiving a minimum of  $12.8 \times 10^6$  motile fresh sperm. Mean ejaculate traits of the pooled sperm samples for AI are summarized in Table 4.1.

### ***Embryo recovery and culture***

On Day 5, overall recovery (total number embryos and oocytes divided by number of CL) was  $68.9 \pm 21.7\%$  in ALT cats and  $67.4 \pm 16.3\%$  in controls. Embryos were recovered from five of six (83.3%) ALT cats and five of seven (71.4%) controls ( $P > 0.05$ ; Fig. 4.2). ALT embryos ranged in developmental stage from two to 16 cells, while control embryos ranged from two to eight cells. The distribution of embryos across developmental stages differed ( $P < 0.05$ ) between treatments, with more consistent development observed in ALT-primed females (Fig. 4.3). UFOs were retrieved in 66.7% of ALT cats and 71.4% of controls, and the mean number of UFOs was similar ( $P > 0.05$ ) in ALT ( $2.5 \pm 1.2$ ) versus control cats ( $2.7 \pm 1.9$ ). Of the total number of UFOs recovered, five of 14 ALT oocytes and 4 of 15 control oocytes had reached metaphase II (Fig. 4.4A). The remaining UFOs were degenerate, with an absence of chromatin and fragmented cytoplasm. Following culture *in vitro*, none of the 2-4 cell embryos progressed to morula. Of the remaining embryos, 86.7% of ALT and 62.5% of control embryos progressed to morulae or blastocysts (Fig. 4.4 B-C). In one spontaneously-ovulating cat, one 2-4 cell embryo was recovered but did not develop in culture. No UFOs or embryos were recovered from the other spontaneous ovulator.

### ***CL progesterone and reproductive histology***

Luteal progesterone was abnormally higher ( $P < 0.05$ ) in controls ( $72.4 \pm 5.8$  ng/mg) than ALT cats ( $52.2 \pm 5.5$  ng/mg), compared to historical data from pregnant naturally-bred cats ( $\sim 50$  ng/mg; Fig. 4.5) (Swanson et al., 1995b). Representative histological sections revealed variable ovarian dynamics in both treatment groups. Many CL on Day 5 had irregular margins with a visible, open central cavity and a mixed population of fusiform and polygonal cells (Fig. 4.6A). The fusiform cells lay perpendicular to and radiated outward from the cavity before transitioning to a population of predominantly polygonal cells. In some females, corpora hemorrhagica (CH) were seen (Fig. 6B). These CH were relatively smaller in size with a blood-filled central cavity (a remnant of the antrum) and predominantly fusiform cells. Regressing CL were observed in both spontaneous ovulators in the control group (Fig. 6C). These older CL were morphologically-distinct from the younger CL, demonstrating prominent lipid vacuoles throughout the structure and irregular borders. Diverse follicular morphology was observed. All females demonstrated varied numbers of primordial and primary follicles populating the ovarian cortex (Fig. 4.7A), and many had at least one tertiary follicle with a large antrum and visible oocyte (Fig. 4.7B). Follicular atresia was observed in some cats (Fig. 4.7C). Representative oviduct and uterine cross-sections also were obtained from each individual (Fig. 4.8).

### **Discussion**

This study provides evidence that oral progestin priming before ovulation induction and AI not only prevents spontaneous ovulation in the cat, but also supports early embryonic development and luteal progesterone production that is comparable to naturally estrual, mated queens. Progestin priming cultivates a maternal environment

that is improved compared to individuals receiving exogenous gonadotropins only. Through the experimental design, we were able to gather important information on the critical pre-implantation period by assessing markers of both ovarian function and fertilization success, while maintaining our ability to continually monitor developmental competence of embryos *in vitro* after recovery. This study also has provided valuable tissue samples that can be used for future gene expression analyses designed to uncover the underlying mechanisms of luteal function and oviductal transport during early pregnancy in the cat.

Determining that ovarian response to exogenous gonadotropins was similar between progestin-treated and control females was unexpected, since previous work demonstrated that ALT-primed cats have an improved ovarian grade following ovulation induction compared to unprimed individuals when using a regimen of 50 IU eCG/37.5 IU hCG (Chapter 3). The relatively low incidence of spontaneous ovulation in the current study (25%) versus the previous study (41.7%; Chapter 3) is one explanation for this disparity, and is likely attributed to random chance. The finding that two of eight cats in the ALT group failed to ovulate in response to exogenous gonadotropins was not observed in the previous study. The two progestin-primed cats exhibiting ovulation failure displayed follicular growth but no pre-ovulatory follicles on Day 2, suggesting that hCG dosage may have been insufficient in these individuals to promote final follicular maturation and ovulation. Alternatively, eCG dosage may have been inadequate to develop these follicles to a point where they were responsive to hCG. Ovarian sensitivity to exogenous gonadotropins is different in induced versus spontaneous ovulators, but this variable sensitivity is mitigated by progestin pre-treatment (Pelican et al., 2007). Thus, it is more likely that subtle individual differences in ovarian sensitivity to near-threshold levels of exogenous gonadotropins caused this unusual response.

Understanding the mechanisms regulating altered ovarian sensitivity will require further investigation.

Whether oocytes exhibit decreased ability to undergo fertilization as a direct result of ovarian stimulation is not well characterized in the cat (Roth et al., 1994). In some domestic livestock and rodents, exposure to exogenous gonadotropins has been linked to poor oocyte quality (Moor et al., 1985; Hyttel et al., 1986; Yun et al., 1987). In the cat, exogenous gonadotropin treatment is associated with more unfertilized oocytes and fewer high-quality blastocysts following AI compared to naturally-bred queens, but good quality embryos still can be obtained and an ~ 50% pregnancy rate achieved after AI (Howard et al., 1992a; Roth et al., 1995). This may explain why litter size in felids undergoing assisted reproduction is generally smaller compared to their naturally-bred counterparts. In the current study, the mean number of embryos and unfertilized oocytes recovered on Day 5 (~ 108 hours post-hCG) was similar between treatments, and data were comparable to numbers recovered from naturally estrual, mated queens at a similar time point (100 hours post-coitus; mean  $3.3 \pm 0.8$  embryos and  $1.1 \pm 0.6$  UFOs) (Swanson et al., 1994). Our observations are in sharp contrast to data in cats receiving higher dosages of exogenous gonadotropins (100 IU eCG/75 IU hCG) followed by AI and pre-implantation ovariectomy, where mean embryo yield is  $17.8 \pm 5.6$  (Roth et al., 1997b). This is more than five times the current findings. Furthermore, cats with these excessive yields had higher proportions of fragmenting or degenerating embryos. This study supports the use of progestin priming and relatively lower gonadotropin dosages for AI, since embryo yields and subsequent viability are likely to be far more consistent with naturally-bred queens.

This study's focus on the oviduct, versus the uterus, was warranted by previous work investigating *in vivo* early embryonic development in both natural-bred and gonadotropin-treated cats (Swanson et al., 1994; Graham et al., 2000). We expected

embryos to be in the oviduct on Day 5 regardless of exogenous hormone treatment, and our findings were consistent with this hypothesis. Indeed, the amount of time the embryo remains in the oviduct before traversing the uterotubal junction is quite long in the cat (144-168 h) compared to species including the rabbit (56-62 h), mouse (72 h) and human (60-70 h) (Croxatto and Ortiz, 1975), making it a critical environment to investigate.

Exogenous gonadotropin treatment has been associated with delayed oviductal transport, where embryos produced by naturally-mated queens reach the uterus sooner than embryos produced following gonadotropin stimulation and AI (Graham et al., 2000). In the current study, we were unable to directly assess oviductal transport, but *in vivo* developmental rates were investigated, which is likely an important indicator of overall oviductal health and function. Development appeared somewhat retarded in both treatments compared to historical data, where 9-16 cell embryos are expected by Day 5. Yet overall embryonic development was more consistent in progestin-primed queens, compared to unprimed individuals.

Uncovering the key factors regulating developmental competence of embryos *in vivo* may be possible with follow-up gene expression studies in oviductal tissue. Of particular interest would be the expression of exogenous steroid receptors, as well as additional factors associated with steroid actions in the oviduct. For example, estradiol is a key regulator of oviductal function (Croxatto and Ortiz, 1975; Roblero and Garavagno, 1979; Bigsby et al., 1986; Zenteno et al., 1989) and can retard embryo transport (Herron and Sis, 1974) and alter oviductal cell function (Bareither and Verhage, 1981) in the cat. Subsequently, understanding the influence of exogenous gonadotropins on estradiol production, alone or concomitant with progestins, may be an important mechanism to investigate. There also is strong evidence of auto-regulatory

actions by the embryo itself on oviductal function in other species (Croxatto, 2002) that are still unstudied in the cat.

Developmental competence *in vitro* following embryo recovery was reduced in this study compared to historical data from naturally-bred cats, where 85-92% of embryos produced *in vivo* progress to morulae or blastocysts (Roth et al., 1994). Based on the total numbers of morulae/blastocysts produced, progestin-primed embryos appeared to perform better in culture than control embryos, but significant variability within individuals did not allow for a statistical difference between treatments. The apparent morula to blastocyst block observed in *in vitro*-produced embryos was not a factor in the current study (Johnston et al., 1991; Roth et al., 1994). In rodents, superovulation is associated with delayed embryo development and abnormal blastocyst formation both *in vivo* and *in vitro* (Molina et al., 1991; Ertzeid et al., 1993; McKiernan and Bavister, 1998; Van der Auwera and D'Hooghe, 2001), but there is no strong evidence for this in cats based on our results.

Luteal function was another key endpoint investigated in this study and will be the subject of significant follow-up work using archived tissue samples obtained during data collection. Results showed a relationship between progestin pre-treatment and luteal progesterone production following ovulation induction and AI. While progestin-primed individuals had CL progesterone concentrations comparable to historical data from pregnant naturally bred queens, controls displayed higher concentrations previously seen only in low fertility cats at a corresponding time point (Swanson et al., 1995b). This could be attributed to decreased consistency of the ovarian response in cats treated with exogenous gonadotropins alone. That higher levels of luteal progesterone are actually detrimental to fertility during early pregnancy is an interesting finding, particularly since these differences are only seen until Day 6 of gestation (Swanson et al., 1995b). Indeed, these observations demonstrate the considerable



effects that subtle differences in the maternal environment can have on fertility. They also serve as the foundation for future studies designed to investigate luteal function more closely in the context of cholesterol biosynthesis, steroidogenesis and hormone receptor expression. Furthermore, these data can be used to guide timing of progesterone supplementation following assisted reproduction.

Taken together, these findings support the incorporation of a short-term regimen of oral progestin before ovulation induction and AI in the cat. The ability to integrate the present results with previous findings in naturally-bred cats permitted several important comparisons that would not have been possible otherwise. While a strong link between progestin priming and improved AI success could not be made in this study, data support that it is no more detrimental than the effects of a gonadotropin-only regimen. At the least, achieving improved ovarian response at the time of AI makes this protocol worthwhile for future use. An important follow-up to this study will be to examine the influence of oral progestin priming on the peri-implantation maternal environment and incidence of implantation following AI.

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Table 4.1. Sperm traits from two males used as artificial insemination donors ( $n = 8$  pooled ejaculates).

<b>Variable</b>	<b>Mean <math>\pm</math> SEM</b>
Sperm concentration/ml ( $\times 10^6$ )	126.6 $\pm$ 15.1
Sperm motility (%)	76.3 $\pm$ 1.6
Sperm forward progressive status <sup>a</sup>	3.9 $\pm$ 0.1
Morphologically-normal sperm (%)	47.7 $\pm$ 1.2
Sperm acrosomal integrity (%) <sup>b</sup>	
Intact	76.5 $\pm$ 1.0
Damaged	18.9 $\pm$ 1.0
Non-intact	4.6 $\pm$ 0.7
Total motile sperm inseminated per female ( $\times 10^6$ )	22.2 $\pm$ 2.1

<sup>a</sup> Forward progressive status rated on a scale of 0-5, with 0 being no movement and 5 being rapid, forward progression.

<sup>b</sup> Acrosomal integrity was assessed using Coomassie blue stain under bright-field microscopy.

Table 4.2. Laparoscopic ovarian response on the day of artificial insemination in altrenogest (ALT)-primed versus unprimed females.

	ALT ( <i>n</i> = 8)	Control ( <i>n</i> = 8)
Ovarian grade	2.3 ± 0.4	2.3 ± 0.4
Total no. of CL/cat	5.5 ± 1.5	6.6 ± 1.5
Proportion of cats w/ fresh CL	6/8 (75%)	7/8 (87.5%)
No. fresh CL/ cat	5.5 ± 1.5	6.0 ± 1.5
Diameter of fresh CL (mm)	3.7 ± 0.1	3.5 ± 0.2
Proportion of cats w/ old CL	0/8 (0%)	2/8 (25%)
No. old CL/cat	0	0.6 ± 0.5
Total no. follicles/cat	4.9 ± 2.3	4.8 ± 1.8
Proportion of cats w/ mature follicles	4/8 (50%)	4/8 (50%)
No. mature follicles/cat	1.3 ± 0.6	3.0 ± 1.5
Ovarian volume (mm <sup>3</sup> )	170.3 ± 11.5	225.0 ± 46.1
Oviduct diameter (mm)	2.7 ± 0.3	3.0 ± 0.2
Uterine diameter (mm)	6.7 ± 0.2	6.6 ± 0.4

With the exception of proportional data, values are means ± SEM. Means and percentages were similar (*P* > 0.05) among treatments.

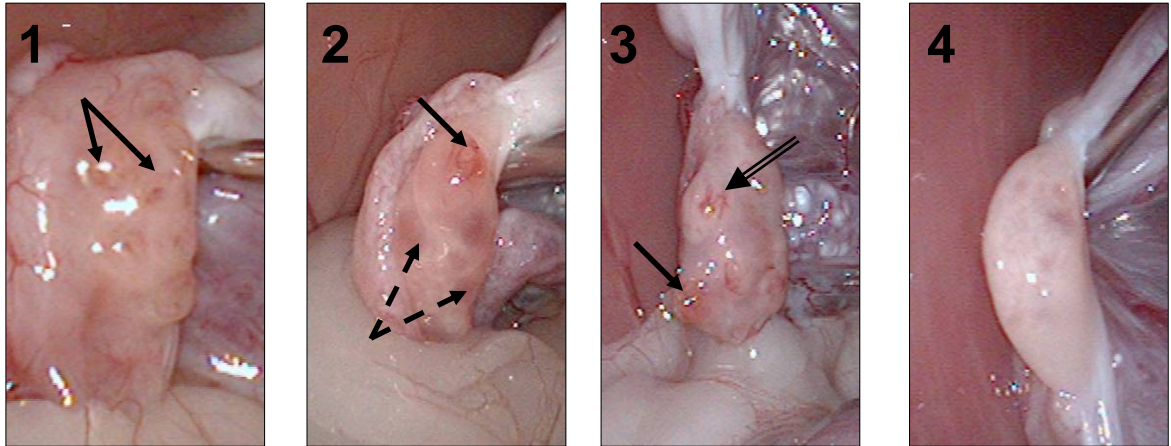


Fig. 4.1. Laparoscopic ovarian grading system used to determine suitability for AI. Ovarian response and gonadotropin-induced ovulation was categorized as (1) excellent, multiple fresh CL and no follicles > 2 mm (Grade 1); (2) good, mixed cohort of fresh CL and follicles > 2 mm (Grade 2); (3) fair, variable-aged CL (Grade 3); or (4) poor, ovulation failure (Grade 4). Solid arrow denotes fresh CL, dashed arrow denotes mature follicles and double-lined arrow denotes old CL from a previous luteal phase. Females displaying a Grade 4 response were not inseminated.

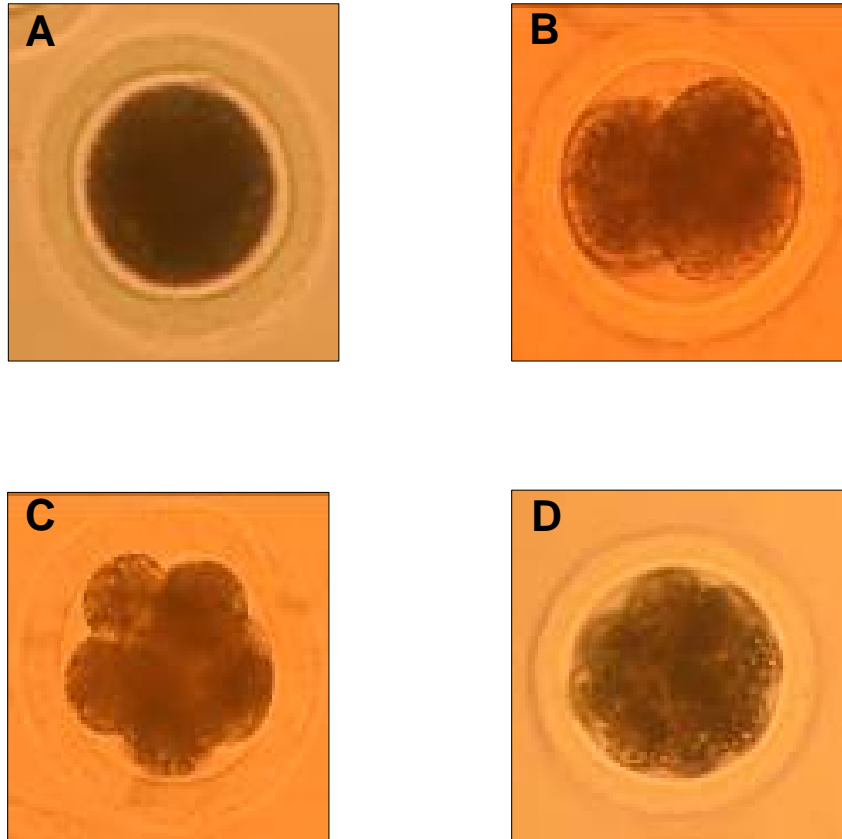


Fig 4.2. Representative photos of (A) an unfertilized oocyte and (B-D) embryos recovered in the oviduct on Day 5. Embryos ranged in developmental stage from (B) 2-4 cell, (C) 5-8 cell and (D) 9-16 cell on the day of flush.

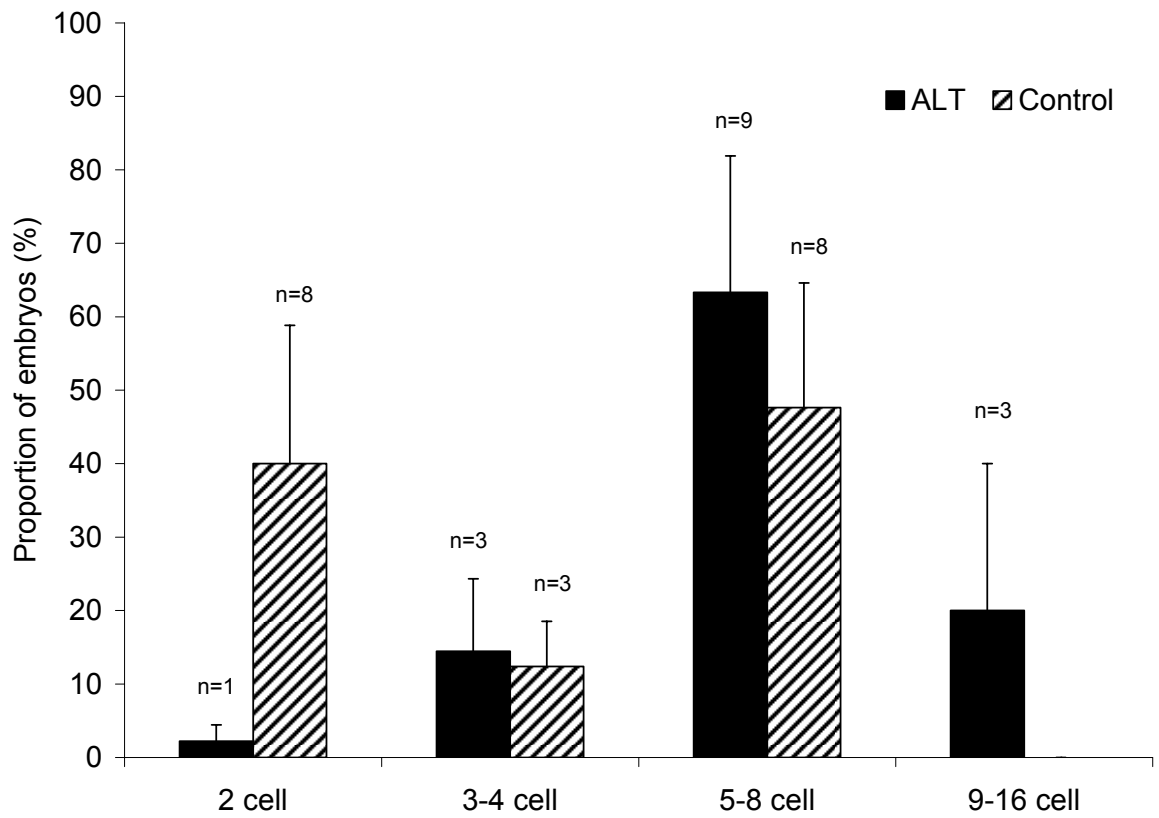


Fig. 4.3. Embryonic stage on the day of oviductal flush (Day 5 post-hCG). The distribution of embryos at each developmental stage differed ( $P < 0.05$ ) between altrenogest (ALT)-primed (solid) and control (hatched) cats.

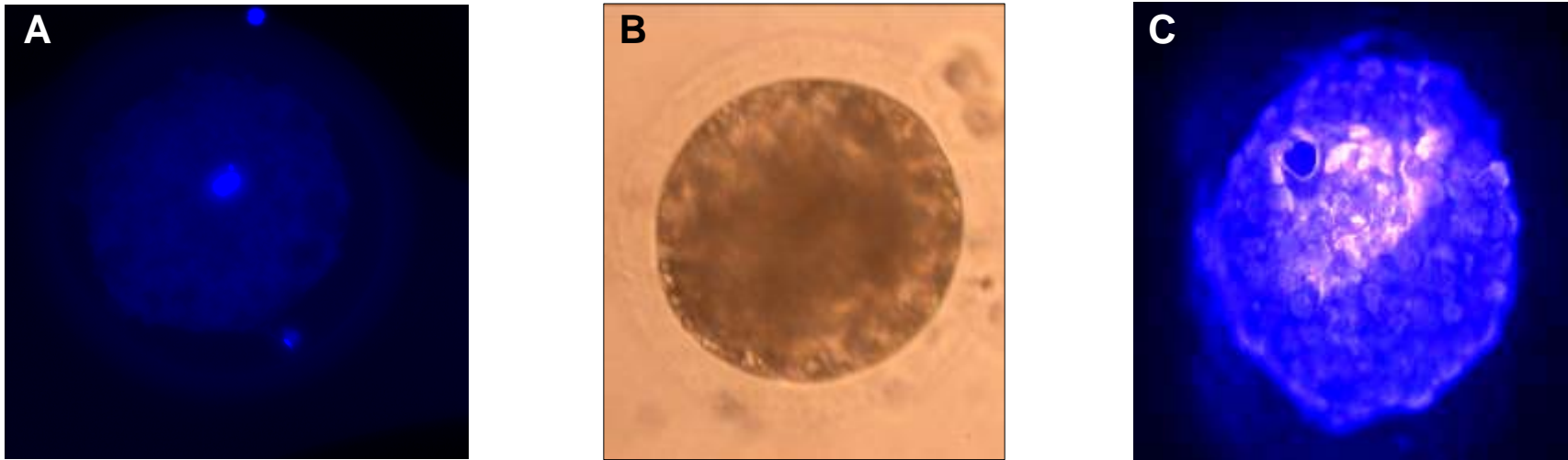


Fig. 4.4. Representative photos of an unfertilized oocyte (UFO) in metaphase II and blastocysts cultured *in vitro*. UFOs were examined with Hoechst fluorescent dye to determine chromatin status at the time of flush (A). Following culture, blastocysts were identified using light microscopy (B) and cell numbers were confirmed with Hoechst staining (C).



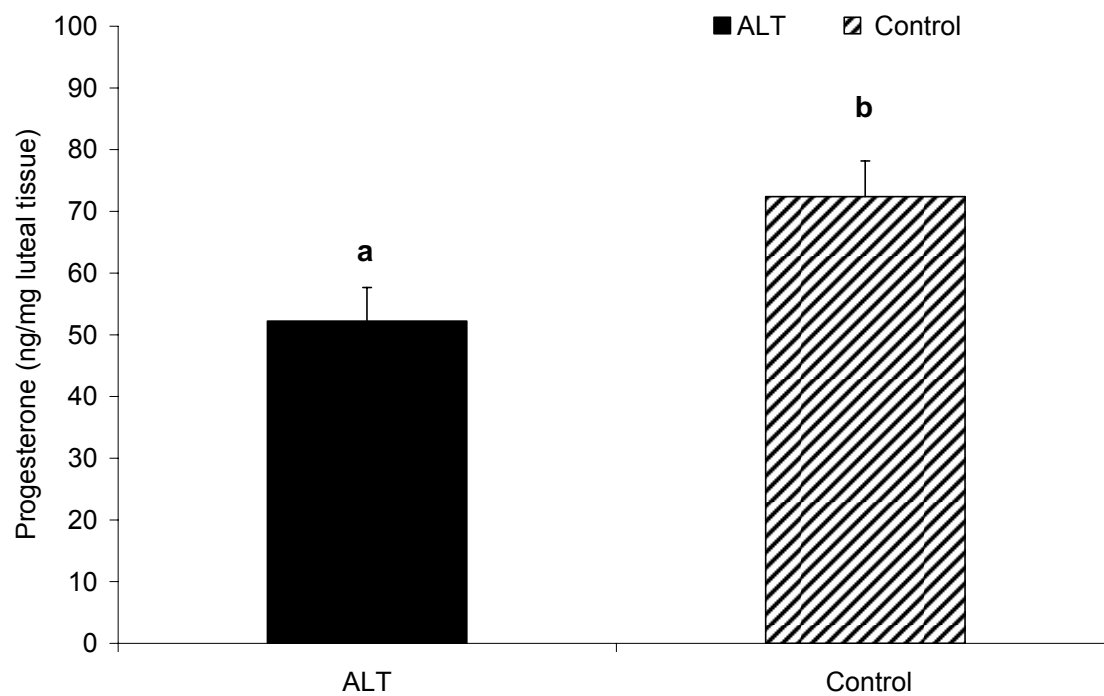


Fig. 4.5. Luteal progesterone concentration in corpora lutea recovered on Day 5 post-hCG in altrenogest (ALT)-primed (solid) and control (hatched) cats. Means with different superscripts differ ( $P < 0.05$ ).

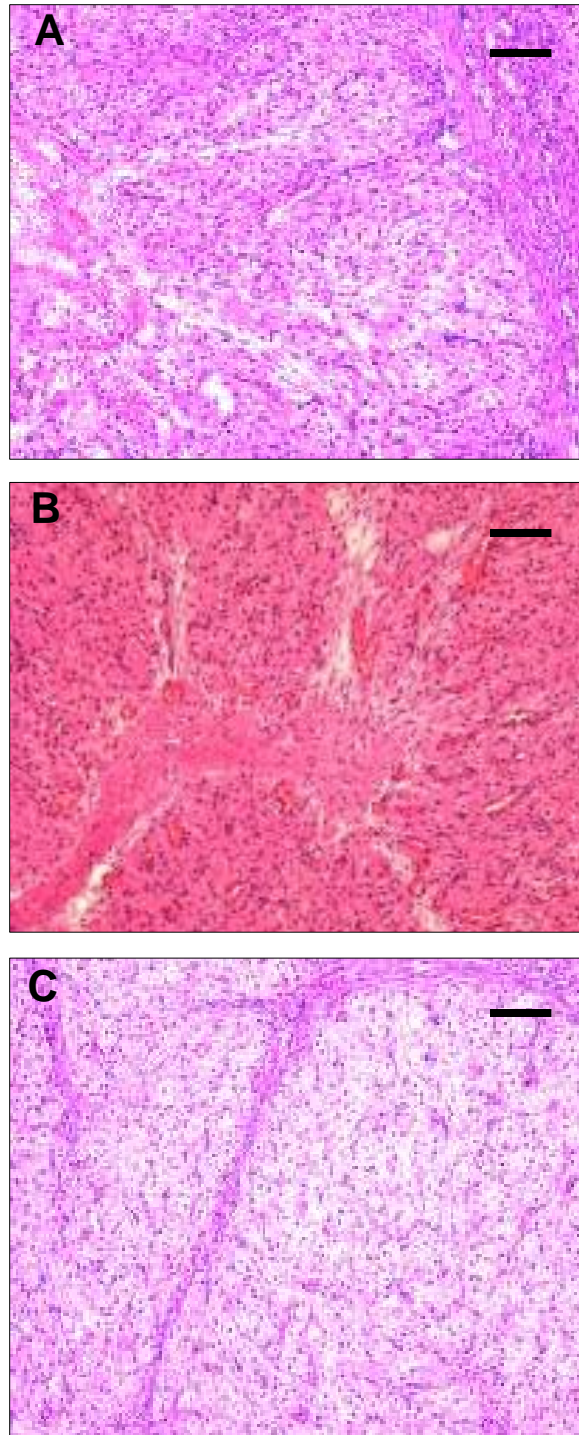


Fig. 4.6. Corpora lutea (CL) histomorphology on Day 5. (A) Most CL had irregular margins, a visible central cavity filled by fibrosis and a mixed population of fusiform and polygonal luteal cells. (B) Corpora hemorrhagica were observed in some females, presumed to be the result of ancillary folliculogenesis and ovulation. (C) Regressing CL were noted in controls demonstrating spontaneous ovulation before ovulation induction. Bar represents 100  $\mu$ m.

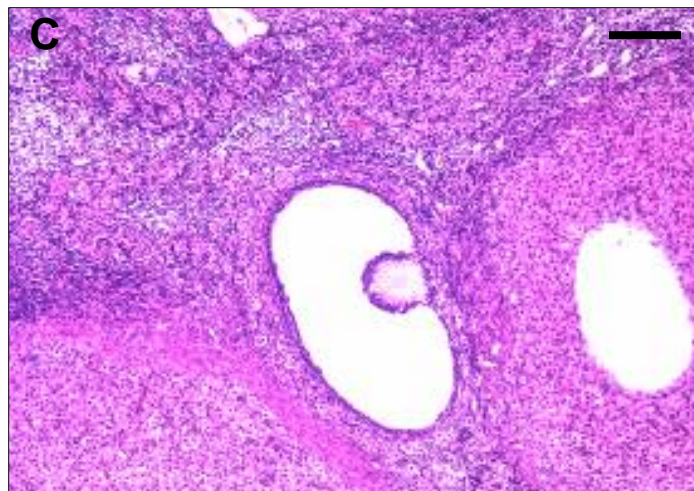
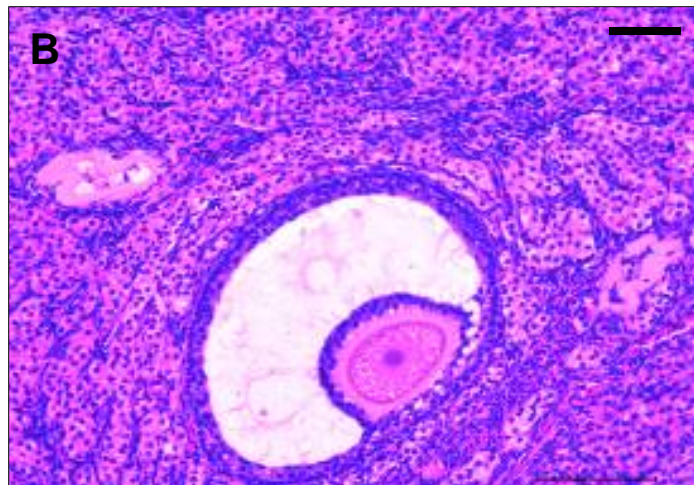
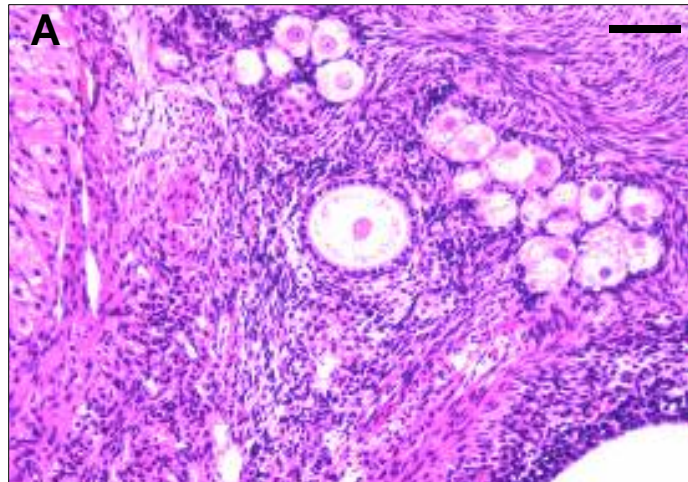


Fig. 4.7. Histomorphology of ovarian follicles observed on Day 5. (A) Mixed populations of primordial and primary follicles were observed in the ovarian cortex. (B) A tertiary follicle with distinct granulosa and theca cells and a large antrum. (C) Follicles undergoing atresia. Note the absence of an ovum and hypertrophy of surrounding cells. Bar represents 100  $\mu$ m.



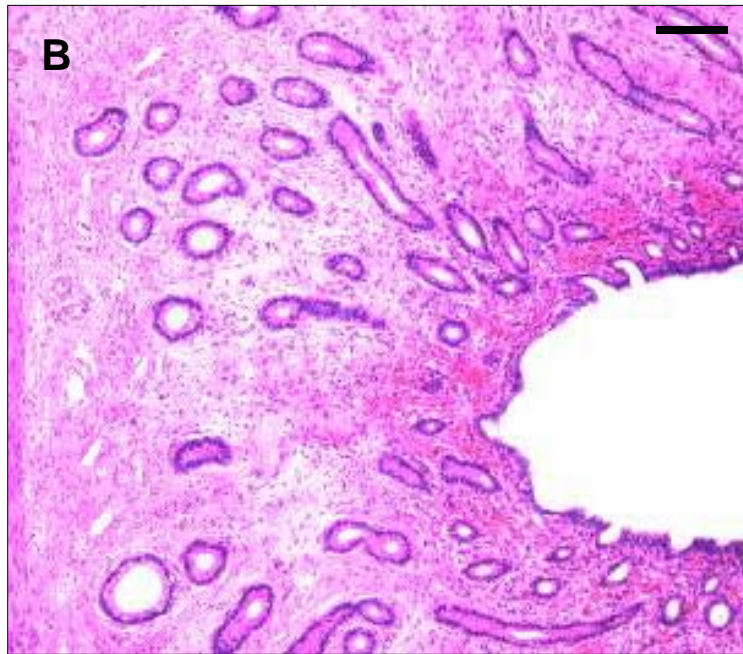
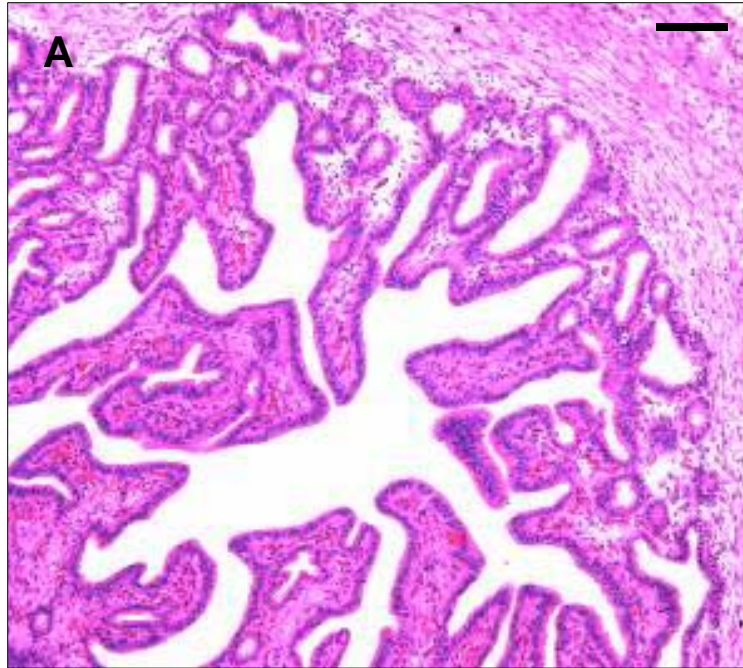


Fig. 4.8. Oviductal and uterine histomorphology of cats on Day 5. Representative cross-sections of oviduct (A) and uterus (B) were obtained from each individual.

## CHAPTER 5

### MOLECULAR AND MORPHOLOGICAL CHARACTERISTICS ASSOCIATED WITH LUTEAL INSUFFICIENCY IN THE CAT

#### **Abstract**

Pregnancy success after assisted reproduction remains low in felids treated with equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) to induce folliculogenesis and ovulation. Exogenous gonadotropins have been linked to abnormal corpora lutea (CL) progesterone production in the cat. However, short-term priming with the oral progestin altrenogest (ALT) before ovulation induction mitigates luteal insufficiency. To characterize this differential response, specific cellular and molecular indicators of luteal function were examined in archived ovarian tissue from queens in Studies 2 and 3 assigned to: (1) ALT + eCG/hCG ( $n = 11$  cats); (2) eCG/hCG only ( $n = 12$ ); or (3) natural estrus/natural breeding ( $n = 6$ ; NB). Ovaries were removed 5 or 17 days post-hCG in ALT + eCG/hCG and eCG/hCG cats, whereas all NB cats were ovariectomized on Day 17 (Day 0 = 1<sup>st</sup> day of breeding). Ovaries were assessed for histological parameters and candidate gene expression in luteal tissue. Ovarian sections contained both primary and atretic follicles, and many individuals (~50% overall) had at least one tertiary, pre-ovulatory follicle. On Day 5, 50% of cats had at least one CL with a residual central cavity, whereas no central cavities were observed by Day 17. CL surface area and luteal cell density were similar ( $P > 0.05$ ) among treatments on Day 5 and 17. Candidate genes associated with luteal function and examined by quantitative real-time RT-PCR included estrogen receptor alpha (ER $\alpha$ ), progesterone receptor (PR), luteinizing hormone receptor (LHR), prolactin receptor (PRLR), steroidogenic acute regulatory protein (sSTAR), cholesterol side-chain cleavage enzyme

(CYP11A1), and 3-beta hydroxysteroid dehydrogenase (3 $\beta$ -HSD). No differences ( $P > 0.05$ ) in gene expression were found among treatments at either time point. Over time, increases ( $P < 0.05$ ) in LHR and decreases ( $P < 0.05$ ) in 3 $\beta$ -HSD gene expression were not correlated with changes in CL progesterone. Overall, this study demonstrates that aberrant CL progesterone production following exogenous gonadotropin treatment is not associated with gross changes in ovarian morphology or altered expression of the candidate genes targeted in this experiment. Moreover, these data suggest that the functional basis for CL insufficiency is complex and related to additional factors that may be revealed by more extensive gene profiling experiments.

## **Introduction**

Artificial insemination (AI) has been used in multiple felid species as a tool for maintaining gene diversity in rare and critically endangered populations (Howard, 1992; Wildt and Roth, 1997). Yet the incidence of pregnancy failure remains high (> 90%) in most species following AI, preventing a significant impact on population genetics (Swanson, 2006). Attempts to determine the underlying basis for AI failure have led to no single causal agent. Instead, multiple contributing factors have been identified, including spontaneous ovulation and inconsistent ovarian response to ovulation induction (Pelican et al., 2007), ancillary folliculogenesis and secondary ovulation following gonadotropin treatment (Swanson et al., 1996a), an abnormal endocrine milieu during early pregnancy (Brown et al., 1994), delayed oviductal transport (Graham et al., 2000) and poor embryo quality (Goodrowe et al., 1988a). All of these observations have been linked to the routine use of equine chorionic gonadotropin (eCG) to induce folliculogenesis and human chorionic gonadotropin (hCG) to induce ovulation before AI in felids.

Pre-treatment (priming) with progestins before ovulation induction and AI has proven effective for mitigating several adverse effects on fertility in the domestic cat. Priming with the oral progestin altrenogest (ALT) temporarily suppresses ovarian activity, improves ovarian response to ovulation induction and supports normal peri-ovulatory steroid hormones, early embryonic development, and oviductal transport (Chapters 3 and 4). These findings are critical for assisted reproduction in felids that spontaneously ovulate, such as the clouded leopard or fishing cat. In these species, ovarian control is required to ensure that AI is timed to coincide with ovulation (Pelican et al., 2006b).

Even queens with excellent ovarian response to ovulation induction still frequently fail to get pregnant, suggesting that a post-ovulatory mechanism, such as luteal malfunction, could be involved. Indeed, previous work has shown that corpus luteum (CL) progesterone production is compromised following exogenous gonadotropin treatment in the cat (Chapters 3 and 4). Similar observations have been made in livestock and humans following ovarian stimulation (McNeilly et al., 1981; Tavaniotou et al., 2001). Interestingly, oral progestin priming before exogenous gonadotropins facilitates CL progesterone production, resulting in levels similar to naturally-bred cats (Chapters 3 and 4). These data provide an interesting dichotomy that warrants further investigation.

The primary function of the CL is to serve as the site of progesterone biosynthesis, and the CL is believed to be the sole source of progesterone during early pregnancy in the cat (Paape et al., 1975; Verstegen et al., 1993). Although it has not yet been documented in the cat, it is likely that the CL also produces androgens and estradiol, similar to other species (Stocco et al., 2007). Furthermore, it is clear that the CL is not an autonomous structure, but one that receives complex, carefully orchestrated endocrine and immune signals which impact progesterone secretion (Niswender, 2002). The CL also is heavily influenced by mediators of luteal formation and regression,

although these agents are poorly characterized in felids (Concannon and Verstegen, 1999). Prolactin is one known luteotrophic agent (Banks et al., 1983). Prostaglandins, which are common luteolysins in other species, do not appear to regulate luteal regression, at least during the first 40 days post-ovulation (Shille and Stabenfeldt, 1979), and can also have luteotrophic activity in the cat (Wildt et al., 1979b).

This study was designed to examine morphological and molecular variables associated with CL regulation and function to understand how progestin priming before ovulation induction maintains normal progesterone biosynthesis. Ovarian samples were assessed from domestic cats spayed during the early (Day 5) or mid (Day 17) luteal phase that were: (1) naturally-bred; (2) treated with oral progestin (ALT) + eCG/hCG; or (3) treated with eCG/hCG only. Histology was used to characterize ovarian morphology and identify differences among treatments. Molecular endpoints examined were limited to candidate gene expression of known or proposed regulators of progesterone biosynthesis, compared using real-time RT-PCR (qRT-PCR). Evaluating the effects of exogenous hormones on luteal function could provide a critical link to improving AI success in endangered felids.

## **Materials and Methods**

### ***Animals and experimental design***

Twenty-nine adult (1-3 year old) female domestic cats were part of an established research population that demonstrates both spontaneous and induced ovulation (Pelican et al., 2005). Queens were housed alone ( $n = 6$  cats) or in pairs ( $n = 23$  cats), while males ( $n = 3$ ) were housed individually in an adjacent room. Cats remained under a 12-h light: 12-h dark artificial light cycle throughout the study and were fed a dry commercial diet (Purina ONE®, Nestlé Purina PetCare Co., St. Louis, MO) with



water ad libitum. All research activities were approved by the Smithsonian's National Zoological Park Institutional Animal Care and Use Committee (Protocol #05-25) and the University of Maryland IACUC (R-06-06).

In Experiment 1, all cats underwent ovariohysterectomy on Day 5 (D5; Day 0 = day of hCG). Queens ( $n = 13$ ) were randomly assigned to: (1) ALT + eCG/hCG ( $n = 6$ ; D5 ALT + eCG/hCG); or (2) eCG/hCG ( $n = 7$ ; D5 eCG/hCG). In Experiment 2, females underwent ovariohysterectomy on Day 17 (D17; Day 0 = day of hCG or first day of breeding). Queens ( $n = 16$ ) were randomly assigned to: (1) ALT + eCG/hCG ( $n = 5$ ; D17 ALT+ eCG/hCG); (2) eCG/hCG ( $n = 5$ ; D17 eCG/hCG); or (3) natural breeding ( $n = 6$ ; D17 NB).

### ***Treatments and tissue collection***

Exogenous hormone treatments and the natural breeding protocol have been described previously (Chapter 3). Briefly, the oral progestin ALT (0.088 mg/kg; Regu-Mate®; Intervet Inc., Millsboro, DE) was administered daily in wet food (Friskies®; Nestlé Purina) for 38 days. Lyophilized eCG (Sigma-Aldrich Corporation, St. Louis, MO) and hCG (Sigma-Aldrich) were solubilized in sterile, preservative-free saline and stored frozen in individual syringes at  $-20^{\circ}$  C until use. Three days after stopping ALT or at a chosen day in unprimed females, cats received 50 IU eCG intra-muscularly (i.m.; Day -3) followed 80 hours later by 37.5 IU hCG i.m. (Day 0). Females assigned to breed naturally ( $n = 6$ ) were monitored daily for signs of behavioral estrus including lordosis, foot treading, vocalization, rubbing and rolling. A proven breeder male was introduced on the second or third day of behavioral estrus and allowed to mate three times a day in 3 hour intervals for 2 consecutive days (Wildt et al., 1981).

Following routine ovariectomy on Day 5 or 17, CL from one ovary were excised and individually weighed. The ovary with more CL was chosen to maximize recovery of luteal tissue. Half of the CL were immediately placed in RNAlater (Ambion Inc., Austin, TX), maintained at 4°C overnight and then stored at -80°C. The second ovary was bisected and one hemi-ovary was fixed in 4% paraformaldehyde (Fisher Scientific Company, Pittsburgh, PA) before paraffin embedding by a commercial company (HistoServ, Rockville, MD).

### ***Ovarian histology***

Paraffin blocks were step-sectioned and four equally-spaced 5 µm sections across the ovary were stained with hematoxylin and eosin by HistoServ. For each section, presence or absence of primary, tertiary and atretic follicles was noted. Primary follicles were identified as an oocyte surrounded by small, poorly-differentiated cell layers; tertiary follicles had a visible oocyte, well-differentiated granulosa and theca cells and a large, distinct antrum; and atretic follicles lacked an oocyte and showed evidence of hypertrophy and irregular follicular margins. Surface area of individual whole CL and CL central cavities (if present) were measured with IP Lab for Windows Version 3.5.1 software (Scanalytics Inc., Fairfax, VA). These structures were outlined with the region of interest (ROI) freehand drawing tool and the number of pixels contained within the outline was recorded. An indirect measure of cell density was obtained by capturing TIFF images of each CL in each section at 200x and then overlaying the image with a uniform square grid in Microsoft Office PowerPoint 2003 (Microsoft Corporation, Redmond, WA). All nuclei contained within the grid were counted, and cell density was expressed as the number of nuclei per unit area.

### ***RNA extraction***

Total RNA was extracted from individual CL samples stored in RNA $\text{/later}$ . Samples were thawed and approximately 20 mg luteal tissue was disrupted and lysed with a guanidine isothiocyanate-containing buffer using a rotor-stator homogenizer. RNA was isolated using a silica-gel membrane column kit according to the manufacturer's protocol (RNeasy Mini Kit; Qiagen; Valencia, CA). DNase digestion was performed during RNA extraction (RNase-Free DNase Set; Qiagen). Following isolation and purification of the RNA, the final product was eluted in 40 $\mu$ l RNase-free water and immediately stored at -80°C. Concentration of individual RNA samples was determined using a spectrophotometer at 320 nm (GeneQuant II; Amersham Pharmacia Bio Tech, Piscataway, NJ). RNA integrity was confirmed by gel electrophoresis, using an agarose formaldehyde gel prepared with 1% agarose, 3% formaldehyde and 1X MOPS buffer (20mM MOPS, 5mM NaOAc, 1mM EDTA) in water. The 1  $\mu$ g RNA samples from 18 randomly-selected samples were diluted in an RNA loading buffer containing ethidium bromide (Sigma); samples and two DNA molecular weight ladders (Sigma) were heated to 95°C for 5 minutes, loaded onto the gel and run at 190 V. The gel was read on an ultraviolet transilluminator (Fig. 5.2).

### ***Primer design***

With the exception of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), primers were designed using published mRNA transcripts in the National Center for Biotechnology Information (NCBI) GenBank database. If a domestic cat sequence was not available for the gene, a canine or bovine transcript was chosen. These sequences were run through BLAST in the Ensembl genome browser ([ensembl.org](http://ensembl.org)) against the whole feline genome, and a novel feline-specific gene transcript was obtained. Primers were designed using PrimerExpress software (Applied Biosystems, Foster City, CA). A

total of 300-500 basepairs of the mRNA transcript close to the 3' end and spanning more than one exon (if possible) were analyzed for suitable primers in PrimerExpress using specified parameters. Forward and reverse primers were chosen based on low penalty, length of primer (19-21 bp), amplicon length (100-120 bp) and low guanine/cytosine content (Table 5.1). Primers were cross-checked in the Ensembl genome browser to ensure that they fell on the forward and reverse strands of the sequence of interest. The cat GAPDH primer sequences had been previously published (Kipar et al., 2001). All primers were commercially obtained (Invitrogen, Carlsbad, CA), reconstituted in nuclease-free water to a 100 $\mu$ M stock solution and stored at -20°C.

### ***qRT-PCR***

Two step quantitative real-time RT-PCR (qRT-PCR) was performed to measure levels of mRNA in extracted CL tissue. Total RNA (~1 $\mu$ g) was reverse transcribed using a 50  $\mu$ M anchored oligo dT primer (Sigma) and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Briefly, RNA was combined with the oligo dT primer and 10mM dNTPs in nuclease-free water. Samples were vortexed, heated to 65°C in a thermocycler (Gene Amp PCR 9700; Applied Biosystems) for 5 min and maintained on ice for at least 1 min. Reverse transcriptase, 0.1 M DTT, 5X strand buffer and 40 U/ $\mu$ l RNase inhibitor (RiboLock; Fermentas Life Sciences, Glen Burnie, MD) were added to the sample and mixed gently with a pipette. A 'no RT control' was produced by combining a pooled RNA sample with all components except the reverse transcriptase. Samples were brought to 50°C in a thermocycler for 60 min followed by 70°C for 15 min to deactivate the reaction. cDNA was diluted 1:5 in nuclease-free water and stored at -20°C.

qRT-PCR was performed for the seven genes of interest using a SYBR green double-stranded DNA detection method (Fig. 5.1). Genes chosen were estrogen

receptor alpha (ER $\alpha$ ), progesterone receptor (PR), luteinizing hormone receptor (LHR), prolactin receptor (PRLR), steroidogenic acute regulatory protein (sSTAR), cholesterol side-chain cleavage enzyme (CYP11A1), 3-beta hydroxysteroid dehydrogenase (3 $\beta$ -HSD) and a housekeeper gene (GAPDH). For each gene, cDNA samples (1  $\mu$ l) were added to individual wells of a 96 well PCR optical plate (Bio-Rad Laboratories, Hercules, CA) in triplicate and a mix containing 10  $\mu$ l iQ SYBR Green Supermix (Bio-Rad), 0.8  $\mu$ l 10  $\mu$ M forward primer, 0.8  $\mu$ l 10  $\mu$ M reverse primer and 7.4  $\mu$ l nuclease-free water was added to each well. The plate was sealed, centrifuged briefly and run in a Bio-Rad iCycler. PCR cycling parameters were 40 cycles of 95°C for 15 sec (denaturation) and 60°C for 1 min (anneal/extend) followed by a melt curve analysis. With the exception of ER $\alpha$ , all assays had a single melt curve peak. Mean intra-assay variation was 2.7% (range, 0.2 to 11.7%). Cycle thresholds (Ct) for each sample were corrected for background contamination using the formula  $Ct_{(no\ RT\ control)} - Ct_{(sample)}$ . No differences in GAPDH housekeeper expression were observed among treatments (mean Ct 19.5  $\pm$  0.1;  $P > 0.05$ ). Thus for each sample, mRNA levels were normalized to GAPDH expression by subtracting the corrected Ct for GAPDH from the corrected Ct value for each gene of interest ( $\Delta$ Ct). Relative mRNA expression then was calculated by transforming the  $\Delta$ Ct values to linear scale ( $2^{\Delta Ct}$ ).

### **Statistical analyses**

For each experiment, data were summarized within treatment and compared using a one-way ANOVA followed by LSD mean comparisons if significant differences ( $P < 0.05$ ) were observed. The experimental design also allowed for comparisons over time (D5 vs. D17) in ALT + eCG/hCG and eCG/hCG only queens, which were performed using a two way factorial ANOVA. Main effects (time and treatment) were interpreted in the absence of a significant interaction. Post-hoc power analysis was performed for

gene expression data sets when trends ( $P = 0.05$  to  $0.2$ ) were observed. By defining power ( $0.95$ ) and using the known treatment means and mean residual error, requisite sample sizes were obtained. All data are presented as means  $\pm$  SEM, and statistical analyses were performed using JMP IN 5.1 (SAS Institute, Cary, NC).

## Results

### *Ovarian histology*

In Experiment 1 (Day 5 ovariectomy), one control queen was excluded due to incomplete ovarian sections, resulting in a total of six cats per treatment for analysis. Females in both groups had primordial and primary follicles in the ovarian cortex (Fig. 5.3A). Tertiary follicles were observed in two of six (33.3%) D5 ALT+ eCG/hCG and three of six (50%) D5 eCG/hCG cats (Fig. 5.3B). All females displayed at least one follicle undergoing atresia (Fig. 5.3C). On Day 5, CL surface area did not differ ( $P > 0.05$ ) between treatments (Fig 5.4A). CL central cavities were observed in an equal number of cats (50%) in each group, and the surface area of these cavities was similar ( $P > 0.05$ ) between treatments (Fig. 5.5). In those cats displaying a central cavity, the cells closest to the cavity were predominantly fusiform, while cells radiating outward from the cavity were primarily polygonal. Conversely, in those CL without a central cavity, nearly all luteal cells were polygonal. Overall luteal cell density also was similar ( $P > 0.05$ ) between treatments (Fig. 5.6A).

In Experiment 2 (Day 17 ovariectomy), one naturally-bred queen was removed due to incomplete sections, allowing for a final comparison of five cats per treatment. Similar to observations on Day 5, all females had primordial and primary follicles in the ovarian cortex, as well as follicles undergoing atresia. Tertiary follicles were observed in 40% of D17 ALT + eCG/hCG and D17 eCG/hCG, and 60% of NB cats.

Central cavities were not observed in any CL on Day 17 and overall CL surface area did not differ ( $P > 0.05$ ) among treatments (Fig 5.4B). Luteal cell density also was similar ( $P > 0.05$ ) among treatments (Fig. 5.6B). When comparing histological parameters over time, luteal cell density decreased ( $P < 0.05$ ) between Day 5 and 17 in ALT + eCG/hCG (88 versus 52 cells per unit area) and eCG/hCG cats (102 versus 54 cells per unit area).

### **CL gene expression**

Of the original 29 queens, five were immediately excluded due to absent or insufficient CL tissue. An additional three females were removed following qRT-PCR analyses due to consistently low or undetectable mRNA expression for all genes of interest, including the GAPDH housekeeper gene. Therefore, data were analyzed from a total of 21 queens as follows: D5 ALT + eCG/hCG,  $n = 4$ ; D5 eCG/hCG,  $n = 5$ ; D17 ALT + eCG/hCG,  $n = 5$ ; D17 eCG/hCG,  $n = 3$ ; and D17 NB,  $n = 4$ .

Within each time point assessed, no differences ( $P > 0.05$ ) in sSTAR, CYP11A1 or 3 $\beta$ HSD expression were observed among treatments (Fig. 5.7). Over time, 3 $\beta$ HSD mRNA decreased ( $P < 0.05$ ) in both treatment groups assessed. ER $\alpha$  could not be accurately detected in any treatment on Day 5 or 17. PR, PRLR and LHR gene expression was consistent among treatments ( $P > 0.05$ ) within each of the two time points (Fig. 5.8). However, there was a trend ( $P = 0.052$ ) towards increased expression of LHR in eCG/hCG cats, compared to ALT + eCG/hCG cats, on Day 5. Power analysis indicated that a sample size of 9 cats per treatment would have been needed to adequately characterize this relationship. Naturally-bred cats showed a trend towards increased PR ( $P = 0.17$ ) and PRLR ( $P = 0.15$ ) on Day 17, compared to eCG/hCG and ALT + eCG/hCG females. In this case, sample sizes of 10 and 12 cats per treatment, respectively, would have been required to detect a significant difference. Over time, LHR expression increased ( $P < 0.05$ ) in both treatment groups assessed.

## Discussion

Studies aimed at understanding the causes of reproductive failure following assisted reproduction in felids generally have focused on poor ovarian responsiveness and reduced gamete/embryo viability following ovulation induction (Pelican et al., 2006b). However, we have shown that CL progesterone biosynthesis is compromised following treatment with exogenous gonadotropins, suggesting that progesterone levels may be insufficient to maintain pregnancy in cats following AI (Chapters 2 and 3). This study represents the first analysis of specific histological and molecular markers of luteal function in the cat after treatment with: (1) a traditional eCG/hCG protocol used before AI; (2) a novel protocol that incorporates ovarian priming with oral progestin prior to eCG/hCG; or (3) natural breeding. By investigating how these treatments differentially act to influence cellular and subcellular characteristics in the ovary, we have gained new insight into how ovulation induction influences subsequent luteal morphology and function in the cat.

In the present study, we determined that altered patterns of luteal progesterone biosynthesis generally do not correlate with changes in ovarian ultrastructure. All cats exhibited varied follicular states throughout the ovarian cortex during the luteal phase, ranging from primordial to antral, that were not influenced by treatment with exogenous gonadotropins or progestins. These dynamic follicular characteristics are consistent with what is seen in naturally-bred, pregnant cats during early pregnancy (Roth et al., 1995). Furthermore, follicular waves of development during the luteal phase are common in other species (Roche, 1996). In cats, it is unknown what influence these follicles play in an environment dominated by the CL. This could be the subject of an interesting follow-up study.



The uniformity in CL surface area and luteal cell density observed among treatments suggests that the eCG/hCG regimen does not impair the process of CL formation and luteal cell differentiation. The predominance of polygonal luteal cells on Day 17 and a mixed population of polygonal and fusiform cells on Day 5 observed across treatments also has been documented in naturally-bred queens at similar time points (Roth et al., 1995). Histologically, small and large luteal cells are not easily differentiated in the cat, as they are in many other species (Fields and Fields, 1996; Niswender et al., 2000). It is interesting that luteal cell density did decrease over time, but only in queens treated with gonadotropins alone. However, in ewes and cows, such shifts in cell density are normal (Niswender et al., 1994). Without a comprehensive, normative database on luteal cell density across time in untreated queens, it is difficult to draw conclusions from this finding.

To our knowledge, this study is the first quantitative assessment of gene expression in the domestic cat CL using real-time RT-PCR. In choosing specific genes to target, we focused on known or presumed regulators of progesterone biosynthesis and general luteal function (Niswender et al., 2000; Stocco et al., 2007). We did not detect estrogen receptor alpha mRNA at either time point, regardless of treatment. It is possible the primer design was flawed; however, circulating progesterone can significantly down-regulate ER (Niswender et al., 2000). Indeed, while ER expression in the ovary is generally up-regulated during follicular growth (Drummond, 2006), detection of ER in the CL during the luteal phase is highly species-specific. For example, ER $\alpha$  is largely undetected during the luteal phase in baboons and monkeys (Hild-Petito and Fazleabas, 1997; Duffy et al., 2000), detected only during the early luteal phase in cows (Berisha et al., 2002) and highly expressed throughout the luteal phase in rats (Telleria et al., 1998). This could be related to the differential role estradiol plays in luteal maintenance across species. ER $\beta$  also is expressed in the CL of some species (Duffy et

al., 2000). Thus, it is entirely possible that this receptor could simply be the more actively expressed ER subtype in domestic cat luteal tissue. A follow-up to this study would be to test the ER $\alpha$  primer in ovarian tissue across the follicular phase of the feline estrous cycle, when there is a strong physiological indication for ER $\alpha$  expression.

Progesterone and prolactin receptor mRNA was detected consistently across treatments on both Day 5 and 17 and their expression was not altered by exogenous gonadotropins or progestin priming. These findings were not surprising, given the critical roles both progesterone and prolactin play in maintenance of luteal function in the cat (Verstegen et al., 1993). We can deduce from this that domestic cats undergoing assisted reproduction are expressing adequate concentrations of PR and PRLR to maintain luteal integrity. Furthermore, detection of PR in the CL demonstrates that progesterone biosynthesis likely involves an autoregulatory mechanism in the domestic cat. Such a mechanism has been characterized in other species (Hild-Petito and Fazleabas, 1997; Berisha et al., 2002). The trend towards increased PRLR and PR in naturally-bred cats on Day 17 could be attributed to pregnancy in three of four NB cats, whereas no cats undergoing exogenous hormone treatment were pregnant. Fetal and/or maternal factors, such as cytokines, could be responsible for the upregulation of PRLR and PR during early pregnancy.

LHR mRNA expression was consistent among treatments during the early and mid-luteal phase. On Day 5, the trend for LHR mRNA to increase in cats treated with exogenous gonadotropins correlates with our previous finding that CL progesterone is abnormally high on Day 5 (Chapter 4). In many domestic animals, LH enhances progesterone synthesis (Niswender, 2002), so an increase in LHR at this time point could be related to this mechanism. This is further supported by the observation that LHR mRNA increases over time, regardless of treatment, which again correlates with

increases in CL progesterone concentrations observed from the early to mid-luteal phase.

Steroidogenic acute regulatory protein mRNA expression was chosen for analysis because it is the rate-limiting step in steroidogenesis, bringing cholesterol from the outer to inner mitochondrial membrane for subsequent enzymatic conversion (Niswender et al., 2000). sSTAR has been detected in the CL of multiple species, including the human (Devoto et al., 2001; Sierralta et al., 2005), cow (Pescador et al., 1996) and rat (Stocco et al., 2001). The present study confirmed that sSTAR mRNA is consistently expressed among treatments during the early to mid-luteal phase, suggesting that the cholesterol substrate is reaching the inner mitochondrial membrane and is available for conversion to progesterone. Thus, exogenous gonadotropin treatment, alone or in combination with progestin priming, does not appear to alter the availability of cholesterol substrate within the CL.

The enzymatic pathway involved in converting cholesterol to progesterone also was not compromised in cats treated with exogenous gonadotropins or progestins. Expression of CYP11A1, the side-chain cleavage enzyme required for pregnenolone formation, was consistent among treatments. 3 $\beta$ HSD, the final enzyme required for progesterone formation, also was consistently expressed. While there was a significant decrease in 3 $\beta$ HSD over time, it did not correlate with progestin priming. Research in rodents has shown that 3 $\beta$ HSD expression is regulated by both prolactin and gonadotropins (Martel et al., 1990; Martel et al., 1994). The current study does not support an influence of eCG, hCG or ALT treatment on sSTAR, or the major enzymes involved in progesterone biosynthesis, in the domestic cat.

In summary, this study provides new insight into the underlying mechanisms dictating maintenance and regulation of luteal function in the domestic cat. Overall, it demonstrates that aberrant CL progesterone production observed following exogenous

gonadotropin treatment: (1) is not associated with gross changes in ovarian morphology; and (2) can not be linked to altered expression patterns of several candidate genes associated with normal luteal function. Post-hoc power analyses suggest that increased sample sizes may be necessary to improve detection of subtle differences in gene expression using the current technique. Overall, these data illustrate the need for more comprehensive gene profiling experiments (e.g. microarrays) in the domestic cat CL, which could be designed to simultaneously investigate gene expression levels for thousands of genes acting alone or in concert to regulate normal luteal function. In doing so, we may be able to increase our understanding of the exact mechanism or mechanism(s) responsible for reproductive failure following assisted reproduction in felids.

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Table 5.1. Gene primer sequences used for qRT-PCR analyses.

<b>GENE</b>	<b>Forward (5'- 3')</b>	<b>Reverse (5'- 3')</b>	<b>Amplicon (bp)</b>
3- $\beta$ HSD	TACCAACCCCCCTTTAACCG	TGAAGAGTGGCTCATACCCCA	103
sSTAR	CGAGCAGAAAGGCATCATCAG	TGAGCAGCCACGTGAGTTTG	101
Cyp11A1	GGATCGCTGAGCTCGAGATG	TGAGGTTGAATATGGTGCCCA	102
PRLR	CAGGATCCGCAAGCTCAAAA	CGTGAAGGAGGCCAGGTCTT	105
LHR	CAATTCTTGCGCCAATCCATT	CCCGATGTTTACAGCAGCCA	101
PR	TTATCCTTTCCCGAGCACTGA	GAGCAGAGGTTTCACCGTCC	101
ER	CACCTACCAAGGAAGATGGCA	TCCCTACCCCATCACTTTTC	106
GAPDH	GCCGTGGAATTTGCCGT	GCCATCAATGACCCCTTCAT	82

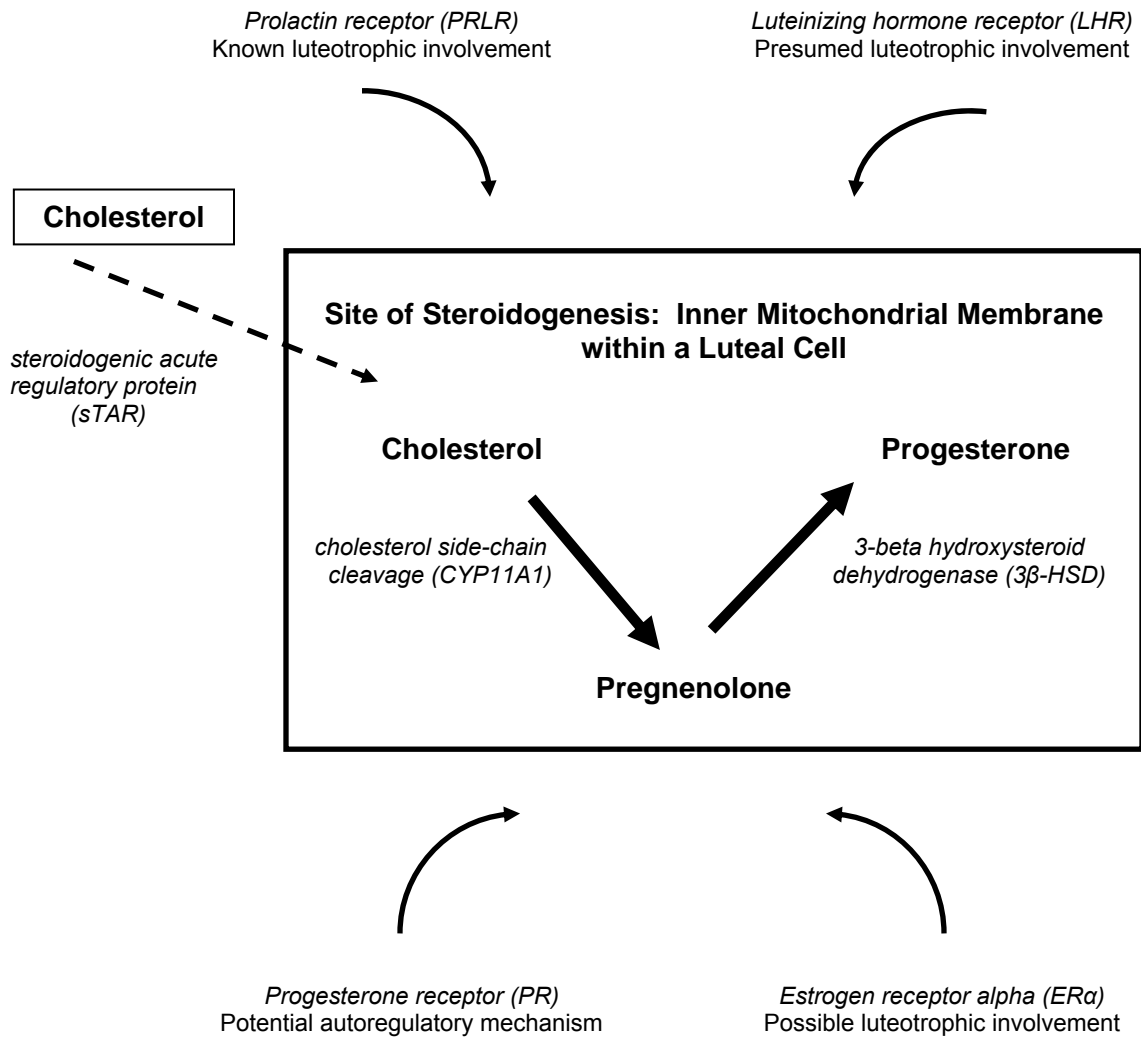


Fig. 5.1. Schematic representation of the genes chosen for qRT-PCR analyses and their involvement in progesterone biosynthesis in the domestic cat.

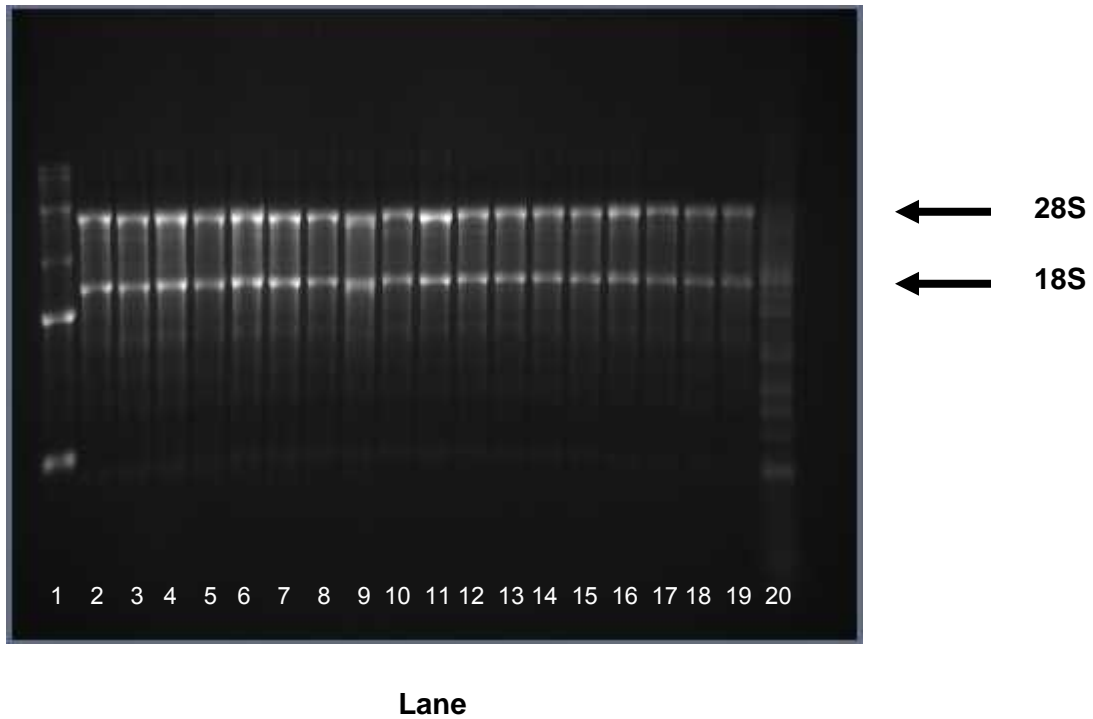


Fig. 5.2. RNA gel electrophoresis of representative domestic cat corpora lutea RNA extracts. DNA molecular weight ladders were loaded in lanes 1 and 20 and individual samples were loaded in lanes 2-19. The two distinct white bands are 28S and 18S ribosomal RNA.

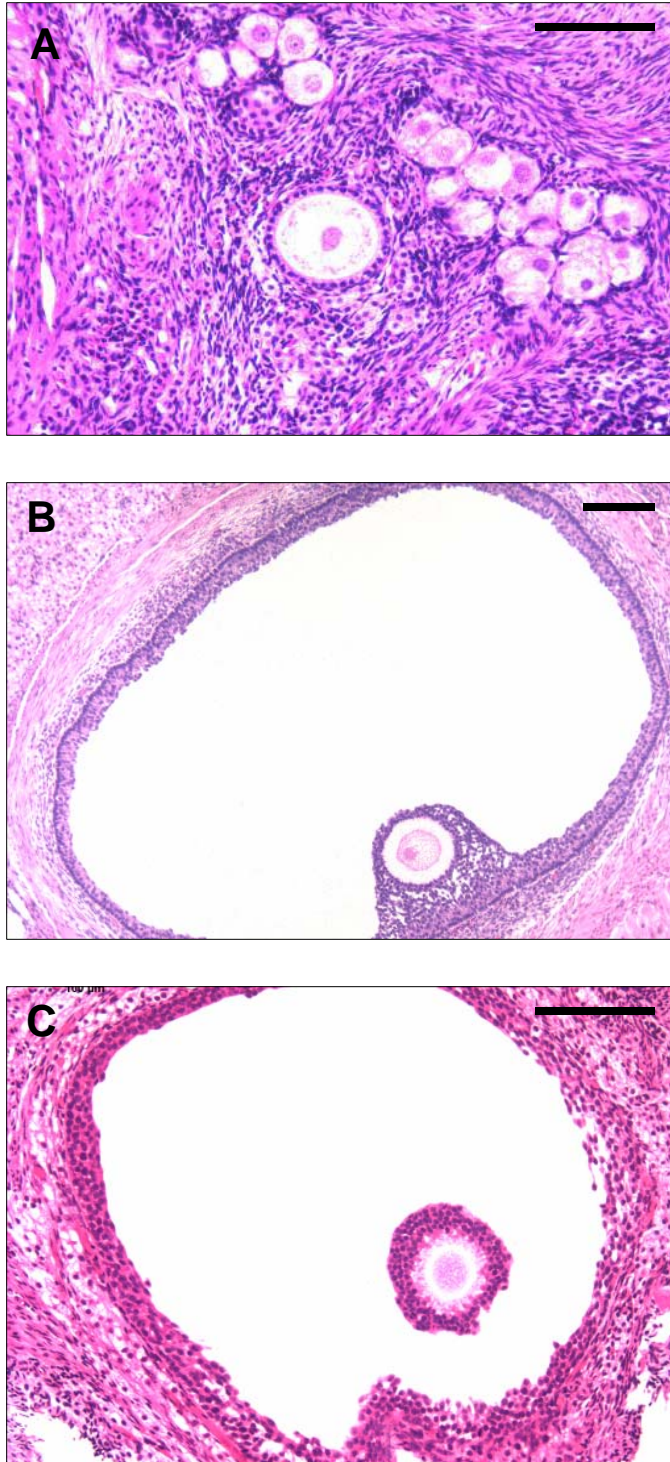


Fig. 5.3. Representative histomorphology of ovarian follicles. (A) Primordial and primary follicles in the ovarian cortex. (B) A tertiary follicle with distinct granulosa and theca cells and a large antrum. (C) Follicle undergoing atresia. Bar represents 100  $\mu\text{m}$ .



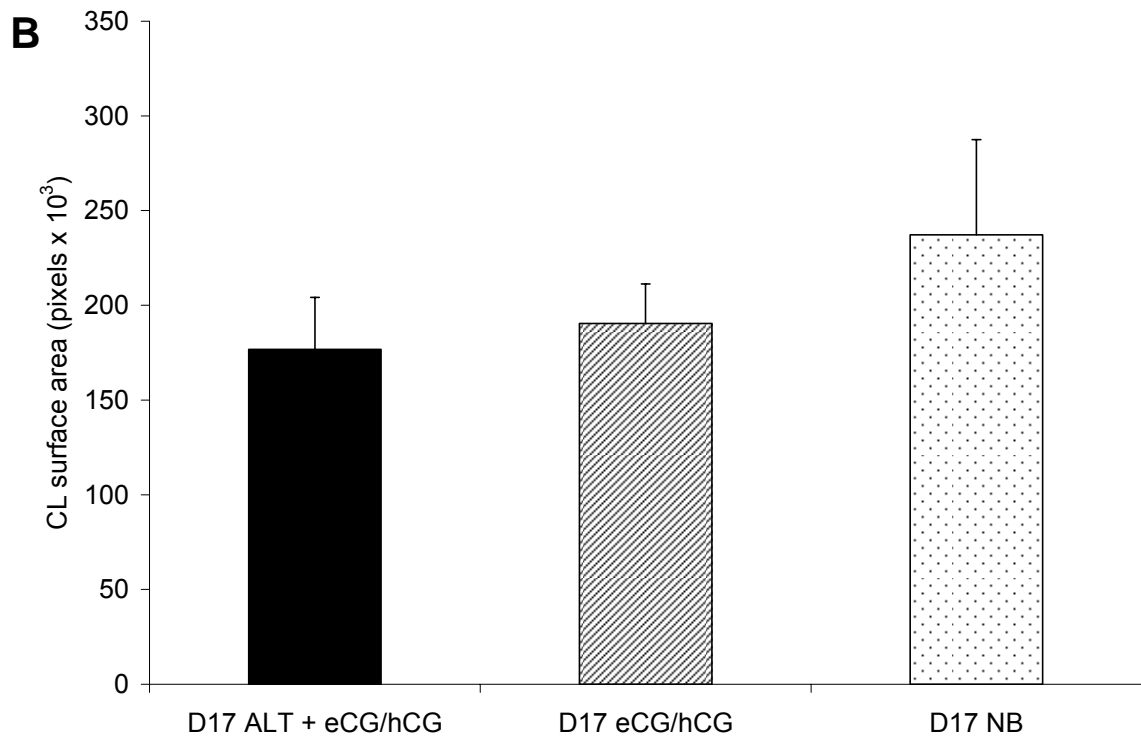
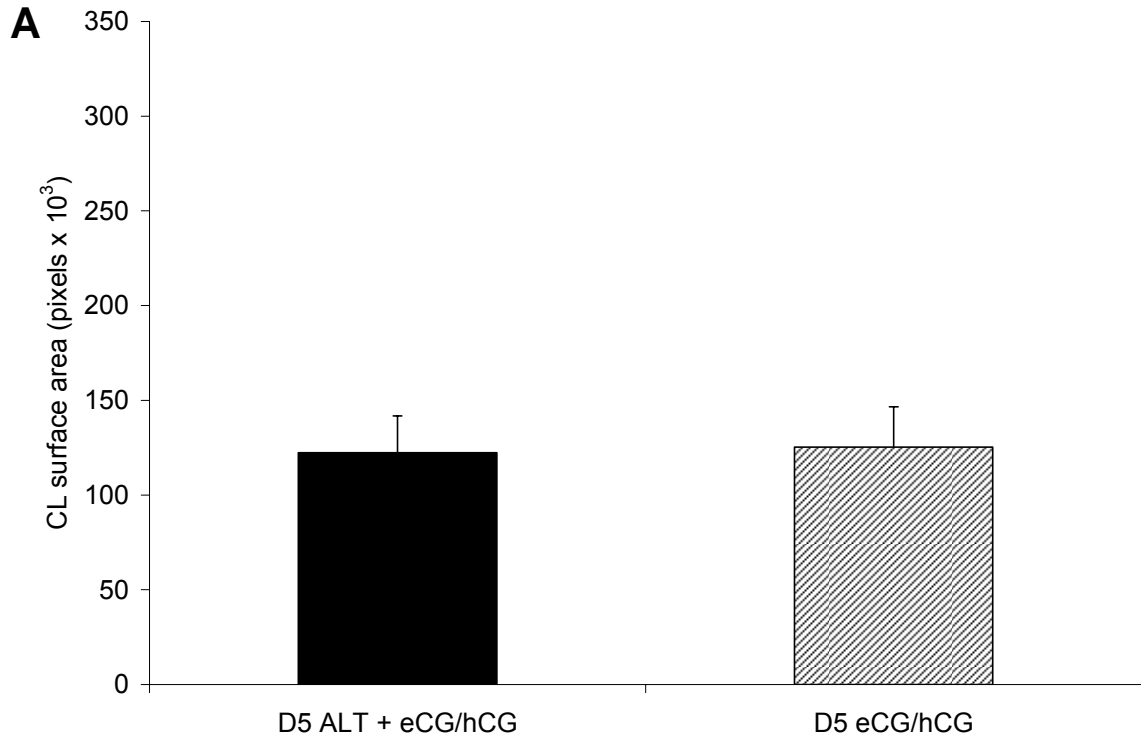


Fig. 5.4. Corpora lutea (CL) surface area expressed in pixels ( $\times 10^3$ ) following ovariectomy on (A) Day 5 or (B) Day 17. No differences ( $P > 0.05$ ) were observed among treatments.

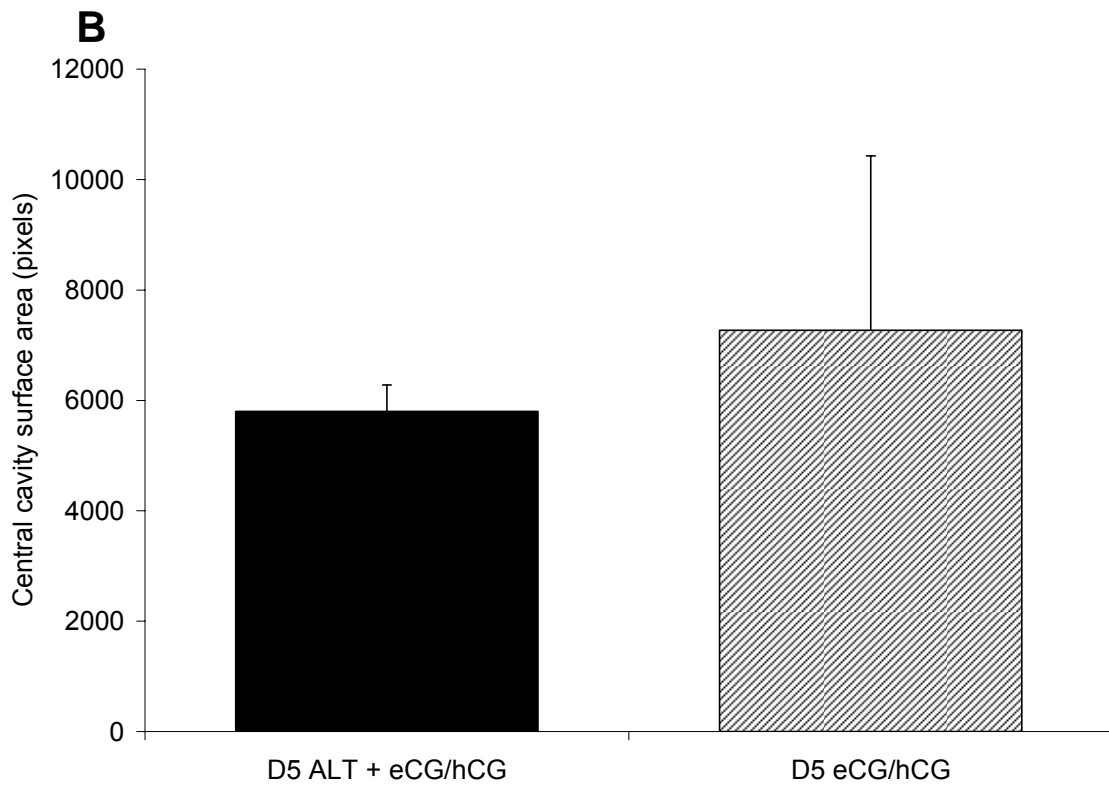
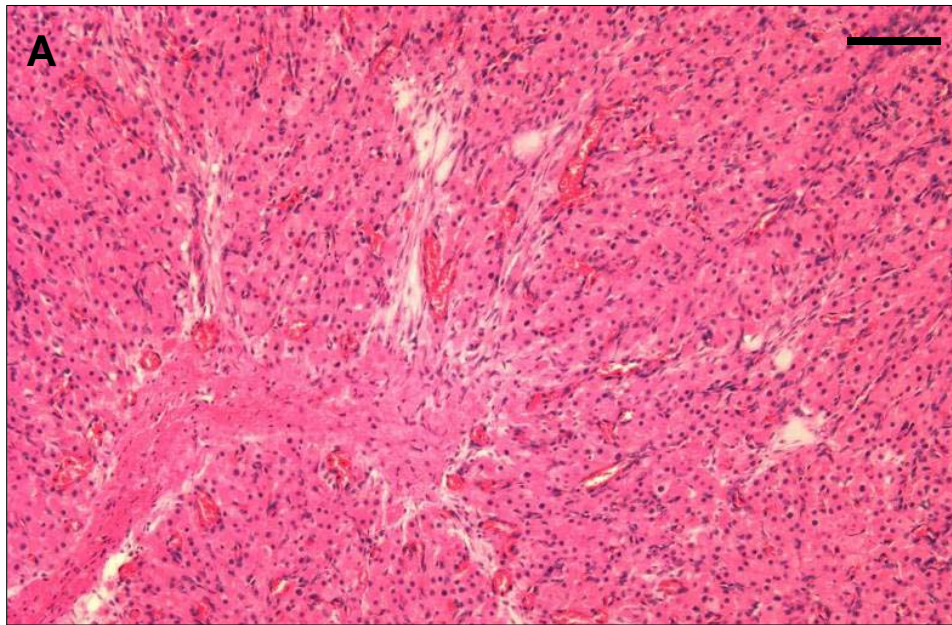


Fig. 5.5. Corpora lutea central cavities observed on Day 5 (A). Central cavity surface area was similar ( $P > 0.05$ ) among treatments (B). Bar represents 100  $\mu\text{m}$ .

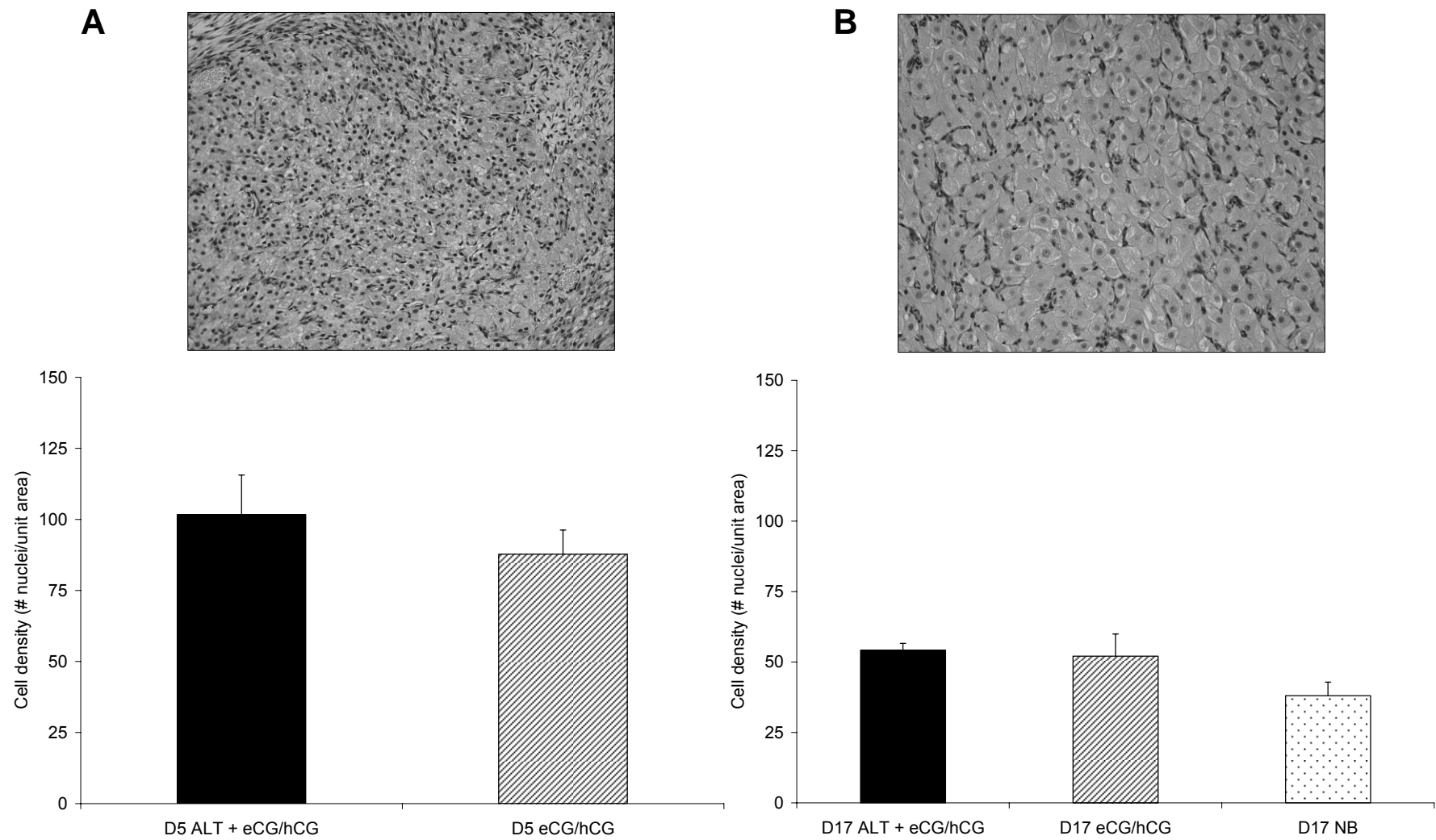


Fig. 5.6. Luteal cell density on Day 5 (A) and Day 17 (B). Within time period, cell density was similar among treatments ( $P > 0.05$ ).





## CHAPTER 6

### SUMMARY AND FUTURE DIRECTIONS

#### Summary

Assisted reproductive techniques, such as artificial insemination (AI), *in vitro* fertilization/embryo transfer (IVF/ET) and gamete cryopreservation, are important tools for retaining gene diversity in endangered felids in *ex situ* breeding programs. They also ensure a contingency plan should something catastrophic occur to the remaining *in situ* population. Yet in practice, the benefits of assisted reproduction remain largely steeped in theory for felids, due to low pregnancy success following AI and IVF/ET using current approaches. While assisted reproduction is not a panacea for wildlife conservation, it does serve an important purpose in the management of captive populations. Indeed, there are several examples where the use of assisted reproductive techniques has made positive impacts on genetic health, and in some cases, even species survival (Pukazhenthil and Wildt, 2004).

Over the past 30 years, the diverse Felidae family has taught us many important lessons about its unusual reproductive physiology. It is the only known taxon to display individual differences in ovulation mechanism, where some felid species demonstrate induced ovulation and other species can, but do not always, exhibit intermittent spontaneous ovulation (Bakker and Baum, 2000). Furthermore, ovarian sensitivity to exogenous gonadotropins used before AI or oocyte retrieval is highly variable among felid species and does not appear to be correlated to body weight or inter-species relatedness (Howard, 1999). As a result, translating assisted reproduction protocols developed largely in livestock and humans to cats has been enormously challenging. There have been a few AI success stories, notably in cheetahs and ocelots, but overall

efficiency still remains well below 50% (Pelican et al., 2006b). Much of this failure relates back to inconsistent ovarian response at the time of AI or oocyte retrieval, observed most often in spontaneous ovulators. Overall, these lessons have taught us that the prerequisite to improving assisted reproduction in felids is to increase our fundamental understanding of their unique reproductive mechanisms.

This dissertation project used a domestic cat research model to characterize reproductive parameters in response to a novel ovulation induction protocol, developed to combat several adverse effects associated with the currently accepted regimen. The primary objective was to understand how priming with oral progestin before ovulation induction influences endocrine dynamics, ovarian responsiveness, fertilization success, early embryonic development and peri-implantation ovarian morphology and function. We hypothesized that oral progestin would suppress ovarian activity, enable a more consistent ovarian response to exogenous gonadotropins and improve reproductive efficiency, compared to unprimed cats treated with gonadotropins alone. Specific objectives were to: (1) assess the influence of oral progestin (altrenogest; ALT) dosage on endocrine function and ovarian cyclicity; (2) evaluate the influence of progestin priming on ovarian responsiveness and sensitivity to exogenous gonadotropin dosage; and (3) characterize how progestin priming before ovulation induction and AI affects fertilization rates, early embryonic development, ovarian morphology and luteal function.

The first step in testing our hypothesis was to evaluate the effect of three targeted oral ALT dosages on ovarian cycle characteristics in the cat using non-invasive fecal hormone analyses. This approach was successful, and we demonstrated that oral ALT provides rapid, reversible inhibition of ovarian activity with no observed side effects. We were not surprised, given the vast body of literature on progestin use in felids. Consistent with studies using progestin implants for ovarian suppression (Pelican et al., 2007), oral progestin did not alter follicular or luteal activity already in progress but did

prevent initiation of folliculogenesis and ovulation. Furthermore, queens treated with the oral progestin showed a more uniform interval of estrous cycle return after removal of the progestin (10-16 days), compared to previous studies with progestin implants. Overall, this study determined an effective ALT dosage (0.088 mg/kg) that provides short-term ovarian suppression with no observed residual effects following return to estrous cycling.

With the oral progestin dosage established, our next objective was to determine whether a progestin-primed ovary displays altered sensitivity to exogenous gonadotropin dosage (eCG/hCG). Laparoscopic reproductive examinations confirmed that ovarian response to eCG and hCG was less variable, and more consistent with a natural cycle, when preceded by short-term (38 day) treatment with ALT. Non-invasive fecal steroid monitoring further substantiated these findings, demonstrating multiple adverse scenarios in unprimed females, such as recent spontaneous ovulation, that were prevented by progestin priming. This study also confirmed that a progestin-primed ovary exhibits increased sensitivity to eCG/hCG dosage. This was an exciting finding, given the long list of adverse effects associated with exogenous gonadotropin use (Graham et al., 2000). It meant that exogenous gonadotropin dosages could be halved (50 IU eCG/37.5 IU hCG) when used in conjunction with ALT, producing an excellent ovarian response while, in theory, reducing adverse fertility effects associated with eCG/hCG persistence in circulation.

Perhaps the most remarkable finding of the second study was the discovery that corpora lutea (CL) progesterone production during the mid-luteal phase (Day 17 post-hCG) is significantly lower in unprimed individuals, compared to ALT-primed and naturally-bred cats. Previous studies have been unable to make this link between exogenous gonadotropin use and luteal insufficiency (Roth et al., 1997b). In contrast, this study showed that the eCG/hCG regimen has a pronounced effect on luteal function



that can be mitigated with progestin treatment, but only in combination with relatively low eCG/hCG dosages (which, in turn, are possible only with progestin priming). This finding supports the hypothesis that pregnancy failure may be occurring well after AI. Overall, results from the second study endorsed the use of oral progestin priming in combination with low exogenous gonadotropin dosages for ovulation induction in the domestic cat.

With the ovulation induction protocol optimized (ALT + 50 IU eCG + 37.5 IU hCG), the next study aimed to examine the impact of this regimen on incidence of fertilization and early embryonic development following AI. Three days following laparoscopic intrauterine AI, embryos were recovered in a high proportion of both progestin-primed and unprimed females treated with low dosages of gonadotropins. While no differences in *in vivo* fertilization or *in vitro* embryonic development could be elucidated, a difference in the distribution of embryonic developmental stages on the day of recovery was observed between treatments. We were surprised that the variation in ovarian response observed between treatments did not translate into more differences in embryonic viability. This could be due to high levels of inherent variability among individuals, or it could be a true physiological phenomenon. In the third study, we also demonstrated that low dosages of eCG/hCG negatively impact CL progesterone production during the early luteal phase (Day 5 post-hCG), and that progestin priming once again mitigates this effect. Taken together, results from this third study supported the routine incorporation of a short-term oral progestin regimen before ovulation induction and AI in the cat.

The final phase of this research was to take one of our most interesting findings, aberrant CL progesterone biosynthesis in gonadotropin-treated cats, and attempt to characterize its underlying mechanism using advanced histological and molecular techniques. This analysis was important because our data suggested that progesterone

levels may be insufficient to maintain a pregnancy in cats following AI using the currently accepted gonadotropin regimen. We wanted to know what was causing this, and whether it was something that could be addressed through a modified protocol. Thus, using archived ovarian tissues from Studies 2 and 3, we compared markers of luteal function in (1) the low dosage eCG/hCG protocol used before AI; (2) the low dosage eCG/hCG protocol that incorporated pre-treatment with ALT; and (3) natural breeding.

This final study provided new insight into the underlying mechanisms dictating maintenance and regulation of luteal function in the domestic cat. Overall, it demonstrated that aberrant CL progesterone production is not associated with gross changes in ovarian morphology. Differences in CL progesterone were not linked to altered expression patterns of six candidate genes associated with general luteal function and progesterone biosynthesis. While differences in LHR and 3 $\beta$ -HSD were seen over time, these changes were not influenced by progestin pre-treatment. However, a post-hoc power analysis showed that sample size was likely too low for the genes assessed to adequately detect significant differences between treatments. Overall, these experiments clearly illustrated the need for follow-up and more comprehensive gene profiling experiments to answer these questions (such as microarrays or differential display) in domestic cat luteal tissue, which could be designed to simultaneously investigate gene expression levels for thousands of genes acting alone or in concert to regulate ovarian function.

The influence of exogenous hormones on ovarian morphology and function in the domestic cat is summarized in Fig. 6.1. The ultimate goal of any ovulation induction and AI protocol is to mimic a natural follicular and luteal phase as closely as possible without affecting the delicate endocrine balance during early embryonic development, endometrial remodeling and implantation. While exact adherence to a natural cycle is impossible, these studies have opened the door for the refinement of improved ovulation

induction protocols in a host of wild felids. We have introduced a new ovulation induction regimen that shows great promise for future applications. These data also could assist contraception research in both feral cats and zoo populations. Finally, because the domestic cat is an emerging model for infertility research, these data may be useful for studying the influence of exogenous hormones on reproductive function in humans.

### **Future Directions**

As the old adage goes, for every question answered, at least 10 more are raised. This dissertation research project was no exception. While we gained new understanding of domestic cat reproductive physiology, we also were frequently reminded that whole animal research is challenging due to high levels of variability among individuals. This research raised many important findings that warrant further investigation. It also demonstrated several key areas of basic research that deserve more attention in the domestic cat. In the context of wildlife conservation, it identified several basic and applied research projects that could strengthen one of the overall missions of our laboratory at the Smithsonian's National Zoo's Conservation and Research Center: to effectively document the diverse reproductive physiology of endangered felids.

The logical next step to this dissertation research project would be to investigate whether cats treated with ALT + eCG/hCG and artificially inseminated can achieve implantation. Concurrent with this, it would be important to confirm that queens primed with ALT can maintain pregnancy following natural breeding on the first cycle following progestin removal. Embryo data from Study 3 demonstrated that even unprimed gonadotropin-treated cats are capable of producing high-quality embryos. This suggests

that luteal function and/or uterine receptivity are the more influential causes of pregnancy failure following assisted reproduction. This link between exogenous gonadotropins and implantation failure has been made in other species (Stein and Kramer, 1989). In doing so, we also would also be able to investigate uterine morphology and function in relation to exogenous hormone treatment, an area of focus that remained largely unstudied in the current project. As part of this uterine study, hormone receptors and growth factors associated with decidualization, vascularization and cell proliferation should be assessed.

It is entirely possible that our modified ovulation induction protocol incorporating ALT priming will not make a measurable improvement on overall assisted reproduction success in the domestic cat. Instead, this protocol may need to be optimized further. It may be necessary to alter intervals between ALT and eCG and/or eCG and hCG in a series of comparative trials. Results from these trials could be assessed with a combination of non-invasive fecal hormone monitoring and laparoscopic examinations. Or, we also may need to revisit alternate gonadotropin regimens for ovulation induction altogether, if follow-up studies indicate that ALT is unable to fully override adverse effects caused by eCG and hCG. There have been efforts to characterize the amino acid sequence of tiger FSH, which may eventually lead to a felid-specific follicle-stimulating gonadotropin (Crichton et al., 2003). While promising, this regimen would need to be modified to ensure minimal injections (particularly important for wild felids that must be darted) and limited residual effects by the exogenous gonadotropins in circulation.

Progesterone supplementation is common in other species after assisted reproduction (Tavaniotou et al., 2001), and has been used successfully in tigers following IVF and ET (Donoghue et al., 1990). Based on our results, a pilot project in domestic cats using exogenous progestin supplementation after AI for luteal support is

warranted. This would be relevant primarily in cats treated with gonadotropins alone, which have a documented deficiency in progesterone biosynthesis, although it also would be interesting to observe the effects of such a regimen on ALT-primed cats. It would require careful consideration of both duration of treatment and dosage. In particular, when to initiate treatment and also when to withdraw treatment to allow for normal parturition would need to be optimized. It is likely that an oral progestin, possibly ALT, would be a good candidate for supplementation because an oral drug is easily administered and does not require anesthesia at the start or end of treatment that is needed for progestin implants.

This project also highlighted several unanswered questions and research needs related to basic domestic cat reproductive physiology. Previous studies have built a strong database of endocrine, embryo and luteal characteristics in the naturally-bred queen during the early luteal phase and after implantation (Roth et al., 1994; Swanson et al., 1994; Roth et al., 1995; Swanson et al., 1995b). Yet there still remains a large gap in knowledge during the critical peri-implantation period and also during folliculogenesis and ovulation. More studies are needed to characterize oviductal transport, factors required for normal oviductal function and the oviduct to uterine transition experienced by embryos around Day 6 in the naturally-bred queen. It also is important to understand maternal-fetal communication in this species, and the direct and indirect roles the embryo likely plays in regulating reproductive function before, during and after implantation.

To answer these questions, a repository of tissues (ovary, oviduct, uterus, CL, follicle, oocyte, embryo) from domestic cats naturally-bred versus treated with exogenous hormones needs to be built for multiple time points surrounding ovulation, fertilization and the window of endometrial receptivity. By performing broad gene expression analyses in these tissues, we would gain new insight into the cellular and

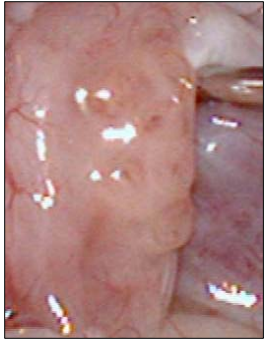
subcellular mechanisms regulating reproductive function in the cat. In addition to steroidogenic enzymes and hormone receptors, areas of study also could include cytokines (e.g. growth factors), embryonic factors and other regulators of cell proliferation and death. Should brain tissue become available from any of these cats, we could explore hormone production in the hypothalamus/pituitary and neuropeptide expression. Furthermore, it is possible that changes in reproductive functionality can be attributed to post-transcriptional and/or post-translational modifications that are not detectable through gene expression analyses. Thus, studies using immunohistochemistry to localize and quantify key proteins involved in reproductive function also would be useful.

Many studies have documented spontaneous ovulation in both domestic and non-domestic felid species, but little is known about the mechanisms regulating this unique difference observed in ovulation strategy. Is there an evolutionary component? Are there other cues (seasonal, auditory, pheromonal, visual, tactile, etc.) involved? Are these cues more prevalent in a captive environment? To answer these questions, an experiment designed to look more specifically at neuroendocrine function and regulation in induced versus spontaneous ovulators throughout the estrous cycle would be needed. A domestic cat colony, like the one at the Conservation and Research Center, would serve as a useful resource for this study because of the differential ovulation strategies displayed among individuals.

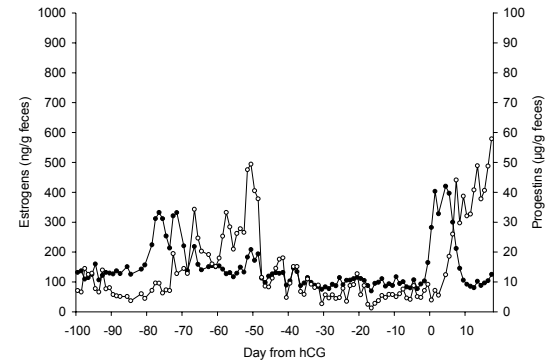
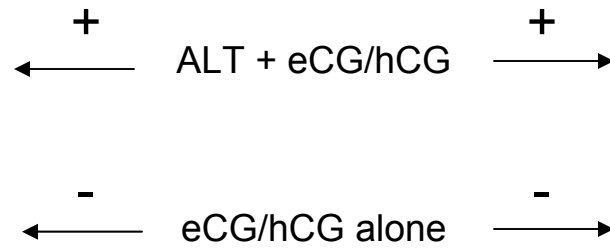
Another factor to consider, which is particularly relevant in captive populations of endangered felids usually skewed towards older individuals, is the influence of age on reproductive function. Felids are believed to reproduce throughout their lifetime, although evidence suggests that this may not be the case and, at the least, it is likely that reproductive efficiency does decline over time. This is evidenced in breeding records from several species of captive wild felids held in North American zoos.

Already, work is being conducted in the cheetah to examine the influence of age on oocyte quality, *in vitro* fertilization success and uterine health. Similar studies are needed in other species, including the domestic cat.

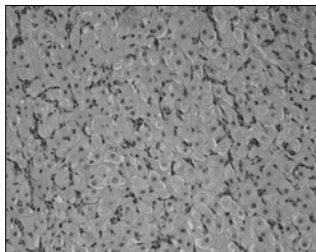
And finally, with the information gathered from our domestic cat research model, we have preliminary information useful for initiating studies in endangered wild felids. Short-term treatment with ALT will need to be tested, both for safety and efficacy, in these species. ALT dosage studies are already underway in two felids of particular interest, the clouded leopard and fishing cat, which both have a documented history of spontaneous ovulation. Furthermore, exogenous gonadotropin dosages previously effective in these species will likely need to be revised to account for the incorporation of progestin treatment, based on our findings of increased ovarian sensitivity in domestic cats to exogenous gonadotropins. Whether these findings translate into success for wild felids remains to be determined, and it will be exciting to see how this field progresses in the coming years. Overall, it is our hope that this dissertation research has provided a strong foundation for ongoing basic and applied research studies in both domestic and non-domestic felids.



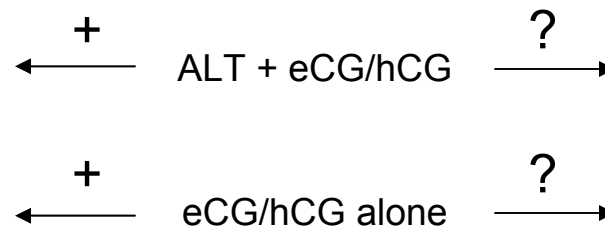
Gross ovarian morphology



Ovarian steroid production (including luteal progesterone)



Ovarian microstructure



Luteal gene expression

Fig. 6.1. Influence of oral altrenogest (ALT) priming and exogenous gonadotropins on ovarian morphology and function in the domestic cat. Arrows denoted with a (+) denote a positive influence, arrows with a (-) denote a negative influence and arrows with a (?) denote an inconclusive influence, compared to normative data from natural estrus/naturally bred queens.



## **Appendix A: Fecal Extraction and Enzyme Immunoassay (EIA) Protocols**

### **Fecal Hormone Extraction**

1. Dry individual bags of feces in a lyophilizer, crush to a fine powder with a rubber mallet and store powder in labeled plastic tubes at -20°C.
2. Whenever possible, include all samples from an individual cat on the same extraction. If multiple extractions are needed (> 108 samples), avoid splitting the extraction near critical time points in the study.
3. Weigh 0.18 to 0.2 g of dried feces into numbered 16x125mm glass tubes. Record the exact weight of each sample. Avoid hair, litter or other debris.
4. Add 4.5 ml absolute ethanol (ETOH) and 0.5 ml distilled water (DW) to each tube and vortex briefly.
5. Boil tubes in a hot water bath (95°C) for 20 min. Add ETOH as needed to prevent the samples from boiling dry.
6. Bring the volume of the extract up to approximate pre-boil levels with ETOH and centrifuge at 500g for 20 min.
7. Pour off the supernatant into a second set of identically labeled 16x125mm tubes.
8. Add 4.5 ml ETOH and 0.5 ml DW to the original tubes (containing the fecal pellets) and vortex each tube for 30 seconds. Centrifuge at 500g for 15 minutes.
9. Add the second supernatant to the first supernatant. Dry supernatants under air until no liquid remains in the tube. You will be left with solid fecal extract adhered to the sides of the glass tube.
10. Resuspend the dried down fecal extracts in 1 ml methanol, vortex briefly and sonicate for 20 min to bring extract adhered to the glass tube into solution.
11. Remove 200 µl extract and add to 1.8 ml EIA dilution buffer (1:10 dilution). Store in individually-labeled polypropylene tubes at -20°C.

### **Estrone Sulfate (E1S) EIA**

1. Plate coating
  - Add 33.3 µl polyclonal anti-EC R583 stock (1:10, -20°C) to 5 ml coating buffer (working dilution, 1:1500).
  - Dispense 50 µl antibody solution per well into 96 well NUNC Maxisorb plates. Do not coat Column 1 (blanks to test for non-specific binding).
  - Gently tap plate, cover with an acetate plate sealer and incubate overnight at 4°C.
2. Plate washing and buffering
  - Wash plate 5 times with EIA wash solution using a Dynatech plate washer.
  - Blot plate thoroughly on a paper towel to remove excess wash solution.
  - Immediately add 25 µl EIA assay buffer to each well and maintain plate at 20°C for 2 to 5 hours.
3. Standards
  - Dilute standard stock (500 pg/well or 10 ng/ml; stored at -20°C) serially 2-fold using 200 µl stock plus 200 µl EIA assay buffer and mix well.
  - Resultant standard values are 500, 250, 125, 62.5, 31.25, 15.12, 7.8, 3.9, and 1.95 pg E<sub>1</sub>So<sub>4</sub>/well. Standards are stable for 2 weeks at 4°C.
4. Samples and controls (26 samples per plate)
  - Dilute samples in EIA buffer to the appropriate dilution.
  - Use prepared house E1S controls (C1 and C2). Controls are designed to bind at ~ 30% and 70%.

5. Enzyme conjugate
  - Just prior to plate loading, prepare estrone-glucuronide-horseradish peroxidase by adding 50  $\mu$ l E1S-HRP stock (1:100, 4°C) to 5ml EIA buffer in a glass scintillation vial (working dilution, 1:20,000).
6. Plate loading
  - Add 50  $\mu$ l standard, sample, or control per well in duplicate as quickly and accurately as possible.
  - Immediately add 50  $\mu$ l E1S-HRP to each well.
  - Cover the plate and incubate at 20°C for 2 hours.
7. Substrate and plate reading
  - After the 2 hour incubation, wash the plate and blot dry.
  - Combine 40  $\mu$ l 0.5 M H<sub>2</sub>O<sub>2</sub>, 125  $\mu$ l 40 mM ABTS and 12.5 ml citrate buffer in a plastic beaker and mix well.
  - Add 100  $\mu$ l substrate to all wells and maintain on a plate shaker.
  - Read optical density of each well using a Dynex MRX plate reader. Take the final reading when zero standard wells reach an OD of 0.9 to 1.

### **Estrogen Conjugate (EC) EIA**

1. Plate coating
  - Add 25  $\mu$ l polyclonal anti-EC R522-2 stock (1:100, -20°C) to 5 ml coating buffer (working dilution, 1:20,000).
  - Dispense 50  $\mu$ l antibody solution per well into 96 well NUNC Maxisorb plates. Do not coat Column 1 (blanks to test for non-specific binding).
  - Gently tap plate, cover with an acetate plate sealer and incubate overnight at 4°C.
2. Plate washing and buffering
  - Wash plate 5 times with EIA wash solution using a Dynatech plate washer.
  - Blot plate thoroughly on a paper towel to remove excess wash solution.
  - Immediately add 25  $\mu$ l EIA assay buffer to each well and maintain plate at 20°C for 1 to 5 hours.
3. Standards
  - Dilute standard stock (200 pg/well or 4 ng/ml; stored at -20°C) serially 2-fold using 200  $\mu$ l stock plus 200  $\mu$ l EIA assay buffer and mix well.
  - Resultant standard values are 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 pg estrone-B-glucuronide/well. Standards are stable for 2 weeks at 4°C.
4. Samples and controls (26 samples per plate)
  - Dilute samples in EIA buffer to the appropriate dilution.
  - Use prepared house EC controls (C1 and C2). Controls are designed to bind at ~ 30% and 70%.
5. Enzyme conjugate
  - Just prior to plate loading, prepare estrone-glucuronide-horseradish peroxidase by adding 33.3  $\mu$ l EC-HRP stock (1:100, 4°C) to 5ml EIA buffer in a glass scintillation vial (working dilution, 1:15,000).
6. Plate loading
  - Add 50  $\mu$ l standard, sample, or control per well in duplicate as quickly and accurately as possible.
  - Immediately add 50  $\mu$ l EC-HRP to each well.
  - Cover the plate and incubate at 20°C for 2 hours.
7. Substrate and plate reading
  - After the 2 hour incubation, wash the plate and blot dry.

- Combine 40  $\mu$ l 0.5 M H<sub>2</sub>O<sub>2</sub>, 125  $\mu$ l 40 mM ABTS and 12.5 ml citrate buffer in a plastic beaker and mix well.
- Add 100  $\mu$ l substrate to all wells and maintain on a plate shaker.
- Read optical density of each well using a Dynex MRX plate reader. Take the final reading when zero standard wells reach an OD of 0.9 to 1.

### **Pregnane (Pg) EIA**

1. Plate coating
  - Add 25  $\mu$ l monoclonal anti-CL425 (1:50, -20°C) to 5 ml coating buffer (working dilution, 1:10,000).
  - Dispense 50  $\mu$ l antibody solution per well into 96 well NUNC Maxisorb plates. Do not coat Column 1 (blanks to test for non-specific binding).
  - Gently tap plate, cover with an acetate plate sealer and incubate overnight at 4°C.
2. Plate washing
  - Wash plate 5 times with EIA wash solution using a Dynatech plate washer.
  - Blot plate thoroughly on a paper towel to remove excess wash solution.
3. Standards
  - Dilute standard stock (200 pg/well or 4 ng/ml; stored at -20°C) serially 2-fold using 200  $\mu$ l stock plus 200  $\mu$ l EIA assay buffer and mix well.
  - Resultant standard values are 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 pg progesterone/well. Standards are stable for 2 weeks at 4°C.
4. Samples and controls (26 samples per plate)
  - Dilute samples in EIA buffer to the appropriate dilution.
  - Use prepared house Pg controls (C1 and C2). Controls are designed to bind at ~ 30% and 70%.
5. Enzyme conjugate
  - Just prior to plate loading, prepare progesterone-3CMO-horseradish peroxidase by adding 25  $\mu$ l Pg-HRP stock (1:100, 4°C) to 5 ml EIA buffer in a glass scintillation vial (working dilution, 1:40,000).
6. Plate loading
  - Add 50  $\mu$ l standard, sample, or control per well in duplicate as quickly and accurately as possible.
  - Immediately add 50  $\mu$ l Pg-HRP to each well.
  - Cover the plate and incubate at 20°C for 2 hours.
7. Substrate and plate reading
  - After the 2 hour incubation, wash the plate and blot dry.
  - Combine 40  $\mu$ l 0.5 M H<sub>2</sub>O<sub>2</sub>, 125  $\mu$ l 40 mM ABTS and 12.5 ml citrate buffer in a plastic beaker and mix well.
  - Add 100  $\mu$ l substrate to all wells and maintain on a plate shaker.
  - Read optical density of each well using a Dynex MRX plate reader. Take the final reading when zero standard wells reach an OD of 0.9 to 1.

*Source: Brown, J., Walker, S., and Steinman, K. 2005. "Endocrine Manual for the Reproductive Assessment of Domestic and Non-Domestic Species". 2<sup>nd</sup> Edition. Conservation and Research Center Endocrine Research Laboratory, Front Royal, VA.*

## Appendix B: EIA Validation

Before relying on EIA results, validation was required. Estrogen and progestin antibodies were chosen based on published HPLC analyses which have characterized the primary steroid metabolites present in domestic cat feces. In the second study (Chapter 3), the estrogen EIA was switched from E1S to EC because E1S was no longer commercially available. For each assay, a parallelism and accuracy/recovery check were performed.

### Parallelism

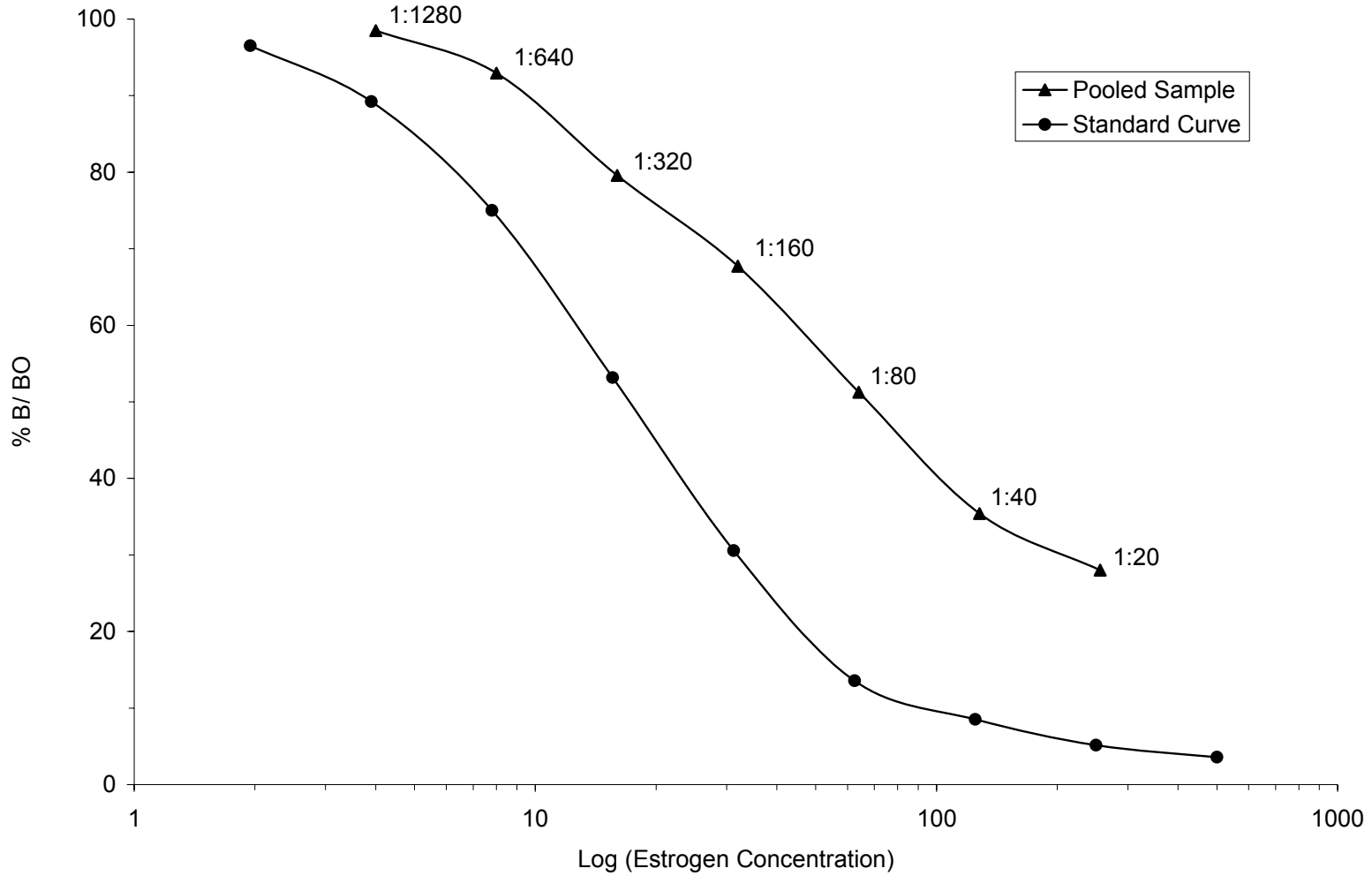
1. Pool an equal amount of fecal extract from multiple samples taken from several cats. Samples should reflect the full range of concentrations expected.
2. Dilute pool serially two-fold in assay buffer (1:10, 1:20, 1:40, 1:80, 1:160, etc.)
3. Run diluted pool samples on the appropriate EIA.
4. Plot the % binding of samples by choosing an arbitrary concentration for the neat sample and then halving the concentration for each subsequent dilution. Plot the standard curve on the same axes.
5. If the sample curve parallels the standard curve, then one can deduce that the hormone(s) contained in the sample is immunologically-similar to the hormone contained in the standards and therefore can be measured proportionately.
6. Parallelism is also used as a starting point for determining what dilution unknown samples should be run at. In general, choose the dilution where the pool bound at approximately 50%.

### Accuracy/Recovery Check

1. Make a sample pool. If possible, use samples that have a low concentration of hormone (e.g., for progestins, use samples from the follicular phase).
2. Spike aliquots of pooled sample (100  $\mu$ l) with an equal amount (100  $\mu$ l) from each standard. Analyze the spiked samples as unknowns using the assay of interest. The sample pool also needs to be analyzed without added standard to determine the amount of endogenous hormone present.
3. Calculate the "Amount Expected" and "Amount Observed" for each standard. "Amount Expected" is (known standard concentration / 2). "Amount Observed" is (concentration obtained from assay results – endogenous hormone concentration).
4. Determine the % Recovery for each standard:  $(\text{Amount Observed}/\text{Amount Expected}) \times 100$
5. Plot "Amount Observed" vs. "Amount Expected" and conduct a linear regression analysis. Slopes  $>$  or  $<$  1 suggests an over or under estimation of hormone mass, respectively. This analysis tests for potential interference caused by substances contained within the biological sample that are independent of specific antigen-antibody binding. This test also indicates the degree to which the measured concentration corresponds to the true concentration of a substance.

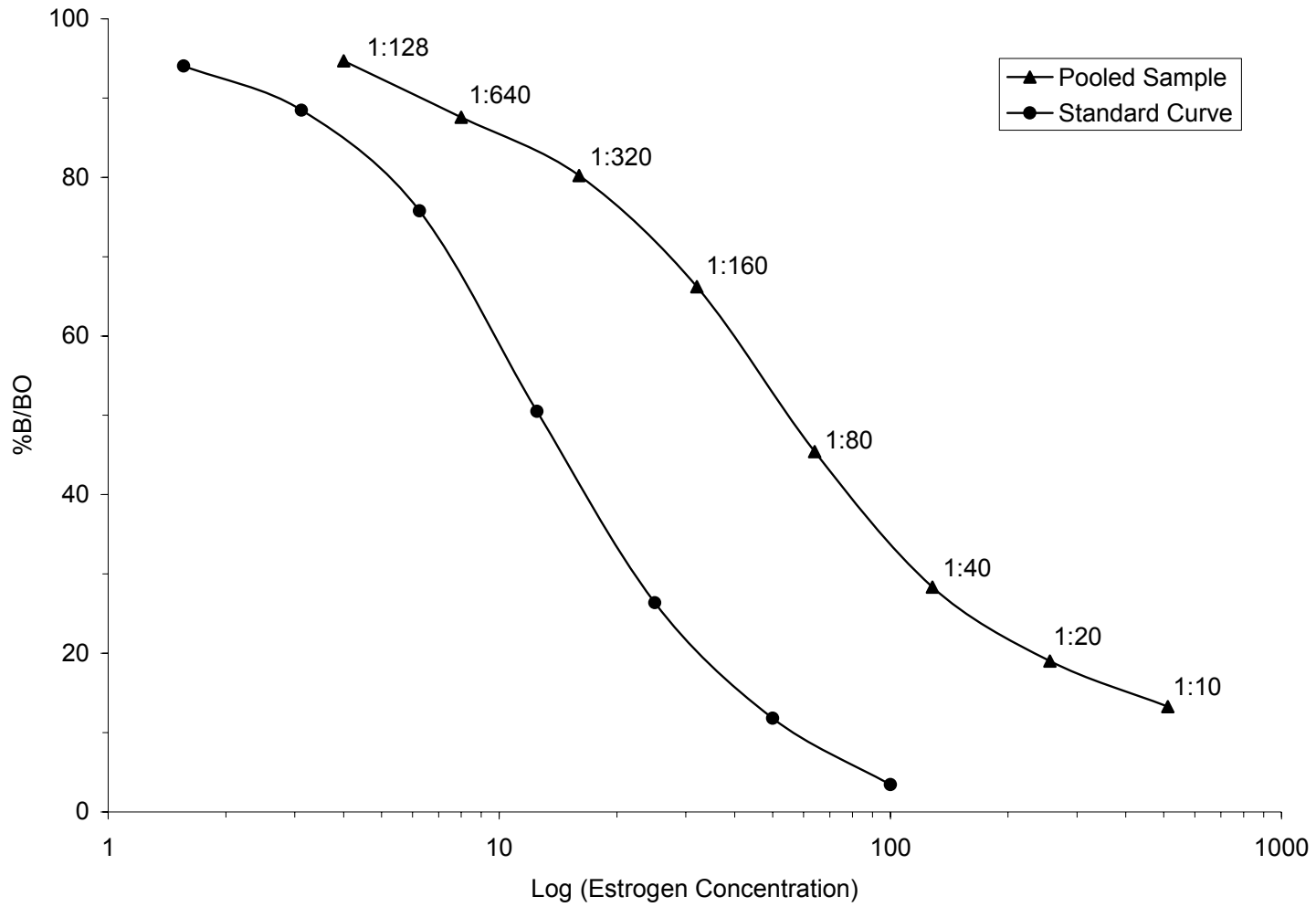
Source: Brown, J., Walker, S., and Steinman, K. 2005. "Endocrine Manual for the Reproductive Assessment of Domestic and Non-Domestic Species". 2<sup>nd</sup> Edition. Conservation and Research Center Endocrine Research Laboratory, Front Royal, VA.

**Domestic Cat Parallelism Results**  
E1S Parallelism



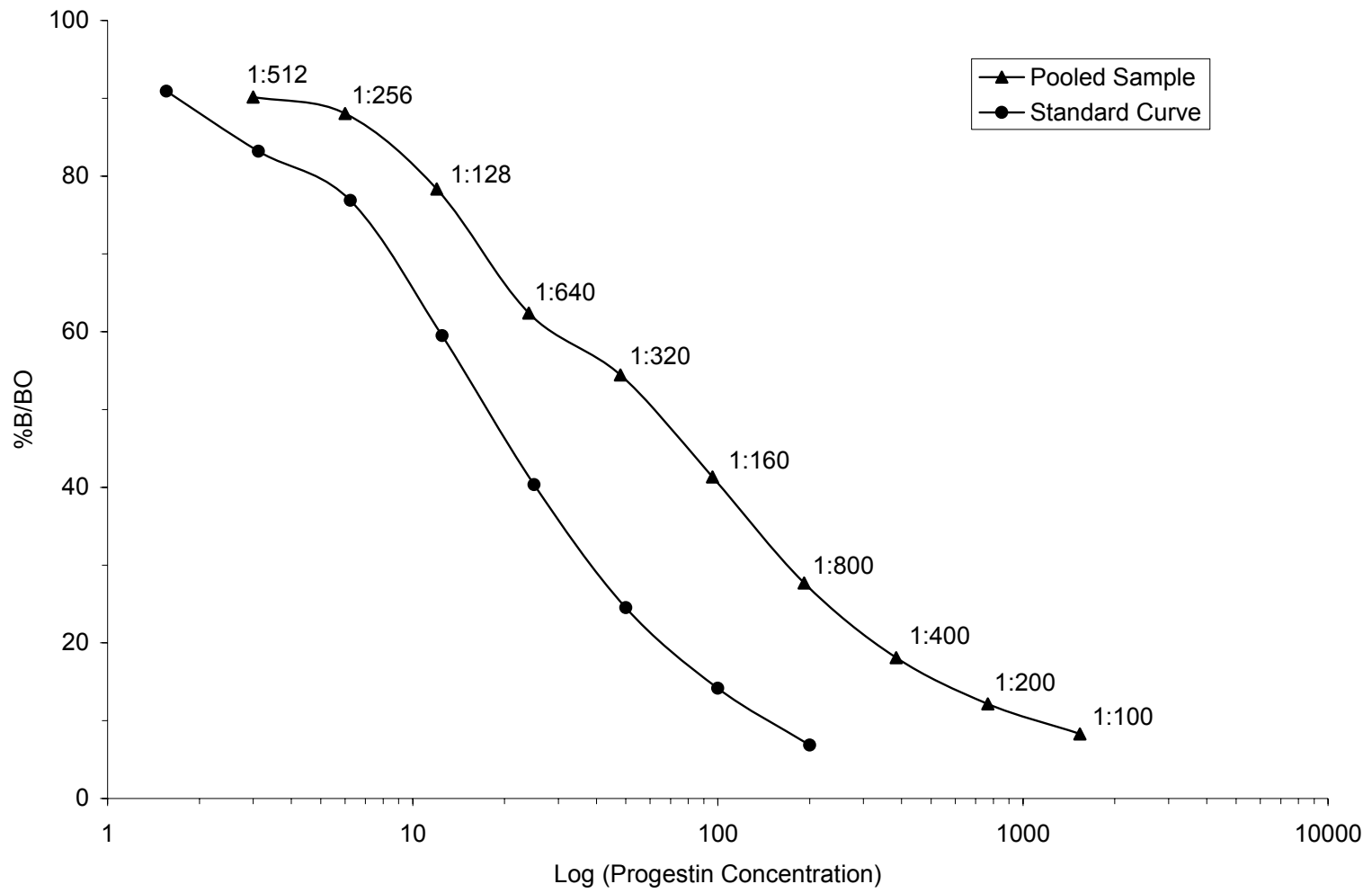
# Domestic Cat Parallelism Results

EC Parallelism

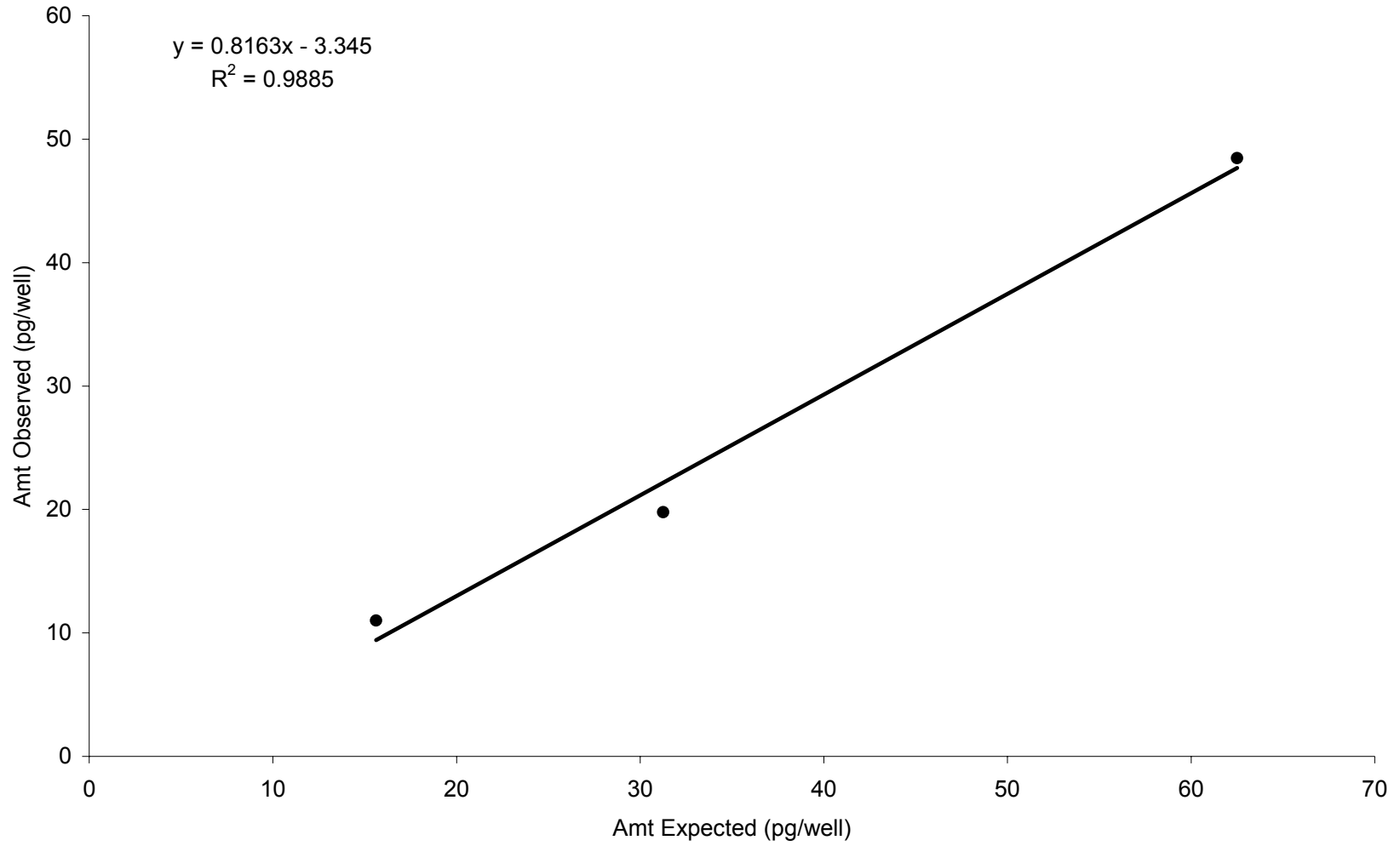


# Domestic Cat Parallelism Results

Pregnane Parallelism

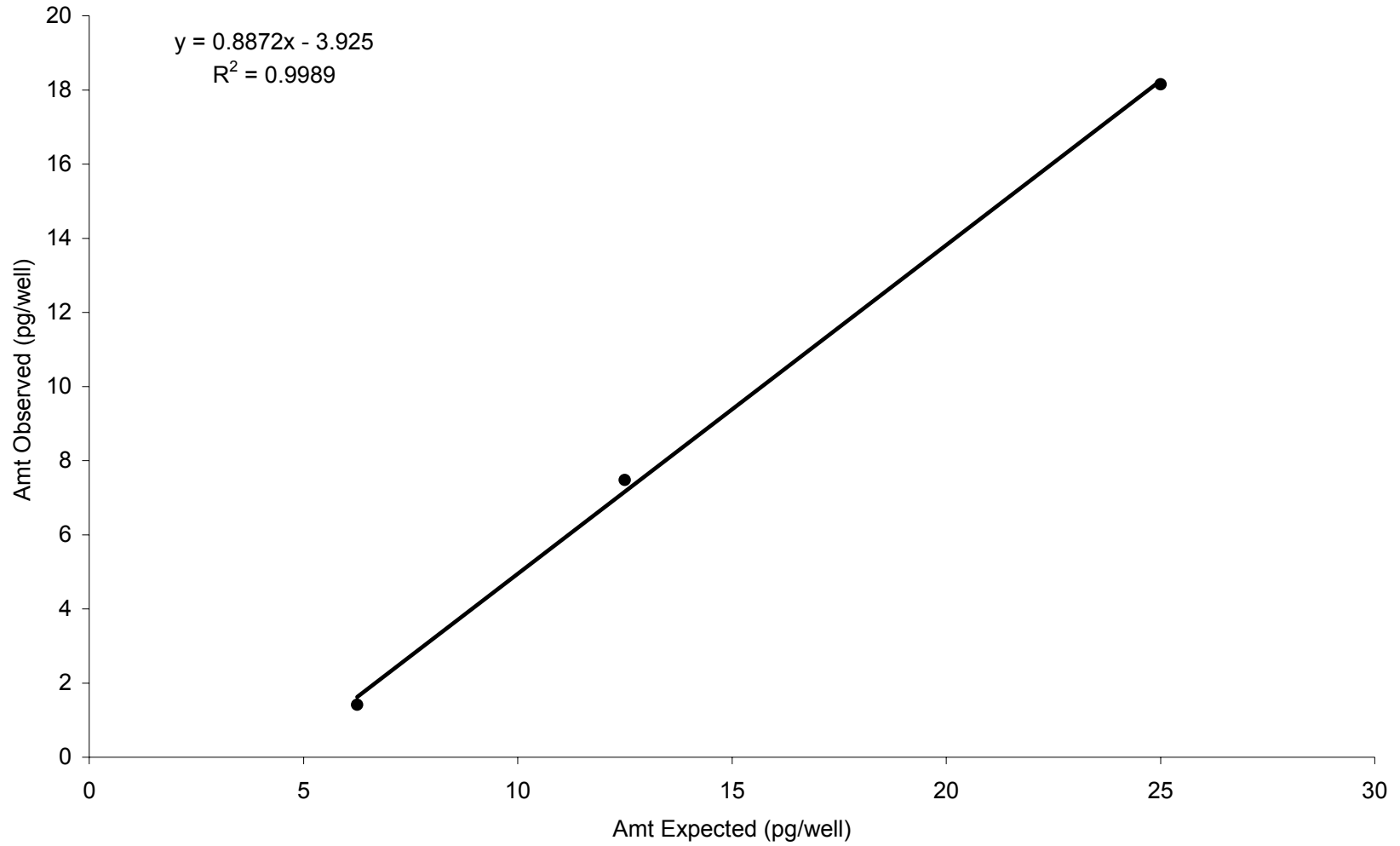


Domestic Cat Recovery/Accuracy Check  
E1S

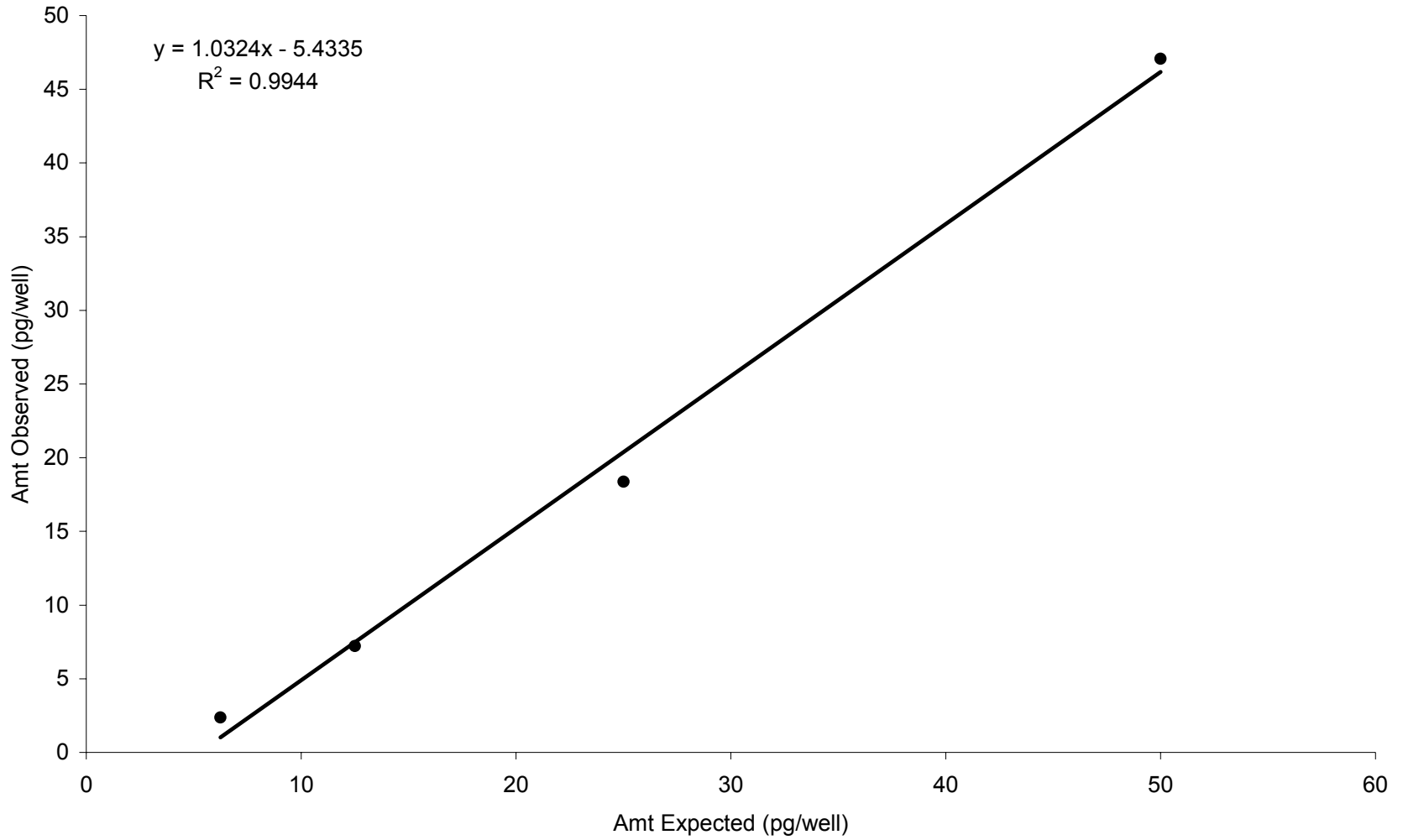




Domestic Cat Recovery/Accuracy Check  
EC



Domestic Cat Recovery/Accuracy Check  
PG



## **Appendix C: Sperm Protocols**

### **Electroejaculation**

1. Prepare HF10 sperm collection media, filter using a 0.22  $\mu\text{m}$  filter into a sterile culture tube and maintain at 37°C.
2. After anesthesia induction, examine testes and measure length and width with laboratory calipers. Calculate testis volume.
3. Prolapse penis and clean with gauze pad moistened with saline. Lubricate a 1 cm rectal probe, insert into the rectum and position the electrodes ventrally. Apply gentle ventral pressure during electroejaculation.
4. Place a warmed, sterile collection vial over the penis.
5. Deliver 3 series of electrical stimulations (30 stimulations/series):
  - a. Series 1 = 10 stims at 2 volt, 10 stims at 3 volts, 10 stims at 4 volts
  - b. Series 2 = 10 stims at 3 volts, 10 stims at 4 volts, 10 stims at 5 volts
  - c. Series 3 = 15 stims at 4 volts, 15 stims at 5 volts
6. Between each series, 'rest' the male for 3-5 minutes and exchange semen vials.

### **Semen Evaluation and Preparation of AI Dose**

1. After each series, measure the total semen volume with a pipettor and transfer into a warmed, sterile Eppendorf tube.
2. Dilute semen slowly (drop-wise) with an equal volume of 37°C HF10. Remove 3  $\mu\text{l}$  and observe several fields under phase contrast microscopy to evaluate sperm percent motility (0-100%) and sperm forward progression status (scale 0-5; 5 = best).
3. For sperm morphology and acrosome assessment, remove 8  $\mu\text{l}$  raw semen from Series 2 and divide between vials of 0.5 ml 0.3% glutaraldehyde (pH 7.4, 340mOsm) and 4% paraformaldehyde. Store fixed samples at 4°C.
4. After electroejaculation is complete, combine all aliquots to determine sperm concentration of the overall ejaculate. Insert 5  $\mu\text{l}$  diluted sperm into an Unopette red blood cell vial and mix well. Allow vial to stand at room temperature ~ 5 minutes to kill sperm. Fill hemocytometer on both sides of unit and allow sperm to settle into one plane. Count sperm in the four large corners of the hemocytometer (16 squares per corner) and multiple by the dilution factor (usually 2).
5. For AI, combine diluted samples from the two males, gently mix and assign an overall percent sperm motility and forward progression status.
6. Centrifuge combined sample for 8 min at 300g. Remove supernatant and re-suspend sperm pellet in fresh Ham's F10. Re-centrifuge the supernatant and add the second sperm pellet to the AI dose. Add HF10 as needed to achieve a final AI volume of ~ 200  $\mu\text{l}$  per cat.
7. Evaluate final AI dose for motility, status and concentration. Fix sperm for morphology and acrosome assessments.

### **Sperm Morphology Assessment**

1. To assess morphology of sperm fixed in 0.3% glutaraldehyde, prepare a wet mount slide with 1-2  $\mu\text{l}$  sperm. Examine 200 sperm/aliquot under oil (1,000x) using phase-contrast microscopy.

2. Categorize sperm as normal or abnormal due to: (1) head defects including macrocephaly, microcephaly, bicephaly, bent neck or damaged acrosome; (2) midpiece anomalies including bent midpiece, bent midpiece with cytoplasmic droplet, midpiece aplasia or damaged midpiece; or (3) flagellar deformities including tightly coil flagella, bent flagella without cytoplasmic droplet or bent flagella with cytoplasmic droplet.

### **Acrosome Assessment**

1. To assess acrosomal integrity in sperm fixed in 4% paraformaldehyde, centrifuge the fixed sperm at 5000 rpm for 8 min, carefully remove and discard the supernatant, add 0.5 ml 0.1M Ammonium acetate to the pellet and gently resuspend the sperm. Repeat this procedure twice.
2. After the last centrifugation, remove the final supernatant, leaving behind 30-50  $\mu$ l of the supernatant. Gently tap the tube to resuspend the sperm mixture.
3. Make short smears with the sperm mixture on 2 labeled, frosted slides. Air-dry or place on a slide warmer until dry.
4. Apply 20-40  $\mu$ l of Coomassie blue stain solution to the slide and incubate at ambient temperature for no longer than 90 seconds.
5. Wash the slides with 4-5 ml of distilled water and air-dry.
6. Apply a drop of Permount mounting medium to the slide and place a clean coverslip over the smear. Allow the mounting medium to dry overnight.
7. Evaluate the slide under oil using bright field optics, counting a minimum of 100 sperm.
8. Categorize sperm acrosome as being: (1) intact; uniform blue stain encompassing the entire acrosomal region; (2) damaged; patchy blue staining pattern; or (3) non-intact; no stain observed in the acrosomal region.

*Source: Howard, J.G. and Pukazhenti, B. 2003. "Protocol for Reproductive Assessment of Male Felids". Smithsonian's National Zoo Gamete Laboratory, Washington, DC.*

## Appendix D: Protocol for Reproductive Tissue Processing

1. Following ovariectomy, cover tract with saline-moistened sponge and transport to lab. Quickly photograph tract and take measurements with handheld calipers.
2. Dissect out mesosalpinx tissue to elongate oviduct. If flushing oviduct (Day 5 spay), place hemostat at the UTJ and flush each oviduct retrograde into Petri dish (25g needle- 5 ml oviduct flushing medium). Repeat 4-6 times in alternating order.
3. Under the dissecting scope, search each dish for oocytes/embryos.
4. If unfertilized oocytes present, fix for staining:
  - a. Wash 3x in embryo culture medium and transfer to a clean, labeled slide with minimal liquid.
  - b. Space oocytes/ embryos around the slide and AIR DRY for several minutes.
  - c. Immerse in 100% ethanol at RT.
  - d. Store slides at 4°C (sample side up) until staining.
5. If embryos present, immediately transfer to culture. Based on the total number of embryos recovered (maximum 5 embryos/drop; minimum 10µl media/embryo), prepare 10-50 µl culture drops under mineral oil using equilibrated embryo culture medium. Wash embryos three times in culture medium, transfer to drops and place in an incubator (38.5°C; 5% CO<sub>2</sub> in air).
6. Process the following oviduct samples, being sure to label each sample specifically:  
Oviduct Transverse Cross Sections (right and left side)
  - a. Place in Cassette: 4% Paraformaldehyde
  - b. Place in Tissue Mold: TBS cryoprotectant
  - c. Place in cryovial: Flash freeze
  - d. Place in cryovial: RNA Later
7. Cut through the uterine bifurcation over a Petri dish. Trim any rough edges and dissect out the mesometrium if necessary to straighten each uterine horn.
8. Process the following uterine samples, being sure to label each sample specifically:  
Uterine Transverse Cross Sections (right and left side)
  - a. Place in Cassette: 4% Paraformaldehyde
  - b. Place in Tissue Mold: TBS cryoprotectant
  - c. Place in cryovial: Flash freeze
  - d. Place in cryovial: RNA LaterEndometrium (right and left side)
  - a. Place in cryovial: Flash freeze
  - b. Place in cryovial: RNA Later
9. Record number of follicles and CL on each ovary and take ovarian measurements. Take the ovary with fewer CL and bisect longitudinally. Place one hemi-ovary in 4% Paraformaldehyde and the other in TBS cryoprotectant.
10. Dissect out CLs from second ovary and weigh. Flash-freeze half of the CLs and place the other half in RNAlater.

## Appendix E: Protocol for Corpora Lutea (CL) Progesterone Assay

### CL Extraction

1. Following ovariectomy, recover one half of the total number of CL from one ovary using curved dissection scissors to gently tease out and release the CL.
2. Weigh CL, place in 2 ml cryovial and plunge into liquid nitrogen. Store at -80° C.
3. Thaw CL at ambient temperature and place in ground glass homogenizer. Add 1 ml PBS buffer and gently homogenize into solution for 2 min. Decant into a labeled 16x125 mm glass extraction tube. Rinse sides of homogenizer with 1 ml buffer to recover any residual tissue and decant into same extraction tube. Rinse homogenizer with 100% ethanol and dry before proceeding to the next CL.
4. Add 3 ml absolute ethanol to each extraction tube and boil for 20 min. Add 100% ethanol as needed during boiling to maintain pre-boil volumes.
5. Centrifuge at 500g for 20 min and decant supernatant into a new extraction tube.
6. Resuspend luteal pellet in 2 ml absolute ethanol, place in sonicator for 20 min to free adhered tissue from the glass tube and vortex for 1 min.
7. Re-centrifuge at 500g for 15 min.
8. Combine both supernatants, dry completely under air and then redissolve in 1 ml methanol. Place in sonicator for 20 min.
9. Dilute CL extract to a concentration of 1:10 (200 µl sample in 1.8 ml buffer). Store at -20° C until analysis.

### P4 RIA

1. Remove Coat-a-Count® progesterone RIA kit from storage at 4°C so it is at ambient temperature before use.
2. Dilute CL extracts as needed in PBS.
3. Label 2 antibody-coated tubes each for the following: standards (A, 0.05, B, C, D, E, F, G), controls (DPC 4-6) and each sample. Label 2 non-coated tubes each for the total count and NSB.
4. Add 50 µl of Standard A to the NSB and A tubes.
5. To create the additional standard, add 70 µl of both A and B standards to a glass tube, and vortex. (this tube will be the 0.05 standard).
6. Add 50 µl each of standards (0.05, B, C, D, E, F, G), controls and samples to the appropriately-labeled tube.
7. Add 500 µl of the <sup>125</sup>I progesterone tracer to each tube, mix well and incubate at ambient temperature for three hours.
8. Pour off all tubes, except totals, making sure there is no liquid left in the tubes.
9. Load tubes into gamma counter (CRC Protocol 5).

*Source: Swanson WF, Roth TL, Brown JL, Wildt DE. 1995. Relationship of circulating steroid hormones, luteal luteinizing hormone receptor and progesterone concentration, and embryonic mortality during early embryogenesis in the domestic cat. Biology of Reproduction 53(5):1022-1029.*

## Appendix F: Protocol for RNA Extraction of Luteal Tissue

1. Prepare the area where the RNA extraction will take place. Clean all equipment and hands with RNAase Zap® prior to starting and throughout the procedure.
2. Prepare the Qiagen RNeasy® Mini Kit. Add 44 µl molecular grade 100% ethanol to the Buffer RPE concentrate. Also, prepare the working Buffer RLT by adding 10 µl β-Mercaptoethanol per ml of Buffer RLT stock in the fume hood. This working solution is stable at RT for 1 month.
3. Prepare the Qiagen DNase stock solution by dissolving DNase I in 550 µl of the RNase free water provided. Mix gently- do not vortex. DNase stock can be stored in single-use (90 µl) aliquots at -20°C for up to 9 months. Thawed aliquots are stable for 6 weeks at 2-8°C- do not re-freeze.
4. For each run (8 samples per run), remove the domestic cat CL samples stored in RNAlater from the -80°C freeze and maintain on ice.
5. Isolate ~ 20 mg of tissue from the RNAlater and record the exact weight.
6. Place tissue in a 2 ml flat bottom microcentrifuge tube. Add 600 µl Buffer RLT and homogenize tissue immediately for 40 seconds using a rotor-stator homogenizer.
7. Proceed to the next sample. Before first use and between each sample, clean the homogenizer by running the blade thru 1) 50 ml DEPC water; 2) 50 ml 70% ETOH; and 3) 50 ml chloroform. Wipe and dry blade.
8. Centrifuge for 5 minutes at maximum speed (13,000 RPM).
9. Pipet supernatant to a new 2 ml microcentrifuge tube. Do not transfer the pellet or any lipid layer on the surface of the supernatant.
10. Add 1 volume (~600 µl; adjust for any lost volume) of molecular grade 70% ethanol and mix by pipetting. Continue immediately to the next step.
11. Add 700 µl ethanol mixture to an RNeasy mini column placed in a 2 ml collection tube. Close the tube gently and centrifuge for 30 seconds at >8,000g. Discard the flow-through. Repeat as needed with the additional volume using the same column and collection tube.
12. Perform DNase Digestion. Pipet 350 µl Buffer RW1 into the column and centrifuge for 15 s at > 8,000g (> 10,000 RPM). Discard the flow-through.
13. Add 10 µl DNase I stock solution to 70µl Buffer RDD. Mix gently by inverting the tube.
14. Pipet 80 µl DNase I incubation mix directly onto the RNeasy silica-gel membrane and maintain at RT for 15 minutes.
15. Pipet 350 µl Buffer RW1 into the column, centrifuge for 15 s at >8,000g . Discard the flow-through.
16. Transfer the column to a new 2 ml collection tube and pipet 500 µl Buffer RPE onto the column. Close tube, centrifuge for 15 s at >8,000g and discard the flow-through.
17. Add another 500 µl volume of Buffer RPE to the column and centrifuge for 2 minutes
18. Move filter to a new collection tube and discard flow-through tube. Centrifuge at full speed for 1 min.
19. To elute, transfer the column to a final 1.5 ml collection tube and pipet 40 µl RNase-free water directly onto the silica-gel membrane. Close tube and centrifuge at >8,000g for 1 min.
20. Place RNA extract on ice immediately.

Source: RNeasy® Mini Kit protocol, Qiagen Corporation.

## Appendix G: Protocol for Primer Design

1. After identifying the genes of interest, check the NCBI nucleotide sequence database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) to determine whether an mRNA sequence exists for domestic cat. If not, choose the most closely related species (canine, bovine).
2. Take this sequence and BLAST it in the Ensembl Genome Browser ([www.ensembl.org](http://www.ensembl.org)) against the cat genome.
3. From the results page, go to the "Contig" view of the best match (top of the list). Check the sequence for gene homology with other species. Click on the novel gene projection to obtain the cat transcript sequence.
4. Choose the final sequence to be used for primer design. It should be approx 300-500 basepairs, as close to the 3' end of the transcript as possible, and span more than one exon if possible.
5. Copy this sequence into PrimerExpress®. Use the RT-PCR template for primer design and choose the following parameters:
  - a. Temp: Minimum 57°C, Maximum 63°C, Optimal 60°C
  - b. GC Content: Min 40, Max 60; 0 residues
  - c. Primer length: Min 19, Max 21, Opt 20
  - d. Annealing temp: Min 75°C, Max 85°C
  - e. Amplicon length: Min 100, Max 120
6. Search for primers and choose the best pair of forward and reverse primers, based on low penalty, length of primers and amplicons and G/C content.
7. Check the primers in Ensembl's BLAST program. Run a BLAST on both simultaneously. Input into BLAST as follows:

```
>cyp11a1_Forward
GGATCGCTGAGCTCGAGATG
>cyp11a1_Reverse
TGAGGTTGAATATGGTGCCCA
```
8. Cross-check to make sure these primers fall on the forward and reverse strands of the sequence of interest.
9. Order primers (0.025 nm; desalted).
10. Upon arrival, reconstitute in nuclease-free water to a 100µM stock solution and store at -20°C.



## **Appendix H: Protocol for Two-Step Real Time RT- PCR**

### **RNA Quantification Using the GeneQuant II Spectrophotometer**

1. Choose the following in the set up menu:
  - Path Length: 10
  - Read at: 320 nm
  - Dilution factor: 100
  - Setup factor: RNA
2. Dilute samples 1:100 (1  $\mu$ l sample in 99  $\mu$ l water). Prepare 90  $\mu$ l water blank.
3. Start with the blank. Put 90  $\mu$ l in the cuvet, check for air bubbles, press Set/Ref and follow the directions to calibrate.
4. Empty cuvet, rinse with water and pat dry. Be careful not to drop the cuvet or touch the front or back where the windows are.
5. Continue with each sample. Record the absorbance at 260 and 280, the ratio (should be greater than 1.5) and the sample concentration.

### **RNA Formaldehyde Gel Electrophoresis**

1. Prepare gel mold by cleaning components thoroughly and spraying down with RNA Zap. Place the spacers and comb in the mold.
2. Dissolve agarose in water by heating in a microwave. Cool to touch (approx. 50°C).
3. In hood, add 10X MOPS and formaldehyde to agarose mixture. Mix and immediately pour into gel mold. Allow to set and then remove spacers and comb.
4. Prepare running buffer (1X MOPS). Add to entire mold.
5. For each sample, place ~1  $\mu$ g RNA in tube (volume will vary). Add RNA loading buffer containing EtBr (Box 1A, -20°C) 2:1 based on your maximum volume (e.g. if largest sample volume is 3  $\mu$ l, add 6  $\mu$ l loading buffer to all samples). Also prepare 2 ladders in this manner (Box 3I; -80°C). Gently mix and centrifuge briefly.
6. Heat samples to 95°C in a dry warming block for 5 minutes. Load samples onto gel and run at 190V. Stop when the dye is approx 2/3 down the gel. Read on the UV transilluminator. Two distinct bands (28S and 18S rRNA) should be seen.

### **Reverse Transcription Using SuperScript III (Invitrogen)**

1. Add to a nuclease-free 0.5 ml microcentrifuge tube:
  - 0.5  $\mu$ g - 1  $\mu$ g RNA
  - 1  $\mu$ l 50  $\mu$ M anchored oligo-dT primer\*
  - 1  $\mu$ l 10 mM dNTPs
  - nuclease-free water to 13  $\mu$ l.\*Make a premix of primer and dNTPs and add 2  $\mu$ l per tube
2. Quick spin, vortex to mix and quick spin again. Heat to 65°C in the thermocycler for 5 minutes and incubate on ice for at least one minute.
3. Quick spin and add:
  - 4  $\mu$ l 5X first strand buffer\*
  - 1  $\mu$ l 0.1 M DTT
  - 1  $\mu$ l RNase inhibitor (40 U/ $\mu$ l)
  - 1  $\mu$ l SuperScript III RT (200 U/ $\mu$ l)

- \* Make a supermix and add 7  $\mu$ l per tube
4. Prepare a no-RT control by adding all components to a pool of 1  $\mu$ g RNA except the SuperScript III reverse transcriptase.
  5. Mix gently with a pipette and quick spin. In the thermocycler, incubate at 50°C for 60 minutes and then 70°C for 15 minutes to inactivate the reaction.
  6. Add 80  $\mu$ l nuclease-free water to the cDNA to make a 1:5 dilution. Store cDNA at -20°C until use.

### **SYBR Green PCR**

1. In a Bio-Rad 96 well PCR plate, dispense 1  $\mu$ l of each cDNA sample in triplicate. Also prepare 1 water sample and 3 no-RT reaction samples to check for potential contamination.
2. Add 19  $\mu$ l PCR mastermix to each well using an automated repeater:
  - 10  $\mu$ l iQ SYBR Green Supermix (Bio-Rad)
  - 0.8  $\mu$ l 10  $\mu$ M forward primer
  - 0.8  $\mu$ l 10  $\mu$ M reverse primer
3. Seal the plate carefully and quick spin.
4. Set up the iCycler/MyiQ with the appropriate protocol: 95°C for 3 min.; 40 cycles of 95°C for 15 sec then 60°C for 1 min; 95°C for 1 minute, 55°C for 1 minute; melt curve analysis (80 cycles of 55°C,95°C).

*Source: University of Maryland: T.E. Porter Laboratory Protocols*

## Appendix I: Recipes for Commonly-used Reagents

### EIA COATING BUFFER (1 liter; pH 9.6)

Na <sub>2</sub> CO <sub>3</sub> (Anhydrous)	1.59 g
NaHCO <sub>3</sub>	2.93 g
Milli-Q H <sub>2</sub> O	1000 ml

### EIA ASSAY BUFFER (1 liter; pH 7.0)

Stock A (0.2M NaH <sub>2</sub> PO <sub>4</sub> )	27.8 g/l dH <sub>2</sub> O; USE 195 ml
Stock B (0.2M Na <sub>2</sub> HPO <sub>4</sub> )	28.4 g/l dH <sub>2</sub> O; USE 305 ml
NaCl	8.7 g
BSA	1.0 g
Milli-Q H <sub>2</sub> O	500 ml

### EIA DILUTION BUFFER (1 liter; pH 7.0)

Equal to Assay Buffer without BSA

### EIA WASH CONCENTRATE (1 liter; dilute 1:10 for working solution)

NaCl	87.66 g
Tween 20	5 ml
Milli-Q H <sub>2</sub> O	1000 ml

### EIA SUBSTRATE BUFFER (1 liter; pH 4.0)

Citric acid (anhydrous)	9.61 g
Milli-Q H <sub>2</sub> O	1000 ml

### EIA ABTS (40 mM; 25 ml)

ABTS	0.55 g
Milli-Q H <sub>2</sub> O	25 ml

*Wrap in foil- light sensitive*

### EIA HYDROGEN PEROXIDE (0.5M; 8 ml)

H <sub>2</sub> O <sub>2</sub> (30% Solution)	500 µl
Milli-Q H <sub>2</sub> O	8 ml

### RIA PRESERVATIVE-FREE PBS (5 liters; pH 7.0)

Disodium phosphate	23 g
Monosodium phosphate	13.55 g
Sodium chloride	44 g
Milli-Q H <sub>2</sub> O	5 L

**SPERM COLLECTION MEDIUM (10ml; filter sterilize; HF 10)**

Hepes-buffered Ham's F-10	9.2 ml
Pyruvate stock (28.4 mg/ml)	100 $\mu$ l
Glutamine stock (2.6 mg/ml)	100 $\mu$ l
Pen/Strep/Neo stock (in 10ml)	130 $\mu$ l
5% Fetal Calf Serum	500 $\mu$ l

**AMMONIUM ACETATE (600 ml; 0.1 M; pH 9.0)**

Ammonium acetate	4.62 g
Milli-Q H <sub>2</sub> O	550 ml

**COOMASSIE BLUE WORKING STAIN (100ml)**

Methanol	50 ml
Glacial acetic acid	10 ml
Milli-Q H <sub>2</sub> O	40 ml
Coomassie Blue G-250	0.22 g

**OVIDUCT FLUSH MEDIUM (50ml; filter sterilize)**

Hepes-buffered Ham's F-10	50 ml
BSA (embryo culture grade)	0.2 g

**EMBRYO CULTURE MEDIUM (5 ml; filter sterilize)**

Ham's F-10 (no Hepes)	4.6 ml
Pyruvate stock (28.4 mg/ml)	50 $\mu$ l
Glutamine stock (2.6 mg/ml)	50 $\mu$ l
Pen/Strep stock (in 10ml)	50 $\mu$ l
5% Fetal Calf Serum	250 $\mu$ l

**10X MOPS (1 liter; pH 6.5-7.0)**

0.2 M MOPS	42 g
0.05 M NaOAc	16.67 ml of 3M
0.01 EDTA	20 ml of 0.5M

*Bring to a final volume of 1L using dH<sub>2</sub>O. Wrap in foil- light sensitive.*

**FORMALDEHYDE GEL**

<i>Final Concentration</i>	<i>Mini Gel</i>	<i>Medium Gel</i>
1% Agarose	0.3 g agarose	2 g agarose
1X MOPS	3 ml of 10X MOPS	20 ml of 10X MOPS
3% Formaldehyde	2.4 ml of 37%	16 ml of 37%
Water	24.6 ml	164 ml

*Source: Conservation and Research Center: Endocrine and Gamete Laboratory  
Protocols; University of Maryland: T.E. Porter Laboratory Protocols*

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