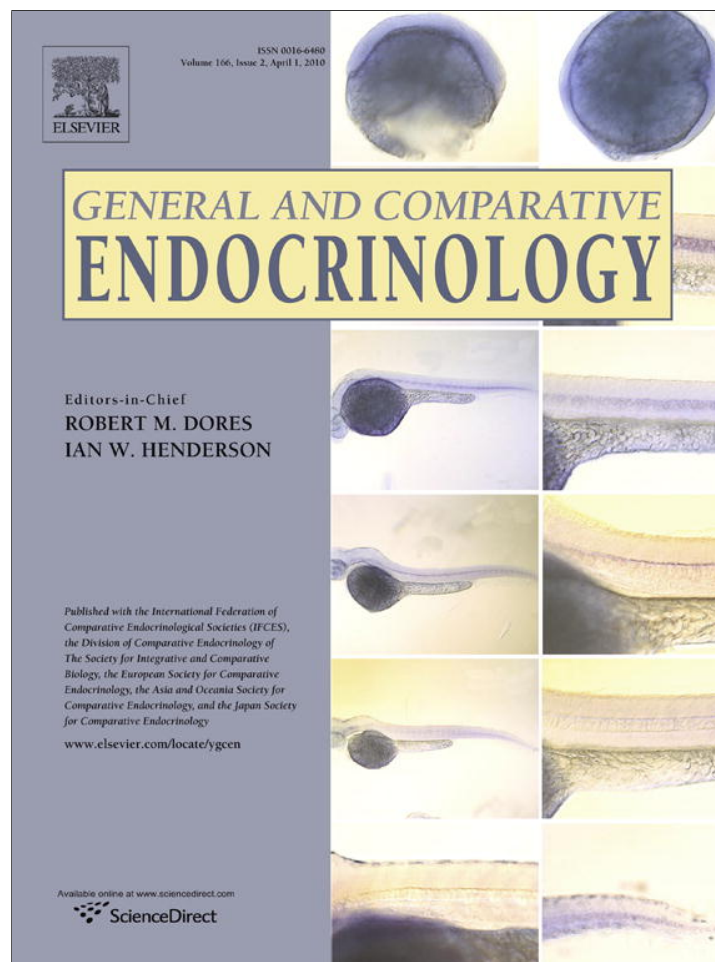


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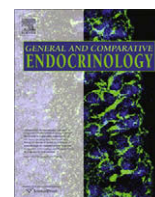
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Oral progestin induces rapid, reversible suppression of ovarian activity in the cat

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ARTICLE INFO

Article history:

Received 28 July 2009

Revised 7 December 2009

Accepted 28 December 2009

Available online 4 January 2010

Keywords:

Cat

Progestin

Altrenogest

Ovarian suppression

Fecal hormone monitoring

Estrous cycle

ABSTRACT

The influence of oral progestin (altrenogest; ALT) on cat ovarian activity was studied using non-invasive fecal steroid monitoring. Queens were assigned to various ALT dosages: (1) 0 mg/kg (control; $n = 5$ cats); (2) 0.044 mg/kg (LOW; $n = 5$); (3) 0.088 mg/kg (MID; $n = 6$); or (4) 0.352 mg/kg (HIGH; $n = 6$). Fecal estrogen and progestagen concentrations were quantified using enzyme immunoassays for 60 days before, 38 days during and 60 days after ALT treatment. Initiation of follicular activity was suppressed in all cats during progestin treatment, whereas controls continued to cycle normally. Females ($n = 6$) with elevated fecal estrogens at treatment onset completed a normal follicular phase before returning to baseline and remained suppressed until treatment withdrawal. All cats receiving oral progestin re-initiated follicular activity after treatment, although MID cats experienced the most synchronized return (within 10–16 days). Mean baseline fecal estrogens and progestagens were higher ($P < 0.05$) after treatment in HIGH, but not in LOW or MID cats compared to pre-treatment values. The results demonstrate that: (1) oral progestin rapidly suppresses initiation of follicular activity in the cat, but does not influence a follicular phase that exists before treatment initiation; and (2) queens return to normal follicular activity after progestin withdrawal. This study provides foundational information for research aimed at using progestin priming to improve ovarian response in felids scheduled for ovulation induction and assisted breeding.

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1. Introduction

Endangered felids have benefited from assisted reproduction strategies designed to maintain or increase genetic diversity in small populations managed *ex situ* (Wildt and Roth, 1997; Wildt et al., 2010). Approaches such as artificial insemination (AI) and *in vitro* fertilization/embryo transfer can improve reproductive efficiency in rare cat species when natural breeding fails (Howard, 1999; Swanson, 2003; Howard and Wildt, 2009). A laparoscopic intrauterine AI technique has been successful, producing offspring in the cheetah (Howard et al., 1992, 1997), clouded leopard (Howard et al., 1996, 1997), tiger (Donoghue et al., 1993), puma (Barone et al., 1994), leopard cat (Wildt et al., 1992), snow leopard (Roth et al., 1997a), tigrina (Swanson and Brown, 2004) and ocelot (Swanson et al., 1996b). However, pregnancy success is variable across species. AI efficiency is ~45% in the cheetah (Howard et al., 1997), but success remains low (<5%) in the clouded leopard (Howard et al., 1997) and tiger (Graham et al., 2006), and multiple

attempts have failed in the fishing cat (Bauer et al., 2004) and Pallas' cat (Brown et al., 2002).

Although a clear etiology for pregnancy failure after AI has not been established, one contributor appears to be inconsistent ovarian response after exogenous gonadotropins are used to stimulate the ovary before insemination (Howard and Wildt, 2009). Most felid ovulation induction protocols involve administering a species-specific dosage of equine chorionic gonadotropin (eCG) to initiate follicular growth followed by human chorionic gonadotropin (hCG) to complete meiotic maturation of the oocyte(s) and ovulation (Howard, 1999). However, eCG can stimulate ovulation if given during estrus, and hCG has folliculogenic properties in the cat (Swanson et al., 1997). Thus, when eCG and hCG are administered in tandem at random intervals in the estrous cycle, a hyper-follicular state can occur (Swanson et al., 1996a), resulting in abnormally high and protracted circulating estrogen profiles, reduced embryo quality and delayed embryo transport through the oviduct (Roth et al., 1997b; Graham et al., 2000). Felids are also notorious for exhibiting unusually high variation in ovulation 'type'. Traditionally considered induced ovulators (Wildt et al., 1980), more recent investigations have confirmed spontaneous ovulation in laboratory-housed domestic cats (Lawler et al., 1993; Gudermuth et al., 1997; Graham et al., 2000; Pelican et al., 2005) and certain non-domestic species, including the fishing cat, margay and clouded leopard (Brown et al., 2001; Moreira et al., 2001; Brown, 2006). Spontaneous ovulation further complicates

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artificial control of the ovary because corpora lutea presence and parallel elevations in circulating progesterone reduce (or prevent) the effective use of exogenous gonadotropins (Pelican et al., 2006, 2008).

For assisted reproduction to be maximally efficient in felids, a 'quiescent' ovary at the time of ovulation induction is required. This environment increases the chance for a consistent and uniform folliculogenic response to ovarian stimulation while greatly diminishing the risk of an altered endocrine milieu. This concept appears especially valid in felids as illustrated by the cheetah, a consistent induced ovulator with frequent periods of acyclicity (Brown et al., 1996). Both these traits no doubt have contributed to 'normal' ovulation numbers post-eCG/hCG and a comparatively high incidence of AI success in the cheetah (Howard et al., 1992, 1997) as well as in another induced ovulator, the ocelot (Swanson et al., 1996b). In non-felids that spontaneously ovulate, including domestic livestock (Lofstedt, 1988; Wood et al., 1992; Deligiannis et al., 2005) and wild ungulates (Monfort et al., 1993; Morrow et al., 2000), progestins are routinely administered prior to assisted breeding to temporarily suppress follicular activity and improve ovarian response. Although the progestins melengestrol acetate (MGA), megestrol acetate and medroxyprogesterone acetate are well-known contraceptive agents in felids (Romatowski, 1989; Munson, 2006), their ability to temporarily suppress ovarian activity for the purpose of assisted reproduction has not been investigated. Our laboratory confirmed the efficacy of the progestin levonorgestrel (Norplant®) delivered as an implant for short-term suppression of ovarian activity in the cat (Pelican et al., 2005). However, this approach requires anesthesia to insert and remove the implants and, more importantly, results in variable returns to ovarian cyclicity after implant removal (ranging from ~2 weeks to >2 months).

The purpose of the present study was to assess the impact of an oral progestin on ovarian function in the cat. We selected altrenogest (ALT), which has been used to synchronize follicular activity before assisted breeding in the horse (Lofstedt and Patel, 1989), pig (Wood et al., 1992), killer whale (Robeck et al., 2004) and bottlenose dolphin (Robeck et al., 2005). The efficacy of three ALT dosages for short-term inhibition of ovarian activity was assessed by non-invasively evaluating fecal steroid profiles. We hypothesized that oral progestin would provide rapid, reversible inhibition in a dose-dependent fashion.

2. Materials and methods

2.1. Animals

Thirteen adult (1–5 year old) female domestic cats were housed at the Conservation & Research Center of the Smithsonian's National Zoological Park. Each queen was maintained individually in a stainless steel cage (0.5 m³) under artificial fluorescent illumination (12 h light:12 h dark) during the ~14-month study. Cats were provided a dry commercial diet (Purina ONE®, Nestlé Purina PetCare Co., St. Louis, MO) and had continual access to water, perch boards, bedding and enrichment items. Each cat was weighed on the day before treatment onset using a digital veterinary scale, and weights (in kg) were used to calculate ALT dosage. All research activities were approved by Institutional Animal Care and Use Committees at Smithsonian's National Zoological Park (05-25) and the University of Maryland, College Park (R-06-07).

2.2. Altrenogest administration

The goal was to achieve an ovarian inhibition interval that coincided with the duration of a normal luteal phase (36–38 days)

in a domestic cat (Brown et al., 1994; Pelican et al., 2005). We presumed that suppression for this interval would ensure lysis of existing corpora lutea (if present) and promote sufficient ovarian quiescence by the end of treatment. Each cat received two different dosages (in Trial 1 versus Trial 2) separated by an 8-month interval. Resumption of normal follicular activity after Trial 1 was confirmed by fecal hormone analyses before beginning Trial 2. Cats were randomly assigned to the two treatments in a balanced order to avoid 'carry-over' effects between the consecutive trials.

Altrenogest oral suspension (Regu-Mate®; 2.2 mg/ml; Intervet Inc., Millsboro, DE) was stored at room temperature (~21 °C) in an opaque container. During each trial, the progestin was mixed into ~5 g of wet food (Friskies®, Nestlé Purina PetCare Co.) and administered daily for 38 consecutive days. In Trial 1, each cat was assigned to one of four ALT treatments: (1) 0 mg/kg (control; wet food only; $n = 3$ cats); (2) 0.088 mg/kg ($n = 3$); (3) 0.176 mg/kg ($n = 3$); or (4) 0.352 mg/kg ($n = 4$). Based on Trial 1 results, the 0.176 mg/kg treatment was removed from the experimental design and replaced with a new test dosage (0.044 mg/kg) to better refine the lowest effective dosage. In Trial 2, each cat was assigned to one of four treatments: (1) 0 mg/kg ($n = 2$); (2) 0.044 mg/kg ($n = 5$); (3) 0.088 mg/kg ($n = 3$); or (4) 0.352 mg/kg ($n = 2$).

2.3. Fecal hormone extraction

Over the course of the study, fecal samples were collected daily, sealed in plastic bags labeled with the individual's name/date and stored at –20 °C. Fecal aliquots from 60 days before, 38 days during and 60 days after each treatment period were extracted to quantify estrogen and progestagen concentrations using assays validated for the domestic cat (Brown et al., 1994). Briefly, each fecal sample was lyophilized, pulverized, and 0.18–0.2 g of dry fecal powder was boiled in 5 ml of 90% ethanol for 20 min. During boiling, 100% ethanol was added, as needed, to maintain approximate pre-boil volumes. After centrifugation (500g, 20 min), the supernatant was recovered, and the pellet was resuspended in 5 ml of 90% ethanol, vortexed for 30 s and re-centrifuged (500g, 15 min). The first and second supernatants were combined, dried under air and reconstituted in 1 ml methanol. Methanol extracts were vortexed briefly and placed in a sonicator for 15 min to free particles adhering to the glass tube. Each extract was diluted 1:10 in steroid dilution buffer (0.2 M NaH₂PO₄, 0.2 M Na₂HPO₄, 0.15 M NaCl, pH 7.0) and was stored in polypropylene tubes at –20 °C until enzyme immunoassay (EIA) analyses.

2.4. 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-reacted with a broad range of estrogen metabolites previously identified in domestic cat feces by high-performance liquid chromatography (Brown et al., 1994). The assay relied on a polyclonal antibody (R583; 1:1500; supplied by C.J. Munro, University of California, Davis, CA) produced against estrone-3-glucuronide in coating buffer (0.015 M Na₂CO₃, 0.035 M NaHCO₃, pH 9.6) added to 96-well, flat-bottomed 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 unadsorbed antibody, and 0.025 ml steroid assay buffer (0.2 M NaH₂PO₄, 0.2 M Na₂HPO₄, 0.15 M NaCl, 2.0 g/L BSA, pH 7.0) was added to each well and maintained at room temperature for 2–5 h. Next, 0.05 ml of diluted sample (range, 1:100 to 1:500) or E₁SO₄ 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; supplied by C.J. Munro).

Following a 2-h incubation at room temperature, plates were washed and 0.1 ml substrate (0.04 M ABTS, 0.5 M H₂O₂ in a 0.05 M citric acid solution) was added to each well. Optical densities (ODs) were read using a microplate reader (MRX, Dynex Technologies, Chantilly, VA) at 405 nm when 0 pg standard wells reached an OD of 0.9–1. Serial dilutions of cat feces yielded a displacement curve that was parallel to the standard curve ($R^2 = 0.99$). Recovery of known amounts of E₁SO₄ standards added to a pool of 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).

2.5. Pregnane EIA

A single antibody progesterone EIA was used to quantify progesterone metabolites in every other fecal sample (Schwarzenberger et al., 1991; Graham et al., 2001). The EIA relied upon a monoclonal antibody (CL425; 1:10,000; supplied by C.J. Munro) that cross-reacted with a broad range of progestagen metabolites previously identified in domestic cat feces by high-performance liquid chromatography (Brown et al., 1994). Procedures and assay reagents were the same as described above for the E₁S assay unless otherwise noted. After overnight antibody incubation, plates were washed, and 0.05 ml of diluted sample (range, 1:2000 to 1:6000) or progesterone standard (range, 0.78–200 pg; Sigma–Aldrich) was added to wells in duplicate immediately followed by 0.05 ml enzyme conjugate (progesterone-3CMO horseradish peroxidase; 1:40,000; supplied by C.J. Munro). Following a 2-h incubation, plates were washed, 0.1 ml substrate was added to each well, and OD was read. Serial dilutions of 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 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).

2.6. Statistical analyses

For each individual, baseline fecal estrogen concentrations were determined using an iterative process whereby all values exceeding the mean plus 2 standard deviations (SDs) were deleted from the data set. The average was then recalculated, and the elimination process was repeated until no values exceeded the mean plus 2 SD (Brown et al., 1994; Pelican et al., 2005). The final average generated using 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 a follicular phase was defined as the number of consecutive days during which 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 progestagens. Baseline progestagen concentrations were determined using a similar iterative process, except the mean plus 1.5 SD was used. Values greater than twice the progestagen baseline were considered elevated for that individual. A metric of 1.5 SD was chosen for progestagens because 2 SD was too sensitive to adequately separate baseline concentrations from elevations. A luteal phase was defined when progestagen levels rose above baseline and remained elevated for at least 3 consecutive weeks. Luteal phase length was the total number of days where progestagens remained above baseline. Following treatment, return to follicular activity was calculated as the number of days from progestin removal until the first day fecal estrogens rose above baseline for that individual.

Data from Trials 1 and 2 were not different ($P > 0.05$) and, thus, were combined for further 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). Differences were evaluated for the 60 days before, 38 days during and 60 days after treatment. Mean 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 progestagens) were calculated for each individual and then the means averaged within each treatment. To normalize the iterative process, only the 38 days before, during and after treatment were used when determining baseline fecal steroid concentrations. Values within treatment across time were evaluated using a mixed model repeated measures ANOVA followed by least significant difference (LSD) mean comparisons. Data among treatments within a single time interval were analyzed using a mixed model one-way ANOVA followed by LSD mean comparisons. When necessary, values 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.

3. Results

3.1. Estrous cycle characteristics before treatment

For the 60 days before treatment onset, the duration of the follicular phase averaged ~5 days (range, 3–17 days) with a wide variation (range, 7–43 days) in estrous cycle length (i.e., interval between consecutive estrogen peaks; Table 1). In comparing metrics among groups prior to treatment, baseline estrogen metabolite concentrations were higher in LOW ($P < 0.05$) than in HIGH cats, and follicular phase duration was longer ($P < 0.05$) in LOW and HIGH cats than in controls. All other metrics, including frequency of the follicular phase, estrous cycle length, mean estrogens/follicular phase, peak estrogens/follicular phase and baseline progestagens, were similar ($P > 0.05$) among groups. During this pre-treatment monitoring phase, two of 13 (15.4%) cats ovulated spontaneously. Luteal phase length, peak progestagen concentration and mean progestagen concentration/luteal phase could not be characterized because progestagens were already elevated on the first day of sample collection.

3.2. Influence of altrenogest dosage on estrous cycle characteristics

Control cats randomly cycled at least twice during the experimental interval (based on a temporal rise and fall in fecal estrogens; Fig. 1A). In contrast, no female receiving oral progestin (at any dosage) initiated follicular activity (Fig. 1B–D). Consequently, the number of estrogen peaks did not change ($P > 0.05$) during or

Table 1

Reproductive traits (before oral progestin administration) in cats assessed by longitudinal fecal steroid metabolite analyses ($n = 13$ females).^a

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 estrogen concentration (ng/g feces)	194.8 \pm 12.5
Peak estrogen concentration (ng/g feces)	401.8 \pm 26.2
Mean estrogen concentration/follicular phase (ng/g feces)	331.5 \pm 16.5
Baseline progestagen concentration (μ g/g feces)	2.8 \pm 0.2

^a Values are means \pm SEM.

after treatment in controls, whereas fewer ($P < 0.05$) peaks were observed during oral progestin treatment at all assessed dosages (Fig. 2). Cats treated with the HIGH dosage produced fewer ($P < 0.05$) estrogen peaks before (1.5 ± 0.4 peaks/60 days) compared to after (2.7 ± 0.2 peaks/60 days) treatment. Baseline estrogens were elevated ($P < 0.05$) in HIGH cats after treatment (195.7 ± 15.4 ng/g dry feces) compared to before treatment (164.1 ± 6.9 ng/g; Fig. 3A). Baseline progestagens were also elevated ($P < 0.05$) in HIGH cats after treatment (3.2 ± 0.3 μ g/g dry feces) compared to before treatment (2.6 ± 0.2 μ g/g; Fig. 3B). In contrast, baseline estrogen and progestagen concentrations 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. 3A).

Females demonstrating follicular activity before treatment, but with baseline estrogens at the onset of exogenous progestin, remained at baseline (see Fig. 4A for a representative profile). Cats exhibiting follicular activity on Day 1 of progestin treatment ($n = 6$) consistently returned to baseline within 6 days and remained inhibited for the duration of treatment (Fig. 4B for a representative profile). In the six females displaying follicular activity at treatment initiation, duration of that follicular phase (7.7 ± 2.0 days), mean fecal estrogens (315.0 ± 21.9 ng/g feces) and peak fecal estrogens (395.2 ± 47.2 ng/g feces) were similar ($P > 0.05$) to estrous cycle characteristics preceding treatment. No females exhibited luteal activity on Day 1 of progestin treatment. After treatment, all cats returned to normal follicular activity in the LOW (8.2 ± 1.8 days post-ALT withdrawal), MID (12.5 ± 0.9 days) and HIGH (16.0 ± 4.0 days) groups (Fig. 5). However, a more synchronized ($P < 0.05$) return to follicular activity was observed in

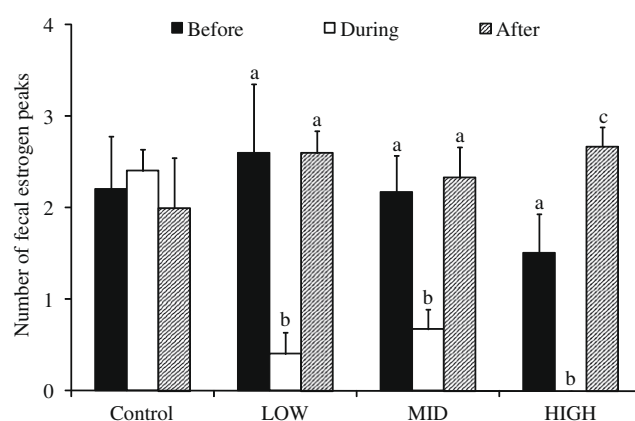


Fig. 2. Influence of oral progestin dosage on number of fecal estrogen peaks per female (mean \pm SEM) before (solid), during (open) and after (hatched) progestin treatment. Within a treatment, means with different superscripts differ ($P < 0.05$).

MID (range, 10–16 days) than in HIGH (range, 9–35 days) cats, with intermediate variation ($P > 0.05$) in the LOW treatment group (range, 2–12 days). Two of 13 (15.4%) cats ovulated spontaneously after treatment.

4. Discussion

Results demonstrated the efficacy of oral progestin for short-term, reversible inhibition of ovarian activity in the domestic cat with no observed side effects. Consistent with previous studies

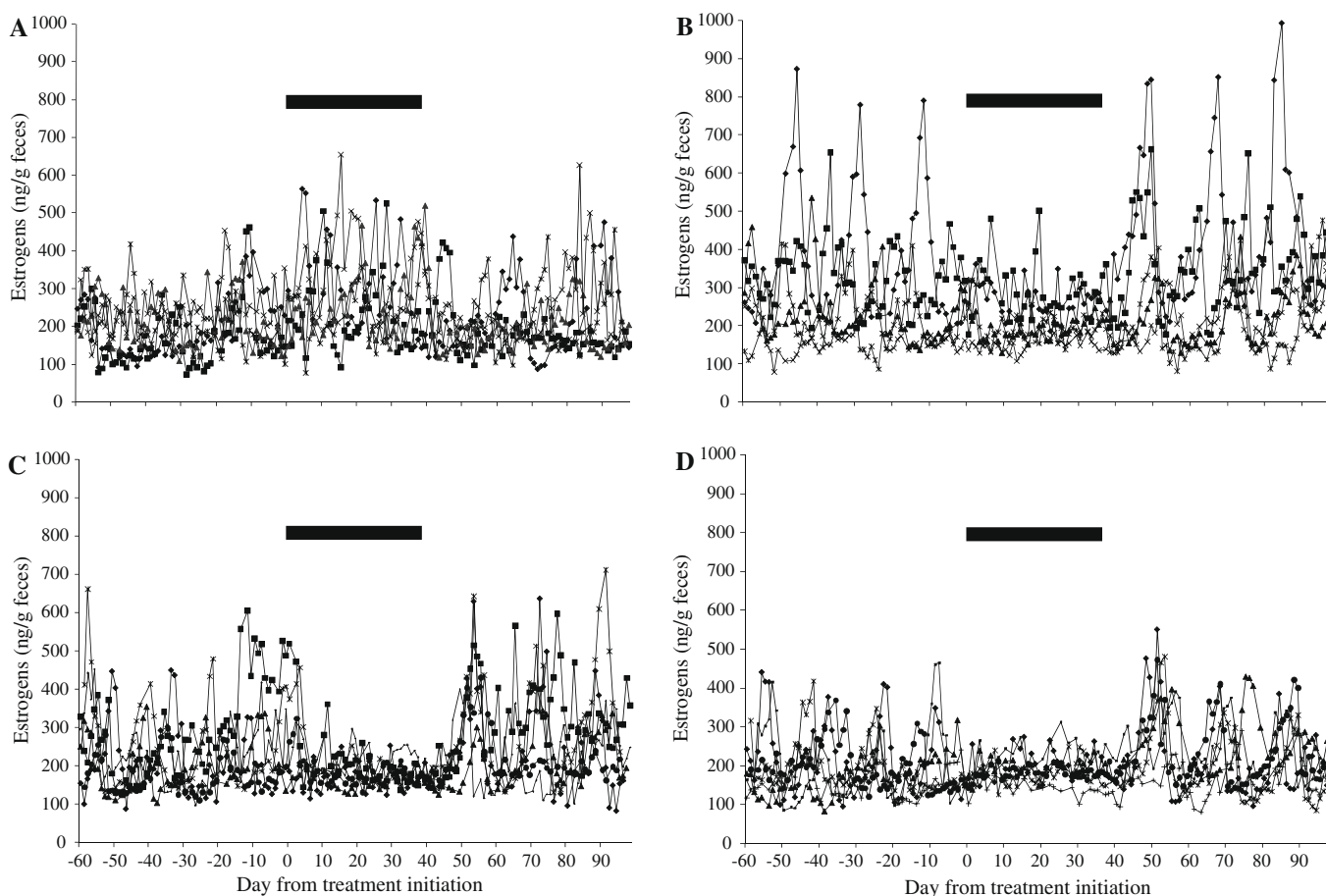


Fig. 1. Influence of oral progestin on longitudinal fecal estrogens. Queens were assigned to (A) 0 mg/kg altrenogest (control), (B) 0.044 mg/kg (LOW), (C) 0.088 mg/kg (MID) or (D) 0.352 mg/kg (HIGH). Each black bar indicates the 38-day treatment period. Individual animals within each treatment are represented by different line markers.

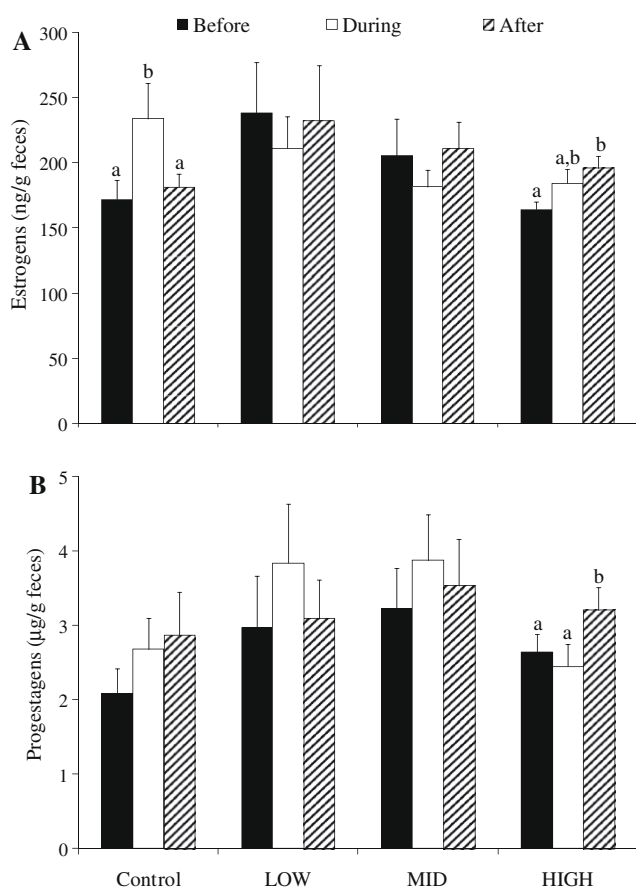


Fig. 3. Influence of oral progestin on (A) baseline fecal estrogens and (B) baseline fecal progestagens before (solid), during (open) and after (hatched) treatment (mean \pm SEM). Within a treatment, means with different superscripts differ ($P < 0.05$).

using progestin implants for ovarian suppression (Pelican et al., 2005, 2008), oral administration of this steroid did not alter follicular activity already in progress. Apparently, once a threshold in folliculogenesis was reached, even the highest dosage of oral progestin tested here was incapable of acutely perturbing final follicular advancement in the cat (although the viability of this structure and its enclosed oocyte remains to be studied). We also demonstrated that oral progestin exerted a dose-dependent effect that was quantifiable via fecal hormone metabolite monitoring. Present results affirmed the advantage of using fecal steroid hormones for detecting even subtle differences among treatments. Most importantly, our observations revealed that, although the ovary was highly sensitive to a progestin challenge, re-initiation of follicular development was resilient and occurred consistently even at the highest progestin dosage tested.

Given the substantial body of literature on progestin therapy for controlling the ovarian cycle in diverse species, it was not surprising that oral altrenogest inhibited follicular activity in our test species. However, it was clear that both progestin dosage and stage of ovarian activity at treatment onset were major regulators of treatment success. Pelican et al. (2005) characterized the effects of another progestin (levonorgestrel; administered via an implant) in the cat and also observed a rapid cessation of ovarian steroidogenesis, but again no impact on the course of an estrogen surge that was initiated before treatment onset. This has been also observed in the mare, where altrenogest is unable to reliably suppress ovulation if given during estrus (Lofstedt and Patel, 1989). Thus, the cat shows consistent ovarian sensitivity (regardless of progestin

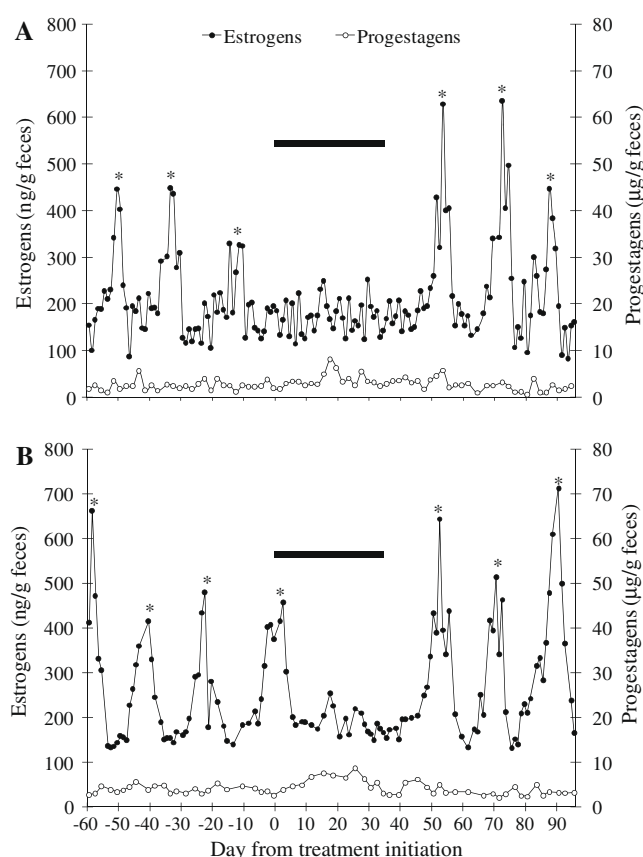


Fig. 4. Representative fecal steroid profiles before, during and after oral progestin treatment. Profiles include a female: (A) exhibiting baseline fecal estrogens at the time of progestin treatment initiation; and (B) experiencing follicular activity at the time of progestin treatment initiation. Asterisks indicate peak estrogens and the solid bar represents the 38-day altrenogest treatment interval.

source) at preventing initial recruitment of small antral follicles, but an inability to override selection and dominance of mature follicles. There is remarkably little information available about the interaction between progesterone and gonadotropins and subsequent effects on folliculogenesis in the cat. In other mammals, follicular recruitment is primarily under the influence of high FSH and low LH, whereas follicular selection is regulated by low FSH and increasing LH (Roche, 1996; Monniaux et al., 1997; McGee and Hsueh, 2000). While endogenous progesterone actions are more dominant during the luteal phase and pregnancy, progesterone receptors have been localized to the follicle, and exogenous progestins regulate granulosa cell function in multiple ways (Drummond, 2006). It is likely that, in this case, the exogenous progestin was simultaneously acting at the pituitary to influence FSH and LH release and at the ovary to alter steroidogenesis and cell remodeling in granulosa cells. Potential actions on activin, inhibin and other regulatory factors must also be considered and warrant future investigation.

Although ovarian suppression with oral progestin was predicted to be dose-dependent, follicular activity was abolished even at the lowest dosage, suggesting that the minimum effective altrenogest dosage for ovarian suppression was at or below 0.044 mg/kg. Other studies have revealed that dosage is not body mass-dependent as a lower dose is required in the horse (0.044 mg/kg) than in the dog (0.088 mg/kg; Lofstedt and Patel, 1989; Root Kustritz, 2001). Interestingly, an evaluation of individual fecal hormone profiles in the cat receiving the lowest dosage (data not shown) revealed modest elevations in estrogens approaching the delineation between baseline and elevated values.

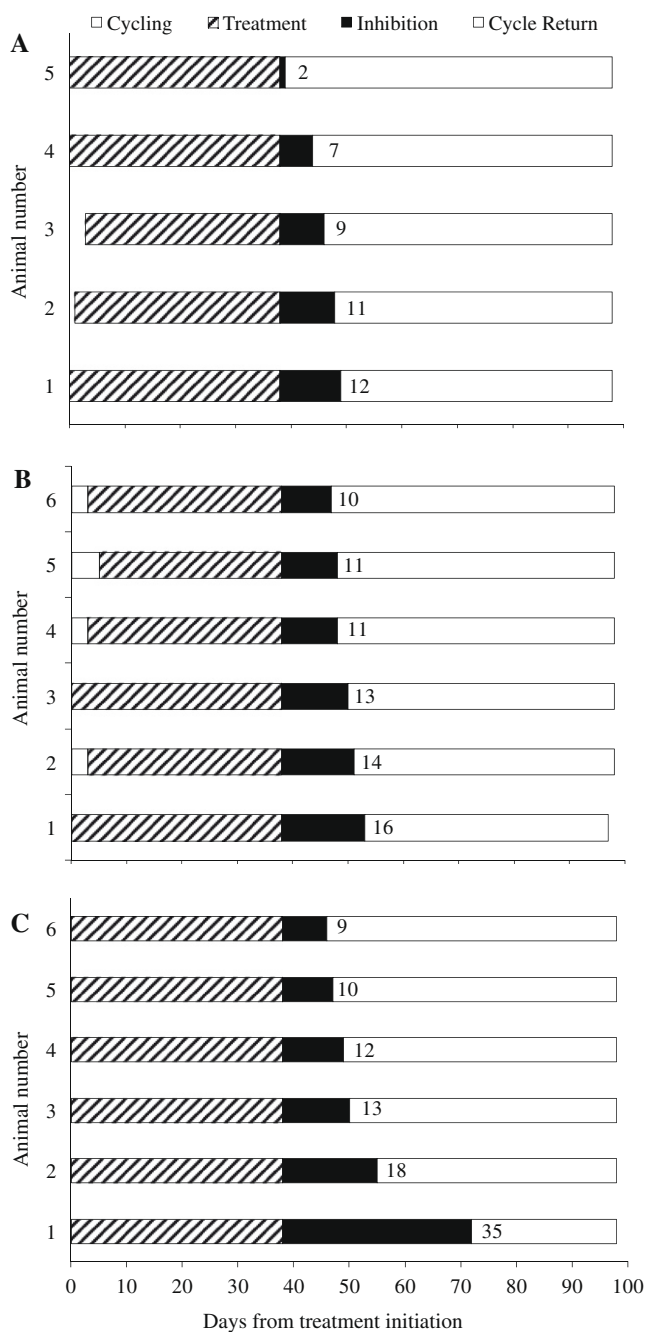


Fig. 5. Comparison of individual inhibition duration following oral progestin treatment. Cats received (A) 0.044 mg/kg altrenogest (LOW), (B) 0.088 mg/kg (MID) or (C) 0.352 mg/kg (HIGH). Bars represent days of inhibition during the treatment period (hatched), inhibition following the removal of oral progestin (solid), and follicular activity (open). Numbers within bars represent interval (days) from progestin removal to first elevation in fecal estrogens.

Therefore, the 0.044 mg/kg treatment may be close to the minimum dosage necessary to suppress follicular activity in this species. The highest dosage assessed (while effective) increased post-withdrawal baseline estrogen and progestagen concentrations. A similar phenomenon for elevated baseline estrogen has been observed in cats after treatment with the progestin implant levonorgestrel (Pelican et al., 2005). The highest dosage also led to an increase in the number of estrogen peaks following treatment. These alterations at the highest dosage could be due to changes in the responsiveness of the brain to endogenous gonadotropins that, in turn, could increase basal estradiol and

progesterone production and also stimulate autocrine regulation of steroidogenesis through negative feedback on the hypothalamic–pituitary–ovarian axis. Although the ramifications for elevated gonadal steroids are not definitively known, it is possible that persistent elevations could influence the peri-implantation period in females undergoing assisted reproduction. Thus, 0.088 mg/kg altrenogest appears to be optimal in the cat, allowing no breakthrough ovarian activity while producing normative baseline estrogen and progestagen concentrations and resumptive ovarian folliculogenesis. The same dosage is used to support pregnancy in the dog (Root Kustritz, 2001).

This study demonstrated two interesting associations between oral progestin dosage and return to follicular activity post-drug withdrawal. First, there was a positive relationship between dosage and mean duration of suppression where the interval from end of treatment to onset of the follicular phase increased with dosage. A similar relationship has been observed in pigs receiving oral altrenogest to synchronize estrus (Kraeling et al., 1981). Although pharmacokinetics of altrenogest have not been studied in the cat, this finding probably can be explained by dose-dependent differences in drug persistence in circulation. Second, we hypothesized that an oral progestin would always provide a more consistent return to ovarian cyclicity compared to previous efforts that relied on an implant formulation (Pelican et al., 2005). Instead, the present results indicated that dosage played a significant 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 counterpart (10–16 days). Synchronization with the oral progestin proved superior to levonorgestrel implants in the cat, particularly when comparing return to follicular activity in the mid-range dosage (10–16 days) to six levonorgestrel implants (11–79 days; Pelican et al., 2005).

Our findings have practical implications because of the comparatively high incidence of spontaneous ovulation in the domestic cat and certain populations of wild felids (Brown, 2006) that, in turn, contributes to ovulation induction inefficiency (Pelican et al., 2006). Interestingly, the particular group of cats studied here had an unusually low prevalence of spontaneous ovulation (15.4%) compared to laboratory-housed queens in earlier studies where 35–87% of cats ovulate without a mating stimulus (Lawler et al., 1993; Gudermuth et al., 1997; Pelican et al., 2005). An increased incidence of this phenomenon 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 did receive olfactory and auditory cues from males, but were housed individually with no visual male contact. An additional hypothesis is that spontaneous ovulation is more prevalent in older females (Lawler et al., 1993). Although not the focus of our investigation and because our study population was too small to evaluate this factor, we nonetheless observed that the two spontaneous ovulators were in the oldest (5 years) age class (data not shown).

In conclusion, short-term treatment with three dosages of the oral progestin altrenogest induced rapid and reversible inhibition of follicular activity in the cat. These findings provide the necessary foundation for ongoing studies evaluating if an ovary 'primed' with oral progestin: (1) expresses altered sensitivity to exogenous gonadotropins; and (2) produces a more normal endocrine milieu that mitigates disruption of ovum transport, fertilization and implantation. Finally, it will also be necessary to ensure that even this short-term progestin treatment does not contribute to other health risks. This is especially important in that the commonly used steroid melengestrol acetate (MGA) has been associated with an increased incidence of endometrial hyperplasia in felids (Munson et al., 2002). Although the latter were associated with long-term (>6 years) administration, it nonetheless is critical to

conduct appropriate safety assessments with altrenogest, especially as most eventual target populations of felids are rare if not endangered.

Acknowledgments

R.A.S. was supported by a Predoctoral Research Fellowship from the Smithsonian's National Zoological Park and a Matching Tuition Scholarship from the University of Maryland Graduate School. Research was supported by a National Institutes of Health SERCA grant to K.M.P. (1KO-01-RR17310-01). We are grateful to Michele Sommers, Cathi Morrison and Stacey Wise for providing excellent animal care. We also thank Ryan Berger, David Kersey, Kate MacKinnon, Bernardo Mesa and Nicole Presley for technical assistance. Dry cat food was donated by the Purina Nestlé PetCare Company.

References

- Barone, M.A., Wildt, D.E., Byers, A.P., Roelke, M.E., Glass, C.M., Howard, J.G., 1994. Gonadotrophin dose and timing of anaesthesia for laparoscopic artificial insemination in the puma (*Felis concolor*). *J. Reprod. Fertil.* 101, 103–108.
- Bauer, R.A., Ottinger, M.A., Pelican, K.M., Wildt, D.E., Howard, J.G., 2004. Challenges to developing an ovulation induction protocol in the fishing cat (*Prionailurus viverrinus*), a felid with a high incidence of spontaneous ovulation. In: Proc. 5th Int. Symp. Canine Feline Reprod., Sao Paulo, Brazil, pp. 168–169.
- Brown, J.L., Wasser, S.K., Wildt, D.E., Graham, L.H., 1994. Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured noninvasively in feces. *Biol. Reprod.* 51, 776–786.
- Brown, J.L., Wildt, D.E., Wielebnowski, N., Goodrowe, K.L., Graham, L.H., Wells, S., Howard, J.G., 1996. Reproductive activity in captive female cheetahs (*Acinonyx jubatus*) assessed by faecal steroids. *J. Reprod. Fertil.* 106, 337–346.
- Brown, J.L., Graham, L.H., Wu, J.M., Collins, D., Swanson, W.F., Wildt, D.E., Howard, J.G., 2001. Understanding the basic reproductive biology of wild felids by monitoring of faecal steroids. *J. Reprod. Fertil. Suppl.* 57, 71–82.
- Brown, J.L., Graham, L.H., Wu, J.M., Collins, D., Swanson, W.F., 2002. Reproductive endocrine responses to photoperiod and exogenous gonadotropins in the Pallas' cat (*Otocolobus manul*). *Zoo Biol.* 21, 347–364.
- Brown, J.L., 2006. Comparative endocrinology of domestic and nondomestic felids. *Theriogenology* 66, 25–36.
- Concannon, P., 1991. Reproduction in the dog and cat. In: Cupps, P.T. (Ed.), *Reproduction in Domestic Animals*. Academic Press, Inc., New York, pp. 517–554.
- Deligiannis, C., Valasi, I., Rekkas, C.A., Goulas, P., Theodosiadou, E., Lainas, T., Amiridis, G.S., 2005. Synchronization of ovulation and fixed time intrauterine insemination in ewes. *Reprod. Domest. Anim.* 40, 6–10.
- Donoghue, A., Johnston, L.A., Armstrong, D.L., Simmons, L.G., Wildt, D.E., 1993. Birth of a Siberian tiger cub (*Panthera tigris altaica*) following laparoscopic intrauterine insemination. *J. Zoo Wildl. Med.* 24, 185–189.
- Drummond, A.E., 2006. The role of steroids in follicular growth. *Reprod. Biol. Endocrinol.* 4, 16.
- Graham, L.H., Swanson, W.F., Brown, J.L., 2000. Chorionic gonadotropin administration in domestic cats causes an abnormal endocrine environment that disrupts oviductal embryo transport. *Theriogenology* 54, 1117–1131.
- Graham, L.H., Schwartzenberger, F., Mostl, E., Galama, W., Savage, A., 2001. A versatile enzyme immunoassay for the determination of progestogens in the feces and serum. *Zoo Biol.* 20, 227–236.
- Graham, L.H., Byers, A.P., Armstrong, D.L., Loskutoff, N.M., Swanson, W.F., Wildt, D.E., Brown, J.L., 2006. Natural and gonadotropin-induced ovarian activity in tigers (*Panthera tigris*) assessed by fecal steroid analyses. *Gen. Comp. Endocrinol.* 147, 362–370.
- Gudermuth, D.F., Newton, L., Daels, P., Concannon, P., 1997. Incidence of spontaneous ovulation in young, group-housed cats based on serum and faecal concentrations of progesterone. *J. Reprod. Fertil. Suppl.* 51, 177–184.
- Howard, J.G., Donoghue, A.M., Barone, M.A., Goodrowe, K.L., Blumer, E.S., Snodgrass, K., Starnes, D., Tucker, M., Bush, M., Wildt, D.E., 1992. Successful induction of ovarian activity and laparoscopic intrauterine artificial insemination in the cheetah (*Acinonyx jubatus*). *J. Zoo Wildl. Med.* 23, 288–300.
- Howard, J.G., Byers, A.P., Brown, J.L., Schwartz, R.J., Evans, M.Z., Barrett, S.J., Wildt, D.E., 1996. Successful ovulation induction and laparoscopic intrauterine artificial insemination in the clouded leopard (*Neofelis nebulosa*). *Zoo Biol.* 15, 55–69.
- Howard, J.G., Roth, T.L., Byers, A.P., Swanson, W.F., Wildt, D.E., 1997. Sensitivity to exogenous gonadotropins for ovulation induction and laparoscopic artificial insemination in the cheetah and clouded leopard. *Biol. Reprod.* 56, 1059–1068.
- Howard, J.G., 1999. Assisted reproductive techniques in nondomestic carnivores. In: Fowler, M.E., Miller, R.E. (Eds.), *Zoo and Wild Animal Medicine: Current Therapy IV*. W.B. Saunders Co., Philadelphia, pp. 449–457.
- Howard, J.G., Wildt, D.E., 2009. Approaches and efficacy of artificial insemination in felids and mustelids. *Theriogenology* 71, 130–148.
- Kraeling, R.R., Dziuk, P.J., Pursel, V.G., Rampacek, G.B., Webel, S.K., 1981. Synchronization of estrus in swine with allyl trenbolone (RU-2267). *J. Anim. Sci.* 52, 831–835.
- Lawler, D.F., Johnston, S.D., Hegstad, R.L., Keltner, D.G., Owens, S.F., 1993. Ovulation without cervical stimulation in domestic cats. *J. Reprod. Fertil. Suppl.* 47, 57–61.
- Lofstedt, R.M., 1988. Control of the estrous cycle in the mare. *Vet. Clin. North Am. Equine Pract.* 4, 177–196.
- Lofstedt, R.M., Patel, J.H., 1989. Evaluation of the ability of altrenogest to control the equine estrous cycle. *J. Am. Vet. Med. Assoc.* 194, 361–364.
- McGee, E.A., Hsueh, A.J., 2000. Initial and cyclic recruitment of ovarian follicles. *Endocr. Rev.* 21, 200–214.
- Monfort, S.L., Asher, G.W., Wildt, D.E., Wood, T.C., Schiewe, M.C., Williamson, L.R., Bush, M., Rall, W.F., 1993. Successful intrauterine insemination of Eld's deer (*Cervus eldi thamin*) with frozen–thawed spermatozoa. *J. Reprod. Fertil.* 99, 459–465.
- Monniaux, D., Huet, C., Besnard, N., Clement, F., Bosc, M., Pisselet, C., Monget, P., Mariana, J.C., 1997. Follicular growth and ovarian dynamics in mammals. *J. Reprod. Fertil. Suppl.* 51, 3–23.
- Moreira, N., Monteiro-Filho, E.L., Moraes, W., Swanson, W.F., Graham, L.H., Pasquali, O.L., Gomes, M.L., Morais, R.N., Wildt, D.E., Brown, J.L., 2001. Reproductive steroid hormones and ovarian activity in felids of the *Leopardus* genus. *Zoo Biol.* 20, 103–116.
- Morrow, C.J., Wolfe, B.A., Roth, T.L., Wildt, D.E., Bush, M., Blumer, E.S., Atkinson, M.W., Monfort, S.L., 2000. Comparing ovulation synchronization protocols for artificial insemination in the scimitar-horned oryx (*Oryx dammah*). *Anim. Reprod. Sci.* 59, 71–86.
- Munson, L., Gardner, A., Mason, R.J., Chassy, L.M., Seal, U.S., 2002. Endometrial hyperplasia and mineralization in zoo felids treated with melengestrol acetate contraceptives. *Vet. Pathol.* 39, 419–427.
- Munson, L., 2006. Contraception in felids. *Theriogenology* 66 (1), 126–134.
- Pelican, K.M., Brown, J.L., Wildt, D.E., Ottinger, M.A., Howard, J.G., 2005. Short term suppression of follicular recruitment and spontaneous ovulation in the cat using levonorgestrel versus a GnRH antagonist. *Gen. Comp. Endocrinol.* 144, 110–121.
- Pelican, K.M., Wildt, D.E., Pukazhenthil, B., Howard, J.G., 2006. Ovarian control for assisted reproduction in the domestic cat and wild felids. *Theriogenology* 66 (1), 37–48.
- Pelican, K.M., Wildt, D.E., Ottinger, M.A., Howard, J.G., 2008. Priming with progestin, but not GnRH antagonist, induces a consistent endocrine response to exogenous gonadotropins in induced and spontaneously ovulating cats. *Domest. Anim. Endocrinol.* 34, 60–75.
- Robeck, T.R., Steinman, K.J., Gearhart, S., Reidarson, T.R., McBain, J.F., Monfort, S.L., 2004. Reproductive physiology and development of artificial insemination technology in killer whales (*Orcinus orca*). *Biol. Reprod.* 71, 650–660.
- Robeck, T.R., Steinman, K.J., Yoshioka, M., Jensen, E., O'Brien, J.K., Katsumata, E., Gili, C., McBain, J.F., Sweeney, J., Monfort, S.L., 2005. Estrous cycle characterisation and artificial insemination using frozen–thawed spermatozoa in the bottlenose dolphin (*Tursiops truncatus*). *Reproduction* 129, 659–674.
- Roche, J.F., 1996. Control and regulation of folliculogenesis – a symposium in perspective. *Rev. Reprod.* 1, 19–27.
- Romatowski, J., 1989. Use of megestrol acetate in cats. *J. Am. Vet. Med. Assoc.* 194 (5), 700–702.
- Root Kustritz, M.V., 2001. Use of supplemental progesterone in management of canine pregnancy. In: Concannon, P., England, G., Versteegen, J.P., Linde-Forsberg, C. (Eds.), *Recent Advances in Small Animal Reproduction*. International Veterinary Information Service, Ithaca (www.ivia.org, document # A1220.0401).
- Roth, T.L., Armstrong, D.L., Barrie, M.T., Wildt, D.E., 1997a. Seasonal effects on ovarian responsiveness to exogenous gonadotropins and successful artificial insemination in the snow leopard (*Uncia uncia*). *Reprod. Fertil. Dev.* 9, 285–295.
- Roth, T.L., Wolfe, B.A., Long, J.A., Howard, J.G., Wildt, D.E., 1997b. Effects of equine chorionic gonadotropin, human chorionic gonadotropin, and laparoscopic artificial insemination on embryo, endocrine, and luteal characteristics in the domestic cat. *Biol. Reprod.* 57, 165–171.
- Schwartzenberger, F., Mostl, E., Bamberg, E., Pammer, J., Schmehlik, O., 1991. Concentrations of progestagens and oestrogens in the faeces of pregnant Lipizzan, trotter and thoroughbred mares. *J. Reprod. Fertil. Suppl.* 44, 489–499.
- Sokal, R.R., Rohlf, F.J., 1994. *Biometry: The Principles and Practices of Statistics in Biological Research*, third ed. W.H. Freeman and Company, New York.
- Stabenfeldt, G.H., Daels, P.F., Munro, C.J., Kindahl, H., Hughes, J.P., Lasley, B., 1991. An oestrogen conjugate enzyme immunoassay for monitoring pregnancy in the mare: limitations of the assay between days 40 and 70 of gestation. *J. Reprod. Fertil. Suppl.* 44, 37–44.
- Swanson, W.F., Graham, K., Horohov, D.W., Thompson, D.L., Godke, R.A., 1996a. Ancillary follicle and secondary corpora lutea formation following exogenous gonadotropin treatment in the domestic cat and effect of passive transfer of gonadotropin-neutralizing antisera. *Theriogenology* 45, 561–572.
- Swanson, W.F., Howard, J.G., Roth, T.L., Brown, J.L., Alvarado, T., Burton, M., Starnes, D., Wildt, D.E., 1996b. Responsiveness of ovaries to exogenous gonadotropins and laparoscopic artificial insemination with frozen–thawed spermatozoa in ocelots (*Felis pardalis*). *J. Reprod. Fertil.* 106, 87–94.
- Swanson, W.F., Wolfe, B.A., Brown, J.L., Martin-Jimenez, T., Riviere, J.E., Roth, T.L., Wildt, D.E., 1997. Pharmacokinetics and ovarian-stimulatory effects of equine and human chorionic gonadotropins administered singly and in combination in the domestic cat. *Biol. Reprod.* 57, 295–302.

- Swanson, W.F., 2003. Research in nondomestic species: experiences in reproductive physiology research for conservation of endangered felids. *ILAR J.* 44, 307–316.
- Swanson, W.F., Brown, J.L., 2004. International training programs in reproductive sciences for conservation of Latin American felids. *Anim. Reprod. Sci.* 82–83, 21–34.
- Wildt, D.E., Seager, S.W., Chakraborty, P.K., 1980. Effect of copulatory stimuli on incidence of ovulation and on serum luteinizing hormone in the cat. *Endocrinology* 107, 1212–1217.
- Wildt, D.E., Monfort, S., Donoghue, A.M., Johnston, L.A., Howard, J.G., 1992. Embryogenesis in conservation biology – or, how to make an endangered species embryo. *Theriogenology* 37, 161–184.
- Wildt, D.E., Roth, T.L., 1997. Assisted reproduction for managing and conserving threatened felids. *Int. Zoo Yearb.* 35, 164–172.
- Wildt, D.E., Swanson, W.F., Brown, J.L., Sliwa, A., Vargas, A., 2010. Felids ex situ: managed programs, research and species recovery. In: Macdonald, D., Loveridge, A. (Eds.), *Biology and Conservation of Wild Felids*. Oxford University Press, Oxford.
- Wood, C.M., Kornegay, E.T., Shipley, C.F., 1992. Efficacy of altrenogest in synchronizing estrus in two swine breeding programs and effects on subsequent reproductive performance of sows. *J. Anim. Sci.* 70, 1357–1364.