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Pouch appearance is a reliable indicator of the reproductive status in the Tasmanian devil and the spotted-tailed quoll

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Abstract

Female Tasmanian devils (TDs) *Sarcophilus harrisii* and spotted-tailed quolls (STQs) *Dasyurus maculatus* were monitored to assess changes in plasma progesterone and faecal oestrogens/progestagens, vaginal smears and qualitative changes in pouch appearance during the oestrous cycle. Pouch condition was characterized based on size, colour and secretions, and was found to accurately reflect reproductive status, being significantly correlated with changes in both sex steroids and vaginal cytology. During the follicular phase, pouch redness and secretions were maximal, and associated with increased sex steroid concentrations, a karyopyknotic index of $>90\%$ and the onset of copulation. Post-ovulation, pouches became wet and deep and developed a glandular appearance; plasma progesterone/faecal progestagen concentrations remained high and sustained throughout the luteal phase. These features were identical during the pregnant and non-pregnant oestrous cycle. This study demonstrated that pouch appearance is a reliable physical indicator of the stage of oestrous in the TD and STQ, and provides an alternative non-invasive method for evaluating the ovarian cycle of these threatened species. This technique can be readily applied to monitor individuals in free-ranging or captive populations, and will aid as a practical tool for improved breeding management.

Introduction

Measurement of plasma sex steroid concentrations or assessment of vaginal cytology are commonly used to monitor oestrous cycles in marsupials (Tyndale-Biscoe & Renfree, 1987). More recently, faecal sex steroid measurement has been developed for several marsupial species including the carnivorous dasyurids (Stead-Richardson *et al*., 2001; Paris *et al*., 2002; Bradshaw *et al*., 2004; Woodd *et al*., 2006; Schwarzenberger, 2007; Hesterman, Jones & Schwarzenberger, 2008*a*,*b*). This contemporary 'hands off' approach confers obvious advantages, but still poses limitations for day-to-day monitoring of reproductive cycles because of the necessity for technical expertise and time required for sample processing. Urinary and vaginal cytology are comparatively intrusive methods, but are accurate and provide immediate results. These techniques are also routinely used to detect oestrus and ovulation (Woolley, 1971; Fletcher, 1985; Tyndale-Biscoe & Renfree, 1987; Millis *et al*., 1999; Stead-Richardson *et al*., 2001), but still require a level of operator skill and access to laboratory equipment for analyses.

In some species such as the small dasyurids, changes in body weight provide an alternative indicator of reproductive status (Tyndale-Biscoe & Renfree, 1987), and this measure has been correlated with changes in plasma progesterone during the oestrous cycle (Fletcher, 1985). Behavioural cues such as characteristic vocalizations and posturing have also been used to detect oestrus in captive dasyurids (Croft, 1982; Fox & Whitford, 1982), but this method is very time consuming (Williams, 1990). More simple and practical methods of detecting oestrous are desirable to improve monitoring and management of *in situ* and *ex situ* marsupial populations.

In dasyurids the pouch area typically undergoes marked development during the breeding season, including an increase in size, intense reddening and secretory activity of the tissues (Woolley, 1966, 1974; Tyndale-Biscoe & Renfree, 1987). For *Antechinus stuartii*, the abundance of urinary epithelial cells at oestrus is accompanied by changes in the appearance of the pouch area (Selwood, 1982), which suggests this measure could serve as a useful external indicator of reproductive status. O'Donoghue (1911) described these progressive changes in the eastern quoll *Dasyurus viverrinus*, and demonstrated a relationship between development of the mammary glands and formation/growth of corpora lutea. However, to date, no studies have investigated changes in pouch condition of dasyurids in relation to hormonal variation during the reproductive cycle.

Dasyurid marsupials are vulnerable to extinction because of their characteristically short life history, which imposes natural constraints on reproductive output (Cockburn,

1997; Jones *et al*., 2003). Maintenance of *ex situ* colonies is thereby an important facet of conservation but intensive management is required to sustain captive founder populations (Jackson, 2003*a*). As with most captive breeding programmes, a reliable and robust method of monitoring the females' oestrous cycle is needed to inform decisions such as the timing of mating introductions. This is of particular importance in solitary species such as devils and quolls because inappropriate grouping can lead to serious injury or death, and loss of young (Guiler, 1971; Collins *et al*., 1993; Roberts & Hutchins, 1993; Jackson, 2003*a*).

Conservation of the world's largest surviving dasyurids – the Tasmanian devil (TD) and the spotted-tailed quoll (STQ) *Dasyurus maculatus* – is a current priority, with free-ranging populations facing significant threat, primarily from disease (Hawkins *et al*., 2006) or habitat loss (Jones *et al*., 2003), respectively. The devil and STQ have a lengthy history of failing to breed reliably in captivity, and reviews concede the primary cause is a lack of reproductive information on these species (Williams, 1990; Carnio, 1993; Jackson, 2003*a*). Detailed information on the reproductive biology and endocrinology of the devil and STQ has recently become available (Hesterman *et al*., 2008*a*,*b*). Similar to other marsupials (Tyndale-Biscoe & Renfree, 1987), both species are seasonally polyoestrous, spontaneous ovulators with equivalent pregnant and non-pregnant oestrous cycles that last *c*. 1 month. Knowledge of the timing and pattern of oestrus and gestation length is helpful; however, further information is required to direct more effective captive breeding. Practical indicators of oestrus and pregnancy have not been established for either the devil or STQ.

The main aim of this study was to evaluate changes in the pouch condition of devils and STQs in relation to changes in sex steroids and vaginal cytology during the oestrous cycle, and to determine whether this is a reliable technique for monitoring the reproductive status in these carnivorous marsupials.

Materials and methods

Study animals and data collection

Data were collected from 14 female TDs of varying ages (0.5–6 years old) and seven STQ of varying ages (1–4 years old) housed at Trowunna Wildlife Park, Tasmania. Details of study animals and husbandry are provided in Hesterman *et al*. (2008*a*,*b*). Devils and quolls were restrained unanaesthetized in a sack during physical examination and collection of blood and vaginal smears, and urogenital opening measurements. Animals were cradled in the lap with the hindquarters exposed and positioned toward the handler to facilitate examination. Sampling was conducted during the period encompassing peak breeding activity for that species: data were collected from devils on a twice-weekly basis from January to April, and approximately weekly thereafter to June/July; and for STQs at ~10-day intervals from June to September. Sampling frequency was increased to every 2–3 days at the onset of oestrus. To reduce any potential impact of handling stress on breeding success, only limited plasma sampling took place after copulation; sampling resumed if subsequent pouch checks confirmed no young were present. Research was conducted with the approval of University of Tasmania Animal Ethics Committee (permit # A5706).

Pouch assessment and morphometrics

Pouches were monitored for changes in appearance, and checked for the presence of young. Data were collected on qualitative factors that is size, colour and type/amount of secretion present and the pouch area was scored (0–4). Urogenital opening colour was graded on a quantitative scale (white = 1, pink = 2, red = 3). Body weight (to 100 g) and urogenital opening measurements (length and width to 0.1 mm) were also measured.

Vaginal (urogenital) smears

Smears were obtained from the posterior vaginal sinus by introduction of a small cotton swab through a glass speculum (quolls: 70 mm long × 3 mm ∅; devil: 5 mm ∅). Smears were air-dried, fixed and stained with acid fuchsin and toluidine blue (Dix & Billings, 1969). Stained smears were examined at ×40 magnification. The percentages of intermediate (IEs) and superficial/cornified epithelials (SEs), leucocytes and parabasal (Pb) cells were calculated. The karyopyknotic index that is % cells with pyknotic nuclei, excluding Pb (Hughes & Dodds, 1968), was determined using a total of 100 cells from five randomly selected fields per slide. In mated females, the presence of spermatozoa was also recorded.

Plasma and faecal collection

A sample of 75–150 μL blood was obtained from a marginal ear vein via a heparinized capillary tube, and centrifuged to recover the plasma, which was stored frozen (−20 °C) until radioimmunoassay. Entire faeces were usually collected in the morning (07:30–09:00 h) or opportunistically when freshly voided throughout the day, and frozen at −20 °C. Plasma and faecal processing and analyses follow methods in Hesterman *et al*. (2008*a*,*b*), and are briefly described below.

Plasma steroid analysis

Duplicate aliquots of plasma were assayed for progesterone by radioimmunoassay as detailed in Hesterman *et al*. (2008*a*,*b*). Cross-reactivities of the 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-deoxycortisol (0.4%) and all others (<0.1%). Assay sensitivity was 3 pg tube^{-1} (0.09 ng mL^{-1}). Intra- and inter-assay coefficients of variation were 9.5% (n = 9) and 14.8% (n = 9), respectively.

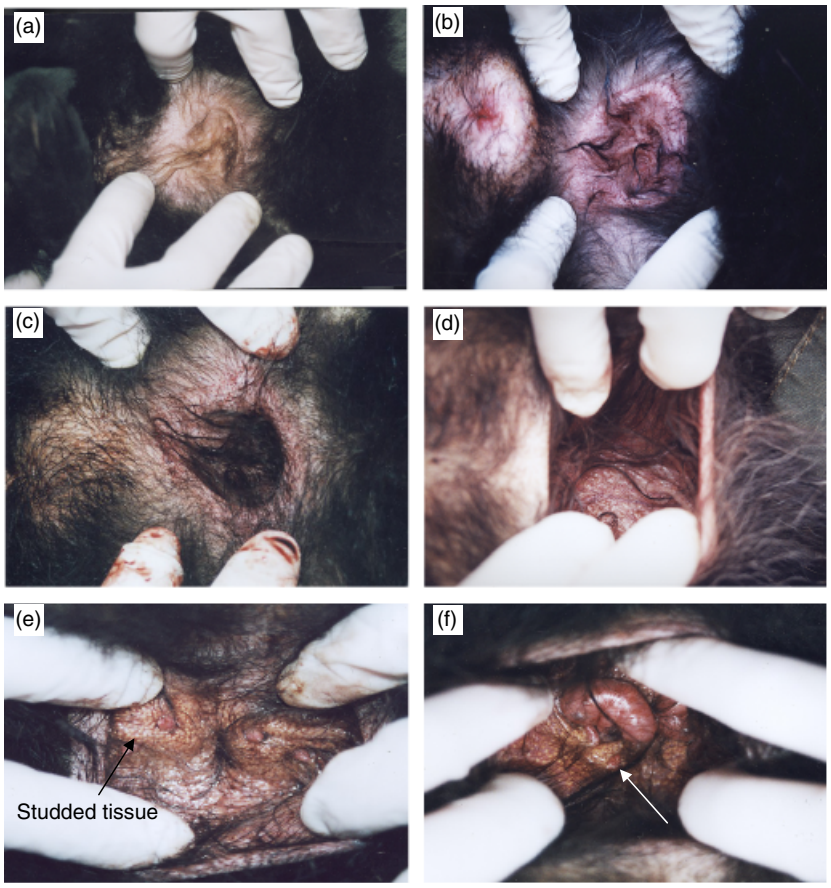

Figure 1 Characteristic stages in pouch development of female Tasmanian devils *Sarcophilus harrisii* during oestrous. Full descriptions with associated changes in vaginal cytology are provided in Table 1. (a) Immature/pre-breeding – pouch is pale and relatively shallow; (b) pro-oestrous – pouch skin is lightly flushed with some red 'greasy' exudate; (c) oestrus – pouch secretes highest levels of red exudate (seen pooling on fingertips of glove: note ring around pouch edge; (d) post-ovulation – pouch very enlarged, deep and damp; exudate thin and clear; (e) late luteal phase – tissue wet and glandular with characteristic white, raised 'studs' and (f) early lactation – studded tissue persists during early pouch occupation. Pouch young shown ~2 weeks of age; note regression of unoccupied posterior teat (indicated by arrow) following loss of another young in litter at early lactation.

Faecal steroid analysis

Samples were analysed for 20α-OH-pregnanes [antibody: 5β-pregnane-3α-20α-diol 3HS:BSA; trivial name pregnanediol (PgD)] and total oestrogens (antibody: oestradiol-17 β-OH 17HS:BSA), as detailed previously (Hesterman *et al.*, 2008*a*,*b*). Assay sensitivity was $2\,ng\,g^{-1}$ both for progestagens and for oestrogens. The intra- and inter-assay coefficients of variation for the assays tested were between 10 and 15%, respectively.

Interpretation of hormone data

Stages of the reproductive cycle determined were anoestrus, oestrus and the luteal phase. These stages have been defined previously, using analyses of group mean concentrations of plasma (progesterone) and faecal (oestrogens and progestagens) sex steroids for the TD (Hesterman *et al.*, 2008*a*) and STQ (Hesterman *et al.*, 2008*b*).

Statistical analyses

Urogenital opening size was standardized by dividing each measurement by the individual's body weight. All data are presented as the mean ± SE, except where indicated otherwise. χ^2 analysis was used for determining the association between pouch or urogenital opening score and reproductive status. One-way analysis of variance with *post hoc* Tukey's tests was used to test for significant differences between mean hormone concentrations or karyopyknotic index with pouch condition/score. The significance level was set at $P<0.05$. Statistics were performed using SPSS (SPSS Inc. 1998, Chicago IL, USA).

Results

Pouch condition

In TDs and STQs, pouch development followed a predictable sequence (Fig. 1a–f; Table 1). Until at least 1 month before onset of the breeding season (TD = January; STQ = May), pouches were pale and shallow (Fig. 1a). In subadult devils (<2 years of age) and quolls (<1 year of age), pouch dimensions were considerably smaller (~20–30 mm ∅, 10 mm depth) (score = 0) than those of adults (~50 mm in ∅, >30 mm deep) (score = 1). Teats were everted and small (<5 mm) in all females, including individuals that had weaned young from the previous season. Post weaning, there was no difference in pouch appearance between females that had reared one or more litters previously, and adults that had never produced young.

At the onset of the breeding season, study animals showed a marked change in pouch appearance, with the

Table 1 Pouch development in female Tasmanian devils *Sarcophilus harrisii* and spotted-tailed quolls *Dasyurus maculatus* during the breeding season

Pouch score/condition	Pouch description
0 (immature)	Round shape. Pale, small and shallow
1 (adult)	Oval shape. Pale and clean, teats may still be elongated if young only recently weaned; occasional yellow 'wax'
2	Skin flushes pink and interior secretes a sticky pink/red, grease-like substance
3	Skin very flushed, marked/copious volumes of thin, red oily secretions produced; area deepens slightly
4	Enlarged: interior very deep and pouch edge thickened; secretion becomes clear and watery leaving interior damp
	Pouch hairs thicken and lengthen
	White 'studs' develop after 1–2 weeks, and persist several weeks in females without pouch young
? Post-reproductive (≥6 years old)	Secretes low-moderate levels of brown, thin/watery liquid

exception of one of the two first year devils. For all other individuals, initially the pouch skin turned pink and the interior began to secrete a pink/red, sticky substance (score = 2; Fig. 1b). During the next stage, the pouch secreted an increasing quantity of the oily exudate and became slightly deeper (score = 3). Production of pigmented pouch 'oil' was more pronounced in devils than in quolls. Devil pouches contained considerable quantities (>1 mL) of the red oily exudate, at times developing a crimson ring around the edge (Fig. 1c). The third stage of pouch development was most visibly conspicuous (score = 4). At this time the pouch enlarged noticeably, becoming at least twice as deep (>50 mm) and with thickened edges. The interior became very vascular and damp, and a thin, clear fluid replaced the red oily exudate (Fig. 1d). After 1–2 weeks, the floor tissue became studded with many small, white raised spots (Fig. 1e). These 'studs' developed in the pouches of non-mated and mated females, and were evident at least a week before birth in pregnant animals (Fig. 1f), persisting for 2–3 weeks in all females (TD: 18.8 ± 3.1 days; STQ: 18.4 ± 2.5 days).

Following this developmental sequence, the pouch remained enlarged and some pink–red secretions continued to be present. An increase in secretions was later observed and the sequence of changes began again, but this was less marked than at the onset of the breeding season. After the breeding season, for females without young, pouch secretions gradually decreased and dried out then ceased, and the area reduced in size. In females that underwent full pouch development (stage 4), the pouch did not revert to its original size (stage 0) regardless of whether young were produced.

Pouch development and sex steroid concentrations

Longitudinal plasma and faecal endocrine profiles for female devils and quolls demonstrated that changes in pouch condition during the oestrous cycle were associated with significant changes in hormone concentrations (Figs 2 and 3). In the 1-year-old devil that underwent only minor changes in pouch appearance (minimal secretions, no enlargement),

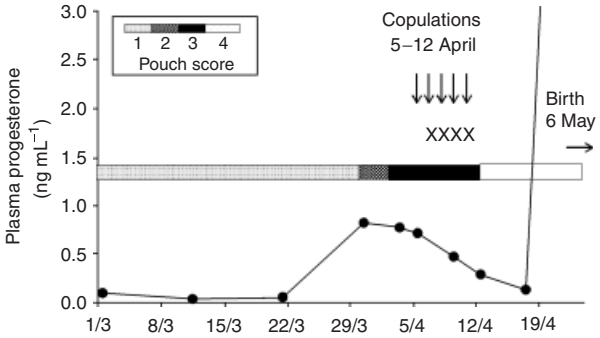

Figure 2 Plasma progesterone profile (ng mL^{-1}) for an individual female Tasmanian devil *Sarcophilus harrisii*, showing associated changes in pouch condition and maturation of vaginal cells during oestrus: 'x' indicates days KI (karyopyknotic index) ≥95%. The pro-oestrous surge in progesterone during the follicular phase is followed by a characteristic nadir before ovulation and the major luteal increase (<10.9 ng mL^{-1}, not shown). Birth occurred 24 days after final copulation.

sex steroid concentrations remained basal, confirming this female failed to attain sexual maturity.

Before the breeding season, when the pouch was pale and shallow (Fig. 1a; score = 1), concentrations of plasma progesterone and faecal progestagens and oestrogens were low. When the pouch flushed strongly pink to red in colour and began to produce an oily red exudate (Fig. 1b and c; score = 2), there was a concurrent increase in plasma progesterone, faecal progestagens and oestrogens, marking the onset of the oestrous cycle (i.e. follicular phase). For females that were paired with males, copulation began when pouch secretions reached maximum (score = 3), and hormone concentrations began to decline (Figs 2 and 3). The interval from distinctive red exudate production by the pouch to onset of hormonal oestrus was 18.7 ± 2.8 days (range 3–32 days) in devils, and 10.9 ± 1.6 days (range 8–17 days) in STQs. Marked deepening of the pouch (Fig. 1d; score = 4) occurred during the luteal phase, when progesterone/progestagen concentrations were highest, and faecal oestrogen concentrations had declined. Individual profiles for devils and quolls showed that substantial enlargement of the pouch began a few days after ovulation. Development of

white 'studs' in the pouch appeared *c.* 1–2 weeks after onset of the luteal phase (TD: 13.8 ± 2.3 days, range 8–22 days; STQ: 9.0 ± 5.4 days, range 4–18 days).

In devils, there was a significant association between pouch score and elevated concentrations of plasma progesterone ($F_{4,44} = 10.5$, $P < 0.01$), faecal PgD ($F_{4,41} = 3.45$, $P = 0.01$) and oestrogens ($F_{4,40} = 3.2$, $P = 0.04$) (Table 2). *Post hoc* comparisons indicated significantly higher mean concentrations of plasma progesterone/faecal progestagens when the pouch was deep and enlarged (score = 4), during the luteal phase (Fig. 1d–f). Similar patterns of hormonal changes and pouch score (PgD: $F_{3,16} = 2.16$, $P < 0.05$; oestrogens: $F_{3,16} = 1.54$, $P = 0.07$) were observed in STQs.

During the inter-oestrous stage and up to 2 months after the breeding season, oestrogen and progestagen concentrations were low with minor fluctuations; pouches remained lax and damp and continued to secrete a low quantity of the red exudate. In aged female devils (>6 years; $n = 2$), the pouch exudate was brown in colour and thin in consistency (Table 1): endocrine profiles for these individuals indicated missing follicular and/or luteal activity.

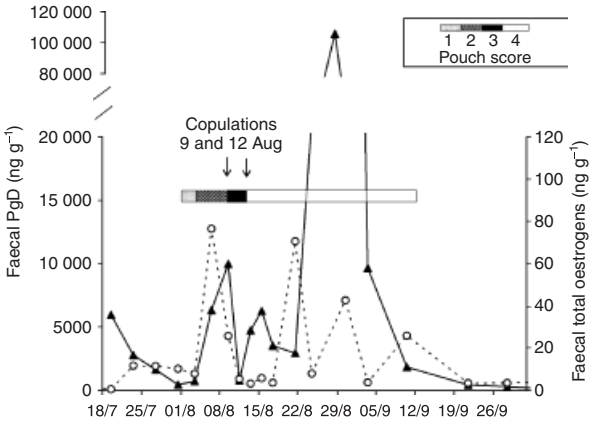

Figure 3 Faecal oestrogen (○) and pregnanediol (PgD) (▲) concentrations (ng g^{-1}) for an individual female spotted-tailed quoll *Dasyurus maculatus*, showing associated changes in pouch condition at oestrus. No pouch young were confirmed. Terminal portion of previous luteal phase is observed at the beginning of profile.

Vaginal cytology

For devils, relatively few cells were present in the vaginal smear before the breeding season. The cell population consisted mainly of IEs in sexually mature devils (≥3 years of age), and predominantly Pb in immature females. In STQs of all ages, the anoestrous smear comprised both IE and Pb cells in females. For both species, there was a proliferation in cell numbers several weeks before oestrus: the smear became inundated with IE cells but a low-moderate presence of SE cells was also observed.

Elevated faecal oestrogen concentrations during the follicular phase (Table 2) were associated with an increased number of mature epithelial cells (IEs and SEs) in the vaginal smear. Faecal oestrogen concentrations were threefold higher when the karyopyknotic index was >90% (TD: 16.67 ± 5.9 ng g^{-1}; STQ: 5.94 ± 1.9 ng g^{-1}) than when it was <50% (TD: 45.26 ± 10.8 ng g^{-1}; STQ: 21.76 ± 10.7 ng g^{-1}) (TD: $F_{2,20} = 1.51$, $P > 0.05$; STQ: $F_{2,11} = 2.61$, $P > 0.05$). Changes in karyopyknotic index and faecal oestrogen concentrations followed the same pattern during the oestrous cycle, rising steadily during the follicular phase, and then dropping sharply at the onset of the luteal phase. The relationship between karyopyknotic index and plasma progesterone/faecal progestagen concentrations was less clearly defined; but in devils, karyopyknotic index and plasma progesterone concentrations were significantly associated ($F_{2,26} = 4.13$, $P = 0.03$), with the highest progesterone concentrations (2.54 ± 0.8 ng mL^{-1}) when smears had a low karyopyknotic index (<50%).

Table 2 Mean concentrations of plasma (progesterone) and faecal (pregnanediol and total oestrogens) sex steroids associated with pouch development in the Tasmanian devil *Sarcophilus harrisii* and spotted-tailed quoll *Dasyurus maculatus*

Species	Pouch score	Plasma progesterone (ng mL^{-1})	Faecal preganediol (ng g^{-1})	Faecal oestrogens (ng g^{-1})	Stage of oestrous cycle
Sarcophilus harrisii	0	0.10 ± 0.0 (12)^{a}	152.8 ± 41.5 (12)^{a}	7.1 ± 1.6 (12)^{a,b}	Immature
	1	0.44 ± 0.1 (9)^{a}	499.2 ± 58.2 (10)^{a}	17.9 ± 5.1 (10)^{a,b}	Anoestrous
	2	0.80 ± 0.2 (11)^{a}	750.7 ± 264.6 (10)^{a,b}	23.7 ± 5.4 (10)^{a,b}	Pro-oestrous; sexually proceptive
	3	1.42 ± 0.6 (11)^{a}	1444.9 ± 296.8 (9)^{a,b}	32.9 ± 7.5 (9)^{a}	Oestrus (follicular phase); sexually receptive
	4	4.72 ± 1.0 (11)^{b}	2201.1 ± 456.8 (11)^{b}	10.9 ± 2.4 (11)^{b}	Post-ovulation; mated and non-mated luteal phase equivalent
Dasyurus maculatus	0	<detection level (1)	–	–	Immature
	1	–	86.4 ± 26.2 (3)	1.1 ± 0.8 (3)	Anoestrous
	2	0.35 ± 0.2 (4)	1390.4 ± 502.2 (6)	16.6 ± 7.7 (6)	Pro-oestrous; sexually proceptive
	3	0.47 ± 0.3 (3)	1307.1 ± 735.4 (5)	10.2 ± 4.7 (5)	Oestrus; sexually receptive
	4	0.74 ± 0.5 (5)	4220.5 ± 1762.6 (6)	4.6 ± 1.4 (6)	Post-ovulation; mated and non-mated luteal phase equivalent

See Table 1 for description of pouch condition/score. Number of animals sampled in brackets; superscript with different letters indicates significant differences between groups ($P < 0.05$).

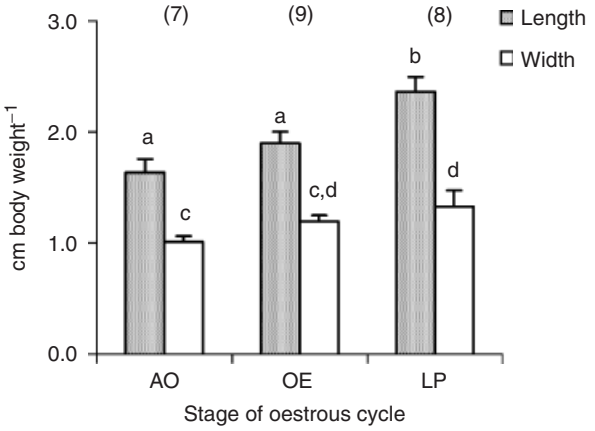

Figure 4 Mean urogenital opening size (standardized cm) during the reproductive cycle in Tasmanian devils *Sarcophilus harrisii*. AO, anoestrous; OE, oestrus; LP, luteal phase. Values with different letters indicate significant differences between groups ($P<0.05$). Numbers of animals for each stage indicated in brackets.

Morphometrics

In devils, the urogenital opening enlarged significantly (length: $F_{2,46} = 10.93$, $P<0.01$; width: $F_{2,46} = 3.79$, $P = 0.03$) during the breeding season and reached maximum size during the luteal phase when progesterone concentrations were high (Fig. 4). For quolls, a similar pattern was observed (anoestrus: $7.5 \pm 0.6 \times 5.8 \pm 1.0\,\text{cm bw}^{-1}$; OE: $9.0 \pm 0.9 \times 6.8\,\text{cm bw}^{-1}$; luteal phase: $8.5 \pm 0.9 \times 5.7 \pm 1.1\,\text{cm bw}^{-1}$); however, sample size was insufficient to perform statistical analyses. For devils and quolls, the urogenital opening was pale in non-breeding females, but became pink to red in colour during the oestrous cycle. The change in urogenital opening colour was significantly associated with the reproductive condition (assigned using hormone concentrations) in both species (TD: $P<0.01$; STQ: $P<0.05$).

Discussion

The present study validates pouch condition as a reliable external indicator of reproductive status for TDs and STQs, reflecting the underlying endocrinology of the ovarian cycle in these species. This method provides a simple, accurate and immediate non-invasive tool for assessment of reproductive activity, which could benefit the monitoring of both captive and free-ranging populations.

As females approached their first breeding season, the pouch began secreting a pink to reddish oily exudate, signifying the approach of puberty, as documented for other dasyurids (Woolley, 1966, 1974). Similarly, in the pubescent brush-tail possum *Trichosurus vulpecula*, it is the appearance of an orange-brown pouch exudate that heralds first oestrus (Bolliger & Carrodus, 1938). As in most other marsupials (Tyndale-Biscoe & Renfree, 1987), puberty in devils and quolls was also associated with rapid growth of the pouch, which serves as a useful indicator of sexual maturity.

Our concurrent evaluation of reproductive endocrinology and vaginal cytology demonstrated that readily identifiable changes in pouch appearance are associated with specific stages of the oestrous cycle. Fleay (1935, 1940) noted reddening and development of the pouch of the devil and STQ during the breeding season, and this feature has been detailed for other dasyurids (Woolley, 1966, 1974). O'Donoghue (1911) determined that similar changes in the eastern quoll pouch result from an increase in size and activity of the cutaneous glands, determining that sweat glands are responsible for producing secretions, whereas hypertrophy of the sebaceous glands results in pouch swelling and enlargement. He suggested that some unknown 'inciting factor' present in blood may be responsible for stimulating glands within the pouch.

In the current study, endocrine monitoring has demonstrated that in devils and STQs the pouch produces pigmented secretions when both oestrogens and progesterone/progestagen concentrations are elevated. As hormone concentrations continue to rise, there is a concurrent increase in the volume of red pouch secretions. Consistent with this finding, Bolliger & Carrodus (1939*b*) demonstrated experimentally that oestrogens play a role in the production of pouch pigment in adult brush-tailed possums. In non-dasyurid marsupials such as the possum, oestrus is associated with elevated oestrogens, and progesterone does not rise until after ovulation (Tyndale-Biscoe & Renfree, 1987), implicating oestrogens as the most likely stimulant of this red exudate from the pouch.

Following oestrus, the pouch interior in devils and STQs became very obviously enlarged and deep – as previously observed in these species (Fleay, 1935, 1940). Similar changes have been reported for other dasyurids (Woolley, 1974; Fletcher, 1985; Soderquist & Serena, 1990; Oakwood, 2000). These visible changes were identical in mated and non-mated females, in agreement with Woolley (1974). We confirmed that this stage of pouch development begins soon after ovulation, in association with the major increase in progesterone/progestagens during the luteal phase. Progesterone is a primary agent of pouch enlargement in the brush-tailed possum (Bolliger & Carrodus, 1939*a*), and is implicated here as the probable cause of pouch expansion in devils and STQs. These results also support early speculations that development of the pouch may be linked to development of the corpora lutea (O'Donoghue, 1911; Marlow, 1961).

The distinctive white spots that appeared later in the cycle, lending a 'granular' appearance to the pouch (Woolley, 1966), have also been observed in other dasyurids. O'Donoghue's (1911) histological analysis of the mammary tissues of the eastern quoll determined that these 'studs' are, in fact, enlarged sebaceous glands. Similar morphological changes have been observed during pregnancy in several non-dasyurids including the numbat *Myrmecobius fasciuatus*, wombats (Vombatidae) and the koala *Phascolarctos cinereus* (Jackson, 2003*b*; Jackson *et al*., 2003; Power & Monoghan, 2003; Finlayson *et al*., 2006). This suggests that pouch appearance may prove to be a useful indicator for monitoring the oestrous cycle in other marsupial species.

The significance of these profound histological developments of the dasyurid pouch during the oestrous cycle is unknown. Given the importance of olfactory signals in dasyurid communication (Croft, 1982), it is possible that secretions contain chemosensory properties that serve in mate attraction or stimulation at oestrus. Male devils have occasionally been observed investigating a female's pouch before mating (HH), and such behaviour has been documented in male macropodids (Tyndale-Biscoe & Renfree, 1987). Pouch secretions may also have a role during the periparturient stage. O'Donoghue (1911) proposed that later secretions may facilitate cleaning of the pouch by the female before giving birth, and there is evidence that antibacterial properties of pouch secretions may confer some immunity to young marsupials (e.g. Old & Deane, 2000).

We also monitored vaginal cytology as an indicator of the reproductive condition in both the devil and STQ. In marsupials, proliferation, maturation and secretory activity of the uterine endometrium are correlated with underlying changes in oestrogens and progesterone (Hughes & Dodds, 1968; Tyndale-Biscoe & Renfree, 1987). The present study showed that the increase in vaginal cell populations and onset of maturation of epithelial cells were mirrored by rapidly rising faecal oestrogen concentrations during the follicular phase: the karyopyknotic index peaked at copulation and declined during the luteal phase in a progesterone-dominant environment. Our findings are consistent with typical changes in vaginal cytology and sex steroid levels in other dasyurids (Selwood, 1982; Woolley, 1982; Fletcher, 1985; Hinds, 1989; Millis *et al*., 1999; Stead-Richardson *et al*., 2001). This confirms that vaginal cytology is a reliable method for the determination of oestrus in the devil and STQ, although the practicality of the technique is limited compared with the simplicity of monitoring changes in pouch appearance.

This is the first study to qualitatively and quantitatively monitor changes in urogenital opening size and appearance during the oestrous cycle in marsupials. We found that enlargement and hyperaemia of the urogenital opening were related to progression of the oestrous cycle and could be used as an external indicator of the reproductive condition. Again, there are some limitations to this method because morphological changes are very gradual, and accurate assessment requires careful monitoring of individual animals.

Consistent breeding of devils and STQs in captivity has continued to prove a challenge (Williams, 1990; Carnio, 1993; Jackson, 2003*a*), which is of concern given the heightened conservation status and naturally limited reproductive potential of these species (Jones *et al*., 2003). Here we have built on new knowledge of the devil and STQ (Hesterman *et al*., 2008*a*,*b*) to provide a practical method for monitoring reproduction, but caution that parameters such as age at sexual maturity, timing and length of oestrus and duration of inter-oestrus must also be taken into account to reduce erroneous judgement. Further, detailed studies of pouch development and simultaneous changes in sex steroids are warranted, to determine whether correlations could similarly aid in determining the reproductive status in other dasyurid and marsupial species.

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