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Reproductive endocrinology of the largest dasyurids: Characterization of ovarian cycles by plasma and fecal steroid monitoring. Part I. The Tasmanian devil (*Sarcophilus harrisii*)

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Abstract

There is a strong body of knowledge on the reproductive endocrinology of macropods, but little detailed information is available on the hormonal control of reproduction in other marsupials. This study used plasma and fecal sex steroid monitoring to characterize the estrous cycle of the largest extant dasyurid—the Tasmanian devil (*Sarcophilus harrisii*). A pro-estrous pulse in plasma progesterone (1.33 ± 0.2 ng/ml) occurred several weeks prior to onset of the luteal phase (LP), resulting in a characteristic biphasic pattern during the estrous cycle. This brief, pro-estrous progesterone pulse was associated with a predominantly cornified vaginal smear, and copulation in females paired with males. Mean luteal progesterone concentrations (5.28 ± 0.8 ng/ml) were sustained and peaked around day 15 from luteal onset; thereafter, concentrations declined precipitously and returned to baseline around day 25. Females that did not produce young returned to estrus after 33.7 ± 5.9 days. Fecal 20α-OH-pregnanes analyzed in a pregnanediol assay (PgD) were excreted in consistently higher levels than 20-oxo-pregnanes, but the pattern was similar for the two metabolites, and significantly correlated with fluctuations in plasma progesterone. Fecal total estrogen concentrations were highest during the follicular phase (FP) and accompanied a pro-estrous pulse in fecal progestagens. The mean duration of the estrous cycle was ~32 days, with a FP of around 14 days (range 8–23 days), and a luteal phase of around 18 days (range 12–25 days). There were no differences in the length of the LP between mated and non-mated cycles. Gestation length was 17.9 ± 1.0 days (range 14–22 days). Fecal steroid monitoring revealed significant differences between the pattern of progestagens and estrogen concentrations during the pregnant and non-mated estrous cycle, suggesting maternal endocrine recognition of pregnancy in the Tasmanian devil.

Keywords: Marsupial; Dasyurid; Reproduction; Progesterone; Progestagens; Estrogens

1. Introduction

The importance of developing knowledge of the reproductive endocrinology of marsupials as a basis for comparative research is well recognised (Tyndale-Biscoe, 1984). There are two primary patterns of reproduction in marsupials, the basic pattern common to most species (*e.g.* dasyurids, possums) and the macropod pattern found in most wallabies and kangaroos (Tyndale-Biscoe and Renfree, 1987). In dasyurids the luteal phase occupies around 60% of the estrous cycle and following parturition, subsequent follicular activity is suppressed by lactation. This contrasts with the macropod pattern, where the luteal phase occupies ~90% of the estrous cycle and follicular growth is not suppressed, permitting post-partum estrus and ovulation to occur. Macropods also differ in that lactation prevents activity of the newly formed corpus luteum, resulting in a period of embryonic diapause. This discovery stimulated the onset of endocrine studies of macropods in the 1960s (Tyndale-Biscoe and Renfree, 1987)—research that has contributed strongly to our considerable

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understanding of their reproductive biology today. Unfortunately, limited attention has been directed toward detailed studies of the hormonal control of reproduction in many other marsupials, despite their diversity of reproductive strategies.

For the majority of marsupials, estrus and ovulation occur spontaneously, with the main hormones estradiol and progesterone acting on the female reproductive system in a manner analogous to that described for eutherian mammals (Tyndale-Biscoe and Renfree, 1987; Hinds et al., 1996). Information is available on the reproductive physiology and breeding patterns for a range of non-macropod species, including the carnivorous dasyurids (Tyndale-Biscoe and Renfree, 1987; Tyndale-Biscoe, 2005), but fundamental knowledge of reproductive and endocrine parameters such as the temporal pattern of hormones and characteristics of the ovarian cycle is lacking. Dasyurids are characterised by a range of different reproductive strategies from semelparity to seasonal polyestry (Lee et al., 1982; Krajewski et al., 2000) but endocrine data is only available for several species (Fletcher, 1985; Hinds, 1989; Hinds and Selwood, 1990; Millis et al., 1999). Our understanding of reproduction in the two largest dasyurids—the Tasmanian devil (*Sarcophilus harrisii*) and spotted-tailed quoll (*Dasyurus maculatus*)—remains relatively rudimentary, and even the most basic information such as timing of breeding, estrous pattern and gestation length (reviews by Lee et al., 1982; McAllan, 2003) is either assumed or inferred.

The Tasmanian devil is the world's largest extant dasyurid, reaching a bodyweight of up to 12 kg (Strahan, 2005). Although the devil was formerly found on mainland Australia ~500 years ago (Jones et al., 2003), wild populations are now confined to the island of Tasmania where, until recently, they were considered a common species of secure status. The outbreak of an apparently contagious and fatal facial tumor disease (Pearce and Swift, 2006) is currently having a devastating impact on wild devil populations, and the species is now listed as Threatened (Hawkins et al., 2006). Despite devils being well represented in zoos for many decades, captive breeding remains inconsistent and limited, highlighting the need for a greater understanding of reproduction in this species (Carnio, 1993; Jackson, 2003). Detailed information of the devil's reproductive biology is now essential to implementing effective conservation programs, and is central to developing a fuller understanding of their natural life history and ecology.

Information is available on the basic reproductive biology (Guiler, 1970, 1971; Hughes, 1982), anatomy (Flynn, 1910, 1911; Pearson and De Bavay, 1953), oogenesis, spermatogenesis and embryology of the Tasmanian devil (O'Donoghue, 1912; Flynn, 1939; Hughes, 1982). There has, however, been no published research on their reproductive endocrinology, leaving important gaps in our fundamental understanding of this species. Most studies of the reproductive hormones during the estrous cycle of marsupials have relied on measuring concentrations of progesterone and/or estradiol in plasma; and have been conducted for a variety of species including macropods (Cake et al., 1980; Walker and Gemmell, 1984; Hinds and Smith, 1992; Jones and Rose, 1992; Rose et al., 1999), possums and opossums (Shorey and Hughes, 1973; Harder and Fleming, 1981; Curlewis et al., 1985; Perret and Atramentowicz, 1989; Hinds et al., 1992; Hinds and Smith, 1992), the koala (Johnston et al., 2000) and several dasyurids (*Dasyuroides byrnei* Fletcher, 1985; *Dasyurus viverrinus* Hinds, 1989; *Antechinus stuartii* Hinds, 1990; *Phascogale tapoatafa* Millis et al., 1999). More recently, fecal steroid monitoring has begun to be applied to monitor female reproductive cycles in marsupials (Stead-Richardson et al., 2001; Paris et al., 2002; Bradshaw et al., 2004; Woodd et al., 2006). Fecal steroid monitoring of reproductive and stress hormones confers obvious advantages over the restraint and handling associated with traditional blood collection, and this non-invasive method is particularly useful for more frequent monitoring, allowing for more thorough longitudinal assessment of endocrine cycles (reviews in: Lasley and Kirkpatrick, 1991; Schwarzenberger et al., 1996).

Knowledge of reproductive biology has been highlighted as being an essential component of conservation programs for Australian marsupials and is identified as having the greatest research priority (Temple-Smith, 2003). The main aim of this study was to provide the first detailed hormonal analysis of the ovarian cycle in a large dasyurid. A secondary aim was to evaluate the application of fecal steroid monitoring (total estrogens and progestagens) for the species as a basis for future applications of such techniques to *in situ* conservation and captive breeding. In a companion paper (Hesterman et al., 2008), we describe the application of these techniques for monitoring ovarian cycles in the closely related spotted-tailed quoll.

2. Materials and methods

2.1. Study animals and husbandry

Samples were collected from 14 captive female Tasmanian devils (0.5–6 years old) housed at Trowunna Wildlife Park (TWP Mole Creek, TAS) during 2001. Devils were provided with a natural diet consisting of kangaroo or wallaby meat and occasionally possum, rabbit, wombat or other native mammals/birds sourced from professional hunters or obtained as roadkill. Meat was provided complete with fur, bones and associated offal (except intestines), and animals were occasionally fed entire carcasses. Additional enrichment food items provided less often included commercially available brands of dog and cat biscuits, and whole raw carrots or apples. Water was available *ad libitum*.

Devils were housed outdoors, in naturalistic enclosures with access to climbing structures, native plants and other natural materials. Dens or nest boxes were available for shelter, with a choice of retreats that met or exceeded the number of animals per enclosure. Grouping of the study animals varied during the year, with devils generally kept in mixed sex or same sex groups and separated for breeding requirements or experimental purposes. Introductions and pairing with males were permitted for selected females during the breeding season, in accordance with experimental design (see below).

2.2. Experimental design

To assess and compare the estrous and pregnant cycles, 11 of the 12 adult females (⩾2 years) were assigned to different groups:

Group A: Females permitted full access to males during estrus (mated; $n = 6$); and
Group B: Females with restricted access to males during estrus (non-mated; $n = 5$).

The first group of animals were housed together in a female-only group for at least 1 month prior to the onset of breeding, and removed for pairing with males at the onset of behavioral and physical cues used by park management to detect estrus. Females removed for mating were placed into a specifically designed enclosure, with free access to a choice of two adult males housed in adjacent wire pens. Copulation was confirmed by behavioral observation (observer presence/video monitoring within dens), or detection of sperm in the vaginal smear. Following mating and/or lack of interest or increased aggression toward males, females were removed to individual enclosures.

The second group of animals was also housed together in a female-only group prior to the onset of breeding season. Between February and March they were periodically exposed to an adult male: three times each week, a male was placed into a cage ($1.5 \times 1.0 \times 0.9$ m) within the females' enclosure for 30–60 min while behavior was recorded for a concurrent study. To compare mated and non-mated estrous cycles in the same individuals, Group B females were housed with unrestricted access to a male throughout April–May.

2.3. Sample and data collection

2.3.1. Plasma collection

Blood samples were collected during routine handling of animals to obtain supplementary data on reproductive status (see below). Devils were captured by hand or use of a large net. They were restrained unanaesthetised in a sack during sample collection and examination. Blood was collected within five minutes of capture.

A peripheral ear vein was pricked with a disposable Stat-Let© lancet and 75–150 μl blood was collected via a heparinised capillary tube. Samples were usually taken between 0730 and 0930 h or 1500 and 1700 h except when individuals were being captured for other husbandry purposes, when blood was collected opportunistically. Samples were kept at 4 °C until centrifuged later that day, and the plasma was recovered and stored frozen (−20 °C) until radioimmunoassay.

Blood was collected from adult devils at intervals of ~5 days during late January–May, with frequency increasing to every 2–3 days from the onset of estrus. For females that were mated, sampling was discontinued 2 weeks post-copulation to minimize any potential stress that might impact on successful rearing of young. Sampling resumed if subsequent pouch checks confirmed no young were present. Subadult devils (1 year olds; $n = 2$) were sampled at ~10-day intervals from late February–May.

2.3.2. Fecal collection

Fecal samples were collected between November 2000 and December 2001. To ensure the identity of individual samples when animals were housed together, small colored plastic beads (3 mm diameter) were mixed into a mincemeat ball and fed to the study animals the day before collection. Color coding was consistent for each animal and up to six different individuals could be identified within a single enclosure. Frequency of collection varied depending on the time of year and breeding status of individual animals. Immediately prior to and during the mating season (February–May), samples were obtained up to three times per week, whereas during the rest of the year samples were collected on a weekly or fortnightly basis.

Entire feces were usually collected during morning servicing (0730–0900 h) or opportunistically when freshly voided throughout the day. When several scats were available from the same individual, the apparently freshest sample was selected. Samples were placed in zip-lock plastic bags and stored at −20 °C for later processing. Frozen samples were freeze-dried (Dynavac FD16, Dynavac High Vacuum Pty. Ltd., Victoria, Australia) and screened through 1 mm^2 plastic mesh to remove fur, bones and other fibrous or undigested matter; screened feces were stored refrozen in ziplock bags. These samples were shipped frozen to Vienna, Austria and kept at −20 °C until analysis.

2.3.3. Additional data and sampling

Vaginal (urogenital) smears were collected from all females paired with males, and also from three non-mated individuals. Smears were obtained from the posterior vaginal sinus by introduction of a small cotton swab through an appropriately sized glass speculum (70 mm length × 5 mm ø). Smears were air-dried, fixed and then stained with acid fuchsin and toluidine blue following methods outlined in Dix and Billings (1969). Stained smears were examined for percentage of intermediate (IE) and superficial/cornified epithelials (SE), leucocytes and presence of spermatozoa. Pouches were monitored for condition/presence of young.

2.3.4. Plasma progesterone analyses

Plasma progesterone was measured by radioimmunoassay (RIA). Forty microliters duplicates of plasma were extracted in 1 ml *iso*-octane (AnalaR grade APS Ajax Finechem, NSW, Australia) by brief vortexing followed by incubation at room temperature for 2 h. Samples were frozen at −20 °C for 30 min to allow phase separation. The solvent was evaporated, the extract redissolved in assay buffer and used for radioimmunoassay. Recovery varied between samples, so extraction efficiency was assessed for individual samples. Average recovery of radioactive progesterone was 85% ±0.47 SE.

For the assay 50 μl [^{3}H]-progesterone ([1,2,6,7] (TRK413 Amersham Pharmacia Biotech, UK)) containing ~12,000 cpm was added to tubes containing sample extracts and progesterone standards (range 3.12–200 pg/50 μl; Sigma–Aldrich Pty, Ltd, Missouri, U.S.A.) and evaporated under air. One hundred microliters of antiserum (P11–192, Endocrine Sciences, California, USA) diluted 1:125,000 in phosphate buffer (PBSG 0.05 M, pH 7.4; 0.1% gelatin) was added to dried extracts and standards. Cross-reactivities of this antiserum with other steroids are: 4-pregnen-20β-ol-3-one (1.3%), 4-pregnen-20α-ol-3-one (0.8%), 17α-hydroxyprogesterone (0.6%), deoxycorticosterone (3.3%), corticosterone (0.6%), 11-desoxycortisol (0.4%) and all others (<0.1%). The tubes were vortexed briefly and incubated at 4 °C overnight. Unbound steroid was separated by addition of 500 μl dextran-coated charcoal (0.5 g/L charcoal (Sigma–Aldrich Pty, Ltd, Missouri, U.S.A.), 0.05 g/L dextran (Dextran T70 Amersham Pharmacia, Buckinghamshire, England) in phosphate buffer and incubation on ice for 15 min, then centrifuging for 15 min (1500*g* at 4 °C). 300 μl of supernatant was counted in 2.5 ml scintillation fluid (Ecolite, MP Biomedicals, Inc. California, USA) for 5 min in a Beckman Coulter Counter LS 5801.

Assay sensitivity was 3 pg/tube (0.09 ng/ml). Serial dilutions of devil plasma ran parallel to the progesterone standard curve. Recoveries of added steroid were determined by spiking pooled plasma for each species with progesterone (0.5, 1.0, 2.0, 4.0 ng/ml) which yielded a mean recovery within 10% of expected values. Multiple aliquots from a pool of female devil plasma were extracted to measure intra-assay variability, whereas commercially available controls (Diagnostic Products Corporation, California, USA) were used to monitor inter-assay variation. Intra- and inter-assay coefficients of variation were 9.5% ($n = 9$) and 14.8% ($n = 9$), respectively. All samples from each individual were included in a single assay.

2.3.5. Fecal sample processing and enzyme-immunoassay of steroids

Lyophilized fecal samples were mixed with distilled water (0.1 g feces in 0.9 ml water), and then extracted in methanol and diethyl ether, as described in Schwarzenberger et al. (2000). After adding 4.5 ml of methanol, the fecal–water–methanol mixture was vortexed (30 min) and centrifuged. Thereafter, 1.0 ml of the methanol supernatant was recovered into a separate vial and 0.5 ml of a 5% $NaHCO_3$ in water solution were added, and then extracted with 3.0 ml of diethyl ether. The ether phase was collected, evaporated to dryness and the extract residue redissolved in assay buffer. After appropriate dilution (1:100 to 1:1000 depending on steroid concentration) immuno-reactive progesterone and estrogen metabolites were assayed using previously

established group-specific enzyme-immunoassays (EIA) (Schwarzenberger et al., 1997). Samples were analyzed for 20α-OH-pregnanes (antibody: 5β-pregnane-3α-20α-diol 3HS:BSA; trivial name pregnanediol), 20-oxo-pregnanes (antibody: 5α-pregnane-3β-ol-20-one 3HS:BSA), and total estrogens (antibody: oestradiol-17β-OH 17-HS:BSA). Preliminary testing showed that assay of 20α-OH-pregnanes (pregnanediol PgD) was most appropriate, with concentrations being excreted in consistently higher levels than 20-oxo-pregnanes throughout all stages of the estrous cycle. EIAs were validated by demonstrating parallelism between standard curves and serial dilutions of the fecal extracts and by showing that fecal values followed the same trend as the values obtained with the plasma progesterone assay. The intra- and inter-assay coefficients of variation for the assays tested were below 10% and 15%, respectively.

2.3.6. Terminology

Various terminology has been used to describe non-conceptive cycles in marsupials—whether not mated, mated or hormonally/mechanically stimulated—as either non-pregnant, pseudopregnant or failed pregnant. To avoid ambiguity, the term non-mated is used here to distinguish between non-pregnant and pregnant cycles. Apart from a single individual for which neither mating nor birth could be confirmed, all females paired with males gave birth: information on that individual was excluded from comparative analyses of pregnant and non-mated cycles.

2.4. Interpretation of hormone data

Stages of the estrous cycle were defined as the follicular phase (FP), luteal phase (LP), anestrous and inter-estrus (period between beginning of FP to onset of next FP). The FP has a secondary stage where ovulation is presumed to occur. This distinct stage reliably follows the hormone surge during the FP, and is distinguished by a trough/return to baseline values for fecal estrogen (and also plasma progesterone concentrations) prior to onset of the LP. Where collection frequency was $\geqslant 7$ days, the durations of successive stages of the cycle were calculated by counting the days elapsed between the two samples, halving the result and adding it to the phase either side.

Onset of the estrous cycle could be determined without monitoring plasma estrogens because plasma progesterone concentrations characteristically rose at pro-estrous. Baseline values for plasma progesterone were generated by averaging values obtained from mature females during anestrous. Increases above the group baseline + one standard deviation (SD) (*i.e.* >0.48 ng/ml) that were maintained for at least 5 days were considered indicative of onset of the FP. Confirmation of the LP was readily identified when plasma progesterone concentrations increased two SDs above baseline levels (*i.e.* >0.64 ng/ml).

Group baseline values were calculated by averaging fecal steroid concentrations in adults during the non-breeding season ($n = 10$ animals, 72 samples). The onset of the estrous cycle was defined when fecal estrogen concentrations rose above the group baseline + one SD (*i.e.* > 25.34 ng/g), and remained elevated for at least 1 week. Onset of the LP was marked by rising and sustained concentrations of pregnanediol which exceeded the group mean (*i.e.* >1036 ng/g). The end of the LP was identified as the time when fecal pregnanes dropped below the mean with subsequent samples remaining low for at least 2 weeks; or, for pregnant females was terminated by birth, indicated by presence of young in pouch (PY). Where date of birth was uncertain (in two cases only), the age of the PY was estimated from measurements and backdating from established growth curves for the species (Phillips and Jackson, 2003; Hesterman, unpublished data).

2.5. Comparison between plasma and feces

To allow comparison between the pattern of excretion between plasma progesterone and fecal progestagens, temporal alignment of samples was necessary. Fecal steroids mimic the pattern of circulating hormone levels in plasma, but incur a lag time due to their passage through the gut (Schwarzenberger et al., 1996), roughly equivalent to passage of digesta (Lasley and Kirkpatrick, 1991; Schwarzenberger et al., 1996). Accordingly, fecal samples were displaced from the plasma results by 24 h, based on the time of appearance of indicators (small, colored plastic beads) fed to individually identify scats.

2.6. Statistical analyses

All data are presented as means ± SE, except where indicated otherwise. Student's unpaired t test was used for comparison of estrous characteristics, and duration between pregnant cycles and mated or non-mated non-parturient cycles. Analyses of variance (ANOVA) were used to detect temporal changes in hormone profiles, and to test for significant differences in hormone concentrations between experimental groups. For comparison of the profiles for plasma progesterone and its fecal metabolites, regression was performed on data that were log-transformed to meet the assumptions of ANOVA. Statistical analyses were performed using SPSS (SPSS Inc. 1998, Chicago IL), Version 13 package.

3. Results

Twelve of the 13 female devils monitored during the study period underwent estrous cycles, and seven pregnancies were recorded. None of the devils that mated and produced young returned to estrus. Five of the eight individuals that were not mated at first estrus underwent a second cycle in the breeding season, which lasted approximately six months from the end of January until late June.

3.1. Estrous cycle characteristics

Characteristics of the estrus cycle are summarized in Table 1. The mean duration of the estrous cycle was approximately 32 days whether assessed by plasma or fecal hormones. There was no significant difference between the length of the FP (~14 days) in non-mated and mated animals ($t_{(11)} = 1.09$, $P = 0.30$). Analyses of plasma progesterone data indicated no difference in the duration of the non-mated FP whether females were physically exposed to males (11.2 ± 1.9 days; $n = 5$) or not (11.0 ± 1.0 days; $n = 2$) at estrus. Duration of the FP based on fecal estrogen concentrations suggested a difference between those two groups (*male exposure* 16.4 ± 1.9 days vs. *no male exposure* 9.25 ± 1.3 days), but sample sizes were too limited for statistical analysis. The inter-estrus period lasted between two to three months (Table 1).

3.2. Plasma progesterone

Plasma progesterone concentrations for adult female devils sampled during the breeding season averaged 1.64 ± 0.3 ng/ml; with the lowest values from an immature (first year) animal that did not cycle, approximating basal levels (<0.5 ng/ml) in all other study animals. The pattern of plasma progesterone concentrations was similar during estrous and pregnancy (Fig. 1a and b). Progesterone concentrations were initially low, but a small well-defined pulse (1.33 ± 0.2 ng/ml) occurred approximately two weeks prior to onset of the LP. Pro-estrous concentrations increased up to a maximum of 3.39 ng/ml about 10 days prior to luteal

Table 1
Characteristics of the estrous cycle in the Tasmanian devil as assessed by changes in fecal (estrogens and progestagens) and plasma (progesterone) sex steroid concentrations

	Feces		Plasma	
	Mean length ± SE (days)	No. of cycles (individuals)	Mean length ± SE (days)	No. of cycles (individuals)
Follicular phase (FP)				
Non-mated	14.9 ± 1.5 (range 8–21)	8 (8)	11.3 ± 1.4 (range 9–17)	9 (7)
Mated	14.9 ± 2.1 (range 8–23)	7 (7)	13. 7 ± 1.9 (range 9–17)	6 (6)
Luteal phase (LP)				
Non-mated	17.9 ± 0.3 (range 12–25)	8 (8)	20.6 ± 1.0 (range 15–29)	10 (6)
Pregnant	16.7 ± 0.8 (range 14–21)	7 (7)	n/a	n/a
Estrous cycle (FP + LP)				
Non-mated	31.4 ± 1.8 (range 21–40)	8 (8)	32.1 ± 1.4 (range 27–42)	10 (12)
Pregnant	32.1 ± 2.7 (range 24–39)	6 (6)		
Inter-estrus (FP to FP)	80.3 ± 7.1 (range 64–102)	6 (6)	73.3 ± 7.3 (range 56–99)	5 (5)

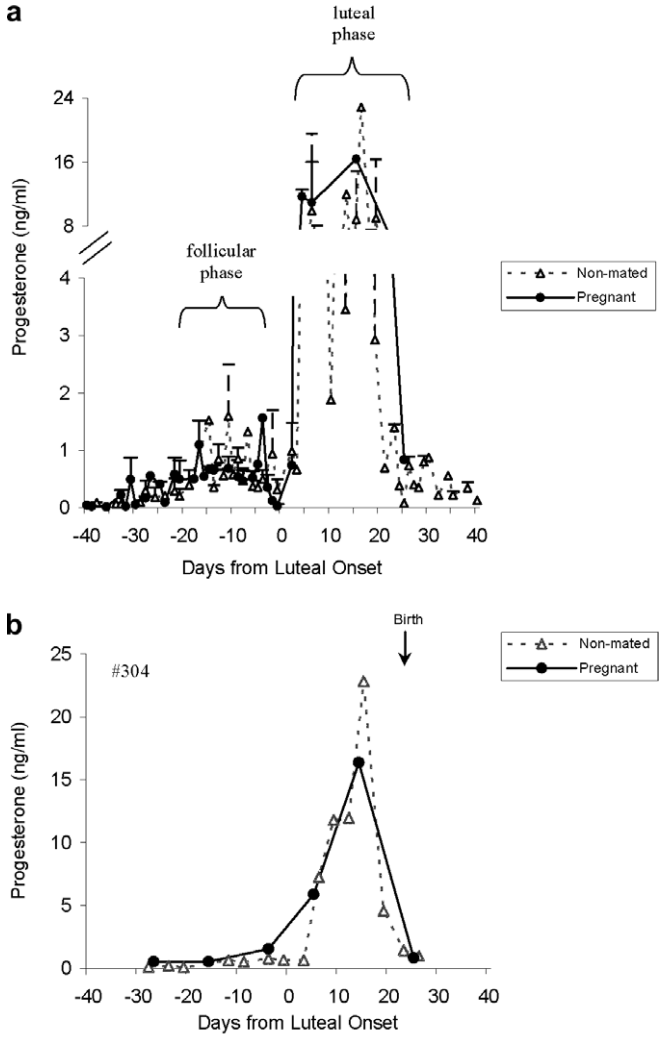

Fig. 1. (a) Mean profile and (b) an individual plasma progesterone (ng/ml) profile for non-mated (–Δ–) and pregnant (●) Tasmanian devils between days −40 to 40 from luteal onset (day 0). Note pro-estrous surge in plasma progesterone concentrations and change in scale of *y*-axis in (a).

onset, and were associated with presence of an estrus vaginal smear (>95% mature epithelial cells) (Fig. 2), and copulation for females paired with males. Following the pro-estrous pulse, plasma progesterone concentrations returned to baseline for 5.0 ± 0.5 days (range 3–9 days), but during the LP mean concentrations reached 5.28 ± 0.8 ng/ml. Progesterone concentrations peaked approximately two weeks after the luteal onset (max 22.82 ng/ml), but dropped rapidly soon afterward. Copulations were recorded over varied intervals, from a single day to recurrent bouts over a period of up to 8 days in length (3.4 ± 0.9 days). Matings occurred immediately prior to, during and after the pro-estrous peak in progesterone. Females gave birth 20–31 days from first copulation. Animals that were not mated returned to estrus by day 65 (range 46–89) (*e.g.* Fig. 2), and

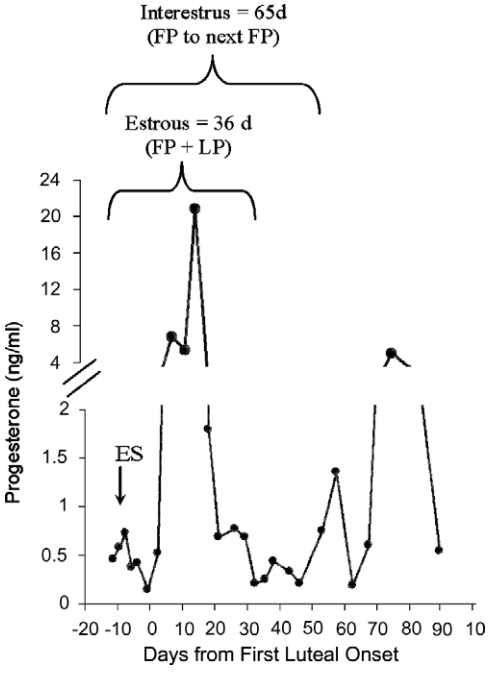

Fig. 2. Individual plasma progesterone profile (ng/ml) in a non-mated female Tasmanian devil (#201) sampled days −20 to 100 from onset of first luteal phase. ES indicates collection of an estrus smear. Note change in scale of *y*-axis. Estrous and inter-estrus interval (duration between follicular phases (FP)) shown.

two of the three females paired with a male in their second cycle produced young.

3.3. Comparisons between fecal metabolites and plasma progesterone

Pregnanediol (PgD) was excreted in consistently higher concentrations than 20-oxo-pregnanes, and during the breeding season PgD concentrations were around 8- to 11-fold higher by comparison. The pattern of excretion was very similar for the two fecal metabolites (Fig. 3), and during the estrous cycle fecal progestagens and plasma progesterone concentrations showed a significant positive correlation ($P < 0.05$; mean slope: $y = 0.91 \pm 0.2$).

3.4. Fecal steroid profiles

There was a highly significant difference in mean concentrations of fecal PgD and total oestrogens during the breeding and non-breeding season (PgD non-breeding: 219.70 ± 29.16 ng/g, breeding: 1634.50 ± 191.56 ng/g ($t_{(215)} = -4.226$, $P < 0.001$; oestrogens non-breeding: 8.56 ± 2.59 ng/g, breeding: 23.84 ± 2.50 ng/g ($t_{(217)} = -3.367$, $P = 0.001$)). Fecal PgD and 20-oxo-pregnanes were significantly positively correlated ($y = 1.05x + 1.21$, $R^2 = 0.54$; $P < 0.01$).

3.4.1. Non-mated estrous cycle

When aligned to days from luteal onset, PgD profiles for non-mated devils during the estrous cycle were strikingly similar (Fig. 4). There was a relatively minor, and brief pro-estrous increase in PgD concentrations upto 2 weeks prior to onset of the LP (<6500 ng/g) and a major increase in total estrogen levels. Immediately after luteal onset (day 0), PgD concentrations surged dramatically to reach a sharp peak within 2–5 days. Elevated PgD concentrations were sustained until around day 15–20, and then declined to near baseline measures (~200 ng/g). Most animals returned to estrus between days 47 and 68. To illustrate the pattern of fecal steroids during the non-mated estrous cycle more clearly, individual profiles from two females are shown in Fig. 5. Elevated fecal estrogen concentrations occurred 1–2 weeks prior to onset of the LP, and timing of peaks related to occurrence of estrus smears (>95% cornified cells) (Fig. 5a). The pattern of estrogens during the LP was inconsistent between individuals. Five of the eight devils observed during non-mated cycles underwent a second cycle, including three females that were paired with males at their second estrus (*e.g.* Fig. 5b).

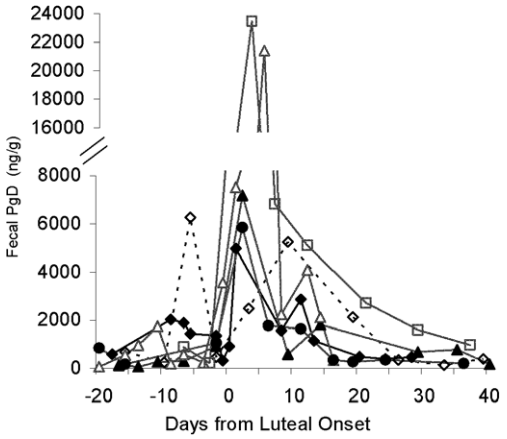

Fig. 4. Fecal pregnanediol (PgD) concentrations (ng/g) in six non-mated female Tasmanian Devils aligned to onset of luteal phase. Note break in *y*-axis.

3.4.2. Pregnant estrous cycle

Fecal hormone profiles for pregnant devils were aligned to day of final mating (day 0) (Fig. 6), due to the variation between individuals in the period between first and final copulation (1–7 days). The biphasic pattern of fecal PgD was similar to that observed during the non-mated cycle (Fig. 4). A brief surge of PgD occurred within five days after last mating, and the major peak occurred on day 18: levels returned to baseline by days 25–30 in most

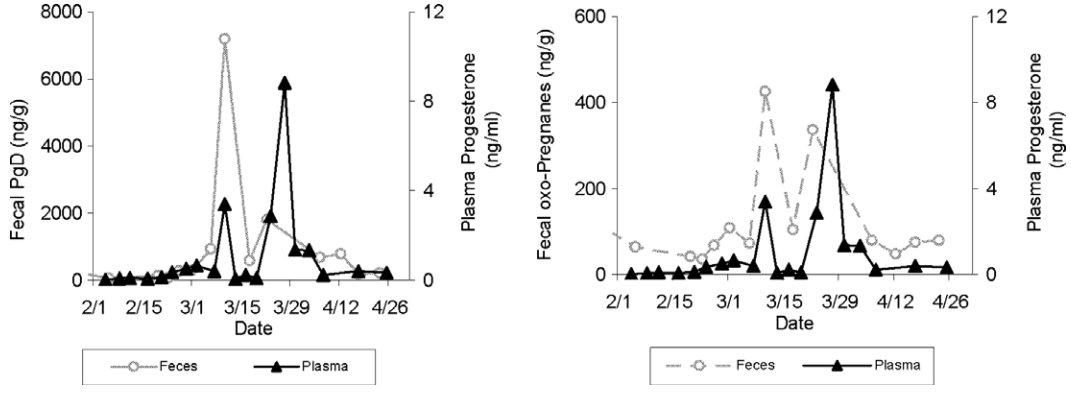

Fig. 3. Fecal progestagens (20-α-pregnanes and 20-oxo- pregnanes) (ng/g) and plasma progesterone (ng/ml) concentrations in a female Tasmanian devil during estrous. Fecal samples were displaced from plasma to correspond with approximate steroid lag time of one day (24 h). Characteristic biphasic pattern due to pro-estrous pulse in progesterone/progestagens during the follicular phase.

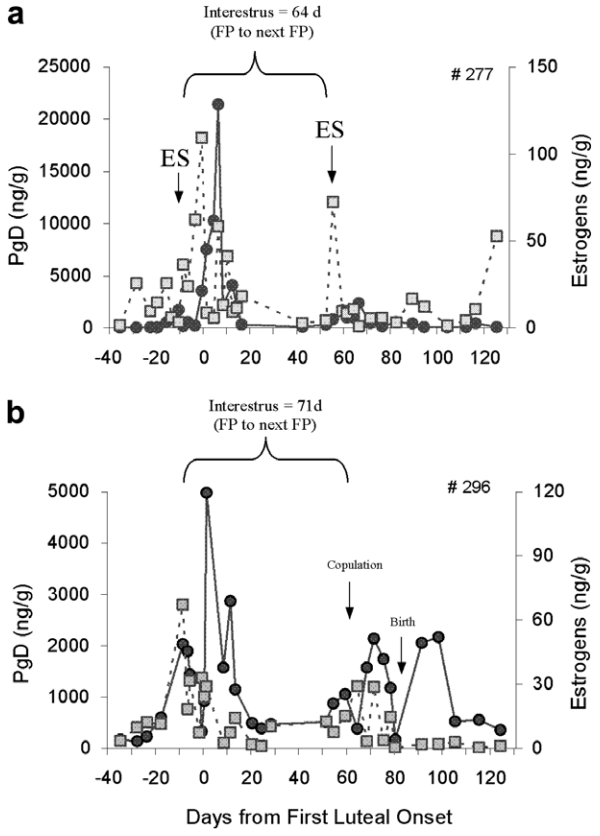

Fig. 5. Fecal pregnanediol (PgD) (●) and total estrogen (□) concentrations (ng/g) in two female Tasmanian devils between days −40 and 120 from first luteal onset (day 0). Female in (a) not mated during either estrus; female in (b) mated during second estrus; copulation and parturition indicated by arrows. ES indicates collection of an estrus smear. Inter-estrus interval shown in brackets for each individual; FP, follicular phase.

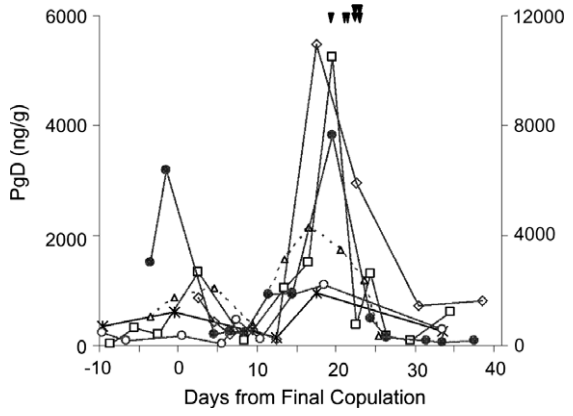

Fig. 6. Fecal pregnanediol (PgD) concentrations (ng/g) between days −10 to 40 days from final mating of that breeding season in six pregnant Tasmanian devils. Note second *y* axis also displays fecal PgD (ng/g), due to elevated concentrations in one individual (●). Births (▼) occurred from days 19 to 27; stacked triangles depict the number of animals giving birth on that day.

animals. Parturition occurred within a few days of the precipitous decline. Births were recorded on days 19, 23, 24, 26(2) and 27(2) from final mating.

Individual profiles for pregnant devils (*e.g.* Fig. 7) show that sustained increases in fecal estrogen concentrations occurred up to three weeks prior to mating; copulation occurred within ~3 days of the concurrent peak in fecal estrogens and PgD. During the LP fecal estrogen concentrations were low, compared to during the FP. Fecal PgD and total estrogen concentrations remained low (<400 and <20 ng/g, respectively) throughout lactation.

3.5. Comparison of the non-mated and pregnant estrous cycle

There were no significant differences between mean concentrations of fecal steroids in pregnant and non-mated devils ((total estrogens $= 16.74 \pm 2.7$ ng/g *versus* 22.72 ± 3.0 ng/g, respectively; $t_{(134)} = -1.42$, $P = 0.16$) (PgD $= 1045.0 \pm 274$ ng/g *versus* 1217.7 ± 175 ng/g, respectively; $t_{(103)} = 1.00$, $P = 0.32$)) (Fig. 8). ANOVA showed a significant difference in fecal PgD and estrogen concentrations over time for both groups (PgD *mated*: $F = 2.641$, $P = 0.02$, *non-mated*: $F_{(7,42)} = 2.777$, $P = 0.02$; estrogens *mated*: $F_{(7,49)} = 2.926$, $P = 0.01$, *non-mated*: $F_{(7,42)} = 3.058$, $P = 0.01$). Fecal estrogens were high in both groups prior to luteal onset (days −15 to −1), but between days 0 to 5 there was a significant difference in estrogen levels between the two groups ($P < 0.05$): estrogens declined briefly in pregnant females but increased significantly in

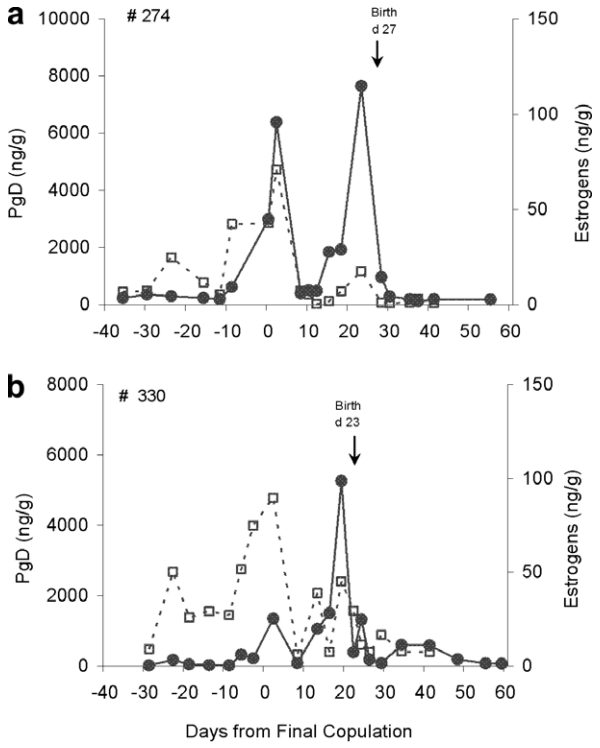

Fig. 7. Fecal pregnanediol (PgD) (●) and total estrogen (□) concentrations (ng/g) in two pregnant Tasmanian devils. Profiles are aligned from day of final copulation during that breeding season, with parturition indicated by arrows.

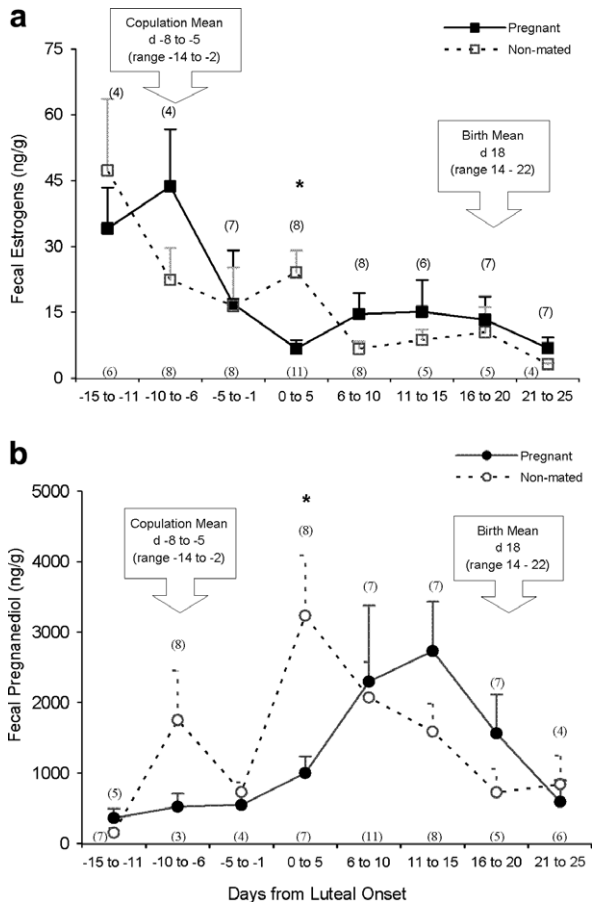

Fig. 8. Grouped mean profiles comparing (a) fecal estrogens and (b) pregnanediol (PgD) concentrations (ng/g) in non-mated and pregnant Tasmanian devils between days −15 to 25 from luteal onset. Mean copulation interval (first to final mating) and range indicated by arrows; mean day of birth (from luteal onset) and range also presented. Sample number indicated in brackets nearest to respective mean. *Significant difference between treatment groups ($P < 0.05$).

non-mated females (Fig. 8a). Between days 6–10 an inverse effect was observed, with estrogens rising in pregnant devils but dropping significantly ($P < 0.05$) in non-mated animals. In both groups, fecal estrogens returned to baseline between days 21–25.

Fecal PgD concentrations were lowest between days −15 to −11 in both groups, but a major increase was observed for non-mated females only between days −10 to −6 (Fig. 8b). Mean PgD levels remained unchanged in females paired with males prior to luteal onset, and were similar between the two groups from days −5 to −1. A significant difference in mean PgD concentrations was observed between the groups on days 0–5 ($t_{(17)} = 2.72$, $P = 0.02$), with non-mated devils having a rapid increase in PgD ($P < 0.05$), which steadily declined throughout the remainder of the LP (days 6–25). In contrast, for pregnant devils PgD levels increased slowly from luteal onset (day 0) and peaked between days 11–15. Births occurred during days 14–22, during the subsequent progestagen decline.

4. Discussion

4.1. Characteristics of the estrous cycle

Through endocrine monitoring we established that the duration of the estrous cycle in the Tasmanian devil is around 32 days. Studies of other dasyurids based on plasma progesterone concentrations have confirmed estrous as ranging from ~37 days in the eastern quoll (Hinds, 1989) to up to ~60 days in the kowari (Fletcher, 1985). Longitudinal profiling confirmed that the devil is a spontaneous ovulator, and is seasonally polyestrous—similar to most other marsupials (Tyndale-Biscoe and Renfree, 1987; Hinds et al., 1996). Due to the lengthy period of lactation in Tasmanian devils (Russell, 1982), females that conceive at first estrus and retain those young do not cycle again during the breeding season. Non-mated devils did not return to estrus immediately following the end of one cycle, and the inter-estrus interval (onset of FP to next FP) was 2–3 months, similar to that reported for kowari (Fletcher, 1985). Longitudinal profiles showed a maximum of two estrous cycles in one year.

4.2. Pattern of the estrous cycle

4.2.1. The follicular phase

The characteristic biphasic pattern of plasma progesterone concentrations during the estrous cycle has previously been reported for the eastern quoll (Hinds, 1989) and the kowari (Fletcher, 1985), but not in non-dasyurid species (Shorey and Hughes, 1973; Curlewis et al., 1985; Gemmell et al., 1987; Johnston et al., 2000; Woodd et al., 2006). The duration of the pro-estrous rise in plasma progesterone in devils was 8–10 days, similar to in the kowari (Fletcher, 1985) and eastern quoll (Hinds, 1989). The source of progesterone at this time is proposed to be developing or luteinized ovarian follicles rather than corpora lutea (CL) (Fletcher, 1985; Hinds, 1989), because the increase precedes the first estrus (Fletcher, 1985).

Multiple, brief peaks of fecal estrogens were sometimes observed during estrus, similar to the wave-like pattern of fecal estradiol-17β reported for chuditch (*Dasyurus geoffroii*) (Stead-Richardson et al., 2001). Increases in fecal estrogens often preceded the onset of estrus, but when they were accompanied by a pro-estrous rise in fecal progestagens, they coincided with fully cornified vaginal smears and copulation. As reported for other dasyurids (Fletcher, 1985; Hinds, 1989; Stead-Richardson et al., 2001), mating occurred immediately prior to, during and/or following these peaks in plasma or fecal hormones. Copulations occurred over a typical 1–2 days time-frame (Tyndale-Biscoe and Renfree, 1987), but in several individuals mating was prolonged for up to 8 days, in accordance with previous reports for the species (Smith, 1993). A relatively protracted estrus period has also been observed in some other dasyurids, including *Antechinus* (Tyndale-Biscoe and Renfree, 1987) and the chuditch (Stead-Richardson et al.,

2001), and is thought to be related to sperm storage and delayed ovulation (Taggart et al., 2003).

After the pro-estrous peak, there was a 3 to 9 day nadir in plasma progesterone and fecal progestagen concentrations. This temporary decline in progesterone is also evident in the eastern quoll (Hinds, 1989) and kowari (Fletcher, 1985), and is presumed to be the period during which ovulation occurs (Selwood, 1982; Fletcher, 1985; Hinds, 1989). Most marsupials ovulate within 1–2 days of estrus, but in dasyurids the interval is longer (Tyndale-Biscoe and Renfree, 1987; Taggart et al., 2003). In the Tasmanian devil, mean duration of this 'ovulatory interval' was approximately 7 days. Ovulation is thought to take place around 4–6 days post-estrus in the eastern quoll and kowari (Hill and O'Donoghue, 1913; Fletcher, 1985), and up to 10 days post-estrus in the brown antechinus (*A. stuartii*) (Selwood, 1980). Considerable variation in time to ovulation occurs in eastern quoll (Hinds, 1989), which could account for the extended interval we observed.

4.2.2. The luteal phase

The pattern of plasma progesterone concentrations during the luteal phase (LP) in Tasmanian devils resembled that described for other non-macropodid marsupials (Tyndale-Biscoe and Renfree, 1987; Hinds, 1989; Hinds and Selwood, 1990; Bradshaw and Bradshaw, 1992; Millis et al., 1999; Johnston et al., 2000). The primary source of progesterone is the autonomous CL (Tyndale-Biscoe and Renfree, 1987; Hinds, 1990; Gemmell, 1995); and in the eastern quoll (Hinds, 1989) and brown antechinus (Hinds and Selwood, 1990) the major sustained increase in plasma progesterone during the LP mirrors CL development. For devils, births occurred within days of a precipitous decline in progesterone/progestagen concentrations, probably in association with the demise and involution of the CL (Hinds and Selwood, 1990).

Temporary elevations in estrogens during the LP have also been noted in the chuditch, squirrel glider (*Petaurus norfolcensis*) (faecal: Stead-Richardson et al., 2001; Woodd et al., 2006) and American opossum (*Didelphis virginiana*) (plasma: Harder and Fleming, 1981). This occurrence probably reflects incomplete suppression of ovarian activity, with the follicles failing to mature (Fleming and Harder, 1983).

4.3. Duration of pregnancy

The period over which matings occurred varied widely (1–8 days) indicating that, as for other dasyurids, ovulation did not occur at a fixed time in relation to estrus. For this reason, the interval from mating to birth (21–62 days) is frequently cited as the gestation period for dasyurids (Tyndale-Biscoe and Renfree, 1987). Previous estimates for devils vary from 19 days (Slater, 1993) to 28–31 days (Guiler, 1970; Guiler, 1971), and we determined a comparative figure of ~24 days (24.6 ± 1.1 days; range 19–27 days) through calculating time from final mating to parturition. Selwood and Woolley (1991) note, however, that the day of copulation is not a useful indicator of onset of embryonic development. In addition to the variable interval from mating to ovulation, the period of sperm storage may also be variable (Taggart et al., 2003). Therefore, the interval from luteal onset to birth provides a more realistic and accurate gestation length of 17.9 ± 1.0 days (range 14–22 days) for the Tasmanian devil.

4.4. Comparison of the mated and non-mated estrous cycle

There was no difference in the mean length of the FP (which at ~15 days is similar to other dasyurids: Tyndale-Biscoe and Renfree, 1987) between mated and non-mated devils. As in most marsupials (Fletcher, 1985; Tyndale-Biscoe and Renfree, 1987; Hinds, 1989; Hinds, 1990; Hinds and Selwood, 1990), there were no differences in the mean duration of the LP or in the magnitude/duration of plasma progesterone profiles between the mated and non-mated estrous cycle. Documenting maternal recognition of pregnancy is difficult in marsupials, not only because there are usually no obvious differences in hormone profiles, but because the conceptus is encapsulated in an extracellular shell membrane prior to implantation (Cruz et al., 2001). However, through monitoring fecal sex steroids, our study revealed intriguing differences in the pattern of estrogen and progestagen excretion between the mated and the non-mated estrous cycle in devils. The signature pattern of sex steroids varied during periods associated (in pregnant animals) with blastocyst expansion and implantation (Hinds and Selwood, 1990): these results suggest maternal endocrine recognition of pregnancy may occur in this species.

This study has provided fundamental information on the reproductive endocrinology of *S. harrisii*, the largest species of dasyurid, and provides a detailed description of the estrous cycle. It has also demonstrated the effectiveness of faecal steroid analyses for monitoring the ovarian cycle in this now-endangered marsupial. This research represents the first analysis of plasma progesterone concentrations in the devil, and the first longitudinal assessment of fecal sex steroids (estrogens and progestagens) in a dasyurid. Correlations between patterns of plasma and fecal sex steroid concentrations and physiological events including copulation, pregnancy and birth confirm the validity of fecal steroid measurements as an alternative, non-invasive application for monitoring female reproduction in this species.

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