

Reproductive activity in captive female Honey possums, *Tarsipes rostratus*, assessed by faecal steroid analysis

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Abstract

Hormonal changes associated with reproductive activity in the unique pollen and nectar-feeding marsupial Honey possum, *Tarsipes rostratus*, have been monitored by the measurement of sex steroids excreted via the faeces. From a radio-metabolism study, 63% of administered [¹⁴C]oestradiol was excreted in the faeces and 37% via the urine. Peak levels in the faeces were reached 6 h after injection and by a mean 12 h, 95% of steroid was eliminated. The principal metabolic products of progesterone that were identified by chromatographic analysis were the isomers 5 α - and 5 β -pregnan-3 β -ol-20-one with only trace amounts of progesterone and the isomers 5 β -pregnan-3 β ,20 β - and 20 α -diols. Extended excretory profiles for faecal progestagens (PM) and oestradiol-17 β (E₂) are reported for the first time in a marsupial. The profiles from 4 females held in indoor cages with an artificial photoperiod suggest that long days inhibit reproductive activity in this species, as is the case in a number of other marsupials. One female appeared to resume cycling after a 5-month period and the time between peak levels of both E₂ and PM suggest that the length of the oestrous cycle in the Honey possum is approximately 25 days. The PM profile suggests that the *corpora lutea* secrete low levels of progesterone for approximately the first 19 days after ovulation, followed by increased rates of excretion during the final 6 days.

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1. Introduction

The Honey possum (*Tarsipes rostratus*) is a tiny marsupial (6–12 g) whose diet consists exclusively of pollen and nectar. Its habitat is restricted to the southwestern corner of Australia where adequate supplies of nectar-rich species of Proteaceae, Epacridaceae, and Myrtaceae occur (Wooller et al., 1984). The long period of adaptation to these Gondwanan species (Hopper et al., 1996) has resulted in not only an extreme specialisation of its dentition and tongue (Gervais and Verreaux, 1842; Richardson et al., 1986) but also in a divergence of the Honey possum from all other extant marsupials, such that its systematic affinities are obscure. As a single species in a mono-typic genus, *T. rostratus* is classified as

the sole member of the family Tarsipedidae within the superfamily Petauroidea (Kirsch et al., 1997).

The reproductive physiology of marsupials has been studied in many species and has been extensively reviewed (Tyndale-Biscoe and Renfree, 1987). Most species are polyoestrous with oestradiol and progesterone appearing to play similar roles in the marsupial as in the eutherian mammal. The oestrous cycle of the macropodid marsupials and some dasyurids is unusual in having a long and biphasic secretion of progesterone (Cake et al., 1980; Fletcher, 1989; Hinds and Tyndale-Biscoe, 1982; Walker and Gemmill, 1983) in which it appears that only the first brief output from the corpus luteum is essential for gestation. The final stages of secretion, however, are essential for the birth process (Renfree and Tyndale-Biscoe, 1973; Tyndale-Biscoe, 1963) and when birth is concurrent with the maturation of a follicle and its subsequent ovulation, fertilisation at the post-partum oestrus results in an embryo, or blastocyst, which is held

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in diapause during the period of lactation (Renfree and Shaw, 2000).

Although the life-history and general aspects of reproduction of the Honey possum have been described, (Renfree et al., 1984; Russell and Renfree, 1989), almost nothing is known of its reproductive physiology, except for the occurrence of embryonic diapause (Renfree, 1980). There appears to be some difference, however, in the control of diapause in the Honey possum compared with that in the macropodid marsupials. In the latter, the act of suckling arrests further development of the blastocyst and it remains as a ball of some 80–100 cells until the inhibitory influence of suckling declines. Although the blastocysts remain in diapause during lactation in the Honey possum, the act of suckling per se does not appear to be the principal inhibitory factor (Renfree, 1984). Diapause may continue for some time after lactation ceases, during which time the blastocysts slowly expand to 3–4 mm and contain some 8000–10,000 cells (Oates et al., in press.) This slow growth during diapause appears similar to that described in the pygmy possum (Ward, 1990) and the feathertail glider (*Acrobates pygmaeus*) (Ward and Renfree, 1988).

There may be some similarity, however, in the seasonal control over diapause between macropodids and the Honey possum. In the tammar wallaby (*Macropus eugenii*), the period of embryonic diapause is extended beyond the cessation of lactation and into the seasonal anoestrous period of this species during October to December. Towards the end of December, during the southern summer solstice, the single blastocyst spontaneously resumes development (Sadlier and Tyndale-Biscoe, 1977) and birth occurs 11 months after fertilisation. Female Honey possums, all housed under identical conditions in a breeding colony, had blastocysts conceived at different times, but all females gave birth simultaneously in mid-February, 1998 (Bradshaw et al., 2000). As a result, the length of the gestation period in these animals ranged between 3 and 5 months and was not highly predictable, in contrast to all other known marsupials.

The limitations imposed by the size and fragile nature of the Honey possum make it an extremely difficult subject to investigate physiological aspects of its reproduction. These investigations involve such traditional methods as the measurement and modulation of hormonal levels in the plasma throughout the various reproductive conditions, as well as surgical intervention. Also, given that reproductive cycles, both pregnant and non-pregnant, cannot be initiated in the Honey possum by removal of the pouch young, other measures are required to establish the beginning of the reproductive cycle. Vaginal swabbing has not been attempted because of its small size and its susceptibility to the stress of handling. The Honey possum is therefore an ideal subject for faecal steroid monitoring.

Faecal steroid analysis is a valuable method for characterising the female ovarian cycle (Schwarzenberger et al., 1997), and has been used widely in the case of rare mammals (Schwarzenberger et al., 1998; Spanner et al., 1998; Wasser, 1996; Wasser et al., 1996) where the stress of capture and venepuncture are ill advised. Oestrogens, including oestradiol-17 β , excreted in the faeces, are for the most part in an un-conjugated form, whereas progesterone is metabolised by the liver and is present in the faeces predominantly as a series of 5 α - or 5 β -reduced pregnanes (pregnanediones, mono- and di-hydroxylated pregnanes). Although the concentration of these steroids in the faeces is an indirect measure of their concentration in the plasma, a single faecal sample represents a pooled plasma fraction and may provide a more integrated measure of the hormonal status of the animal (Goyman et al., 1999). Increases in the concentration of faecal oestradiol-17 β may, or may not, be interpreted as follicular activity, whereas increases in progestagens (Schwarzenberger et al., 1996) should provide information on functional *corpora lutea*.

This preliminary analysis of faecal progestagens is one of the first reported for a marsupial (Curnow et al., 2001; Paris et al., 2002). Together with the pattern of excretion of faecal oestrogen in captive animals throughout the year, the data suggest that Honey possums exhibit ovarian cyclicity that ceases when the animal is in a confined space or exposed to an unfavourable light régime.

2. Materials and methods

2.1. Animals

Four female Honey possums were captured from Scott National Park (34°17' S, 115°13' E), in southwestern Australia, and transferred to the Zoology Department, University of Western Australia. Faeces were collected periodically over three years and Table 1 summarises the previous conditions of each animal. Female number 20 was captured 18th June 1999 and held in an outdoor colony in semi-natural conditions (Bradshaw et al., 2000) in the presence of males. She produced young early in December, which were weaned by March and, as no further young were produced, she entered the experiment on 25th April 2000 with blastocysts aged approximately 5 months. Females number 21 and number 48 were caught 25th July 2001 and placed directly in a controlled temperature room (CTR) before faecal collection commenced 5th August. Female number 3 was captured 18th February 2002 and transferred to the outdoor colony (with males) until faecal collection commenced 1 May 2002 in the CTR. Female number 21 spent 4 months (January–April) in the outdoor colony before a second period of faecal collection commenced 1

Table 1

Experimental conditions of four female Honey possums (*Tarsipes rostratus*) held in captivity for daily collection of faeces

Animal ID BW (g)	Prior location (field or semi-natural)	Date commenced light:dark	CTR light:dark	Light phase transition ^a	Reproductive history
20 (9.4)	Semi-natural	25 April 2000, 11:13	10 h:14 h	S to S	Post-partum oestrus near 7 December 1999. Weaned PY by 1 March. NPY in April.
3 (11.7)	Field	3 May 2002, 10.45:13.15	12 h:12 h	S to L	NPY on capture
21 (2001) (11.9)	Field	5 August 2001 10.30:13.30	12 h:12 h	L to L	NPY on capture
21 (2002) (11.8)	Semi-natural	1 May 2002, 10.53:13.07	12 h:12 h	S to L	Housed with males January–May. Pouch inspected monthly, NPY.
48 (16.8)	Field	5 August 2001, 10.30:13.30	12 h:12 h	L to L	NPY on capture

^a Light phase transition is based on the southern winter solstice occurring between 15 and 25 June, when light:dark phase remains at 10.04:13.56. S, shortening light phase; L, lengthening light phase. NPY denotes the absence of pouch young.

May 2002. The conditions in the CTR did not include a substitute for natural sunlight (see Table 1 for day-length control) and temperature was maintained at 22 °C day and 15 °C night. Each animal was housed separately in a Perspex cage, 160 cm × 80 cm × 100 cm, containing plant cover and provided with 10 ml/day of an artificial diet (Bradshaw et al., 2000) that has supported the gestation and rearing of young. They were weighed weekly and their pouches were checked with an otoscope each month for the duration of the experiment.

Although the presence of pouch young (PY) can be determined, there are no other external signs that identify a particular stage of reproduction. Thus, we have assumed that each female on capture, in the absence of pouch young (NPY), will be carrying blastocysts, rather than ova, although this may not always have been the case. There were no visible PY during the period of collection, either in or discarded from the pouch (although this may not be discounted as the new-born young are only a few millimetres in length). Neither were there any young evident in the pouch for one month after the transfer of the possums to the outside yard.

2.2. Faecal collection and validation of extraction

Faeces were collected each day and stored immediately at –15 °C. Daily collection from cages ensured accurate identification of each sample, which was approximately one-half the amount excreted in 24 h (Bradshaw and Bradshaw, 2001).

Extraction of steroids from a faecal pool (≈1 g combined faecal samples from females, homogenised in 2.5 ml buffer, dried to constant weight at 45 °C, and stored at –15 °C as faecal powder) compared the efficiency of diethyl ether with that of 90% ethanol. Thirty milligrams homogenised faecal powder, representing the dried weight of the daily sample, was vortexed in buffer together with either 167 Bq [³H]progesterone or 167 Bq [³H]oestradiol-17β. For extraction with di-ethyl ether, 2.5 ml were added and the tubes vor-

texed for 1 min, allowed to settle, and the supernatant transferred to a scintillation vial. The process was repeated and the combined supernatants were dried and counted for radioactivity in 3 ml scintillation fluid (Ultima Gold, Packard Technologies, USA) in a Packard Tri-Carb 2300TR Liquid Scintillation Analyser with automatic external standardisation. For the ethanolic extraction, 2.5 ml 90% ethanol (allowing for the volume of aqueous in the homogenate) were added to a similarly spiked series of faecal samples, which were maintained in a water bath at 79 °C for 20 min. After centrifugation, the supernatant was removed to a scintillation vial, the extraction process repeated and the combined supernatants dried and counted. After ether extraction, the recoveries of [³H]progesterone and [³H]oestradiol-17β were 95.4% ± 1.3 (SEM) and 87.8% ± 2.8; and after ethanolic extraction, were 96 ± 1.8% and 95.2 ± 0.6%, respectively. Ethanol (90%) was the preferred solvent because of its high efficiency in extracting both steroids, its established ability to extract water-soluble progesterone metabolites (Wasser, 1996), and the low variation between samples.

2.3. Time course of steroid excretion and proportion of conjugated to un-conjugated steroid in faeces and urine

Two microcurie of [4-¹⁴C]oestradiol (74 kBq) in 0.13 ml sterile saline was injected i.p. into 2 non-lactating female Honey possums maintained in custom-designed metabolism cages (Bradshaw and Bradshaw, 2001) in the CTR. Faeces and urine were collected quantitatively at 2-hourly intervals for 20 h after injection and at 4-hourly intervals for the next 2 days. Urine samples (20 μl) were counted directly for radioactivity. Faecal samples were extracted in 10 volumes of ethanol and 50 μl aliquots were dried and counted for radioactivity. The recovery of radioactivity in the urine and faeces is expressed as a percentage of the total administered radioactivity, after correction for the residual in the injection syringes.

The proportion of ether-soluble to insoluble steroid was assessed by ether–water extraction (Bahr et al., 2000). For urine, buffered aliquots (50 μ l) were extracted with 3 ml di-ethyl ether with a 5 min vortex. The aqueous phase was snap frozen, the ether phase decanted, evaporated to dryness, reconstituted in 150 μ l ethanol, and counted for radioactivity to estimate the amount of unconjugated steroid. The aqueous phase was hydrolysed by incubating overnight at 37 °C with β -glucuronidase (Sigma–Aldrich, St Louis, USA) in PBS buffer (pH 6.8). De-conjugated steroid was then extracted into 10 ml di-ethyl ether with a 5-min vortex and radioactivity counted. The residual radioactivity was assumed to represent that proportion of [¹⁴C]oestradiol in a sulphated, or non-hydrolysable, form. The efficiency of combined hydrolysis and extraction was assessed by subjecting duplicate aliquots of non-labelled urine spiked with [³H]oestradiol or ³H-oestrone glucuronide. The extraction recovery of [³H]oestradiol was 91% and the combined hydrolysis and extraction efficiency of ³H-oestrone glucuronide was 63.5%.

For faeces, aqueous samples (50 mg) were homogenised and extracted 4 \times with 2 ml di-ethyl ether and a 5 min vortex (validated extraction efficiency of >90%). The ether phase was decanted, pooled, dried, reconstituted in 150 μ l ethanol, and counted for radioactivity to estimate the amount of un-conjugated steroid. Following the ether extraction, the faecal samples were dissolved in Dimilume-30 (Packard United Technologies) and counted; radioactivity remaining in the faeces was assumed to be conjugated. Relative proportions of conjugated to un-conjugated steroid were calculated relative to the combined total radioactivity counted in the ether extract and that remaining in the faeces.

2.4. Preparation of faeces for GC/MS analysis

Approximately 2 g of dried female faeces were extracted with 15 ml 90% ethanol and boiled for 20 min. In an attempt to reduce interference from pollen pigments, the dried extract was partially purified by adsorption chromatography on alumina (Raeside, 1963). Briefly, the extract was re-dissolved in 2 ml ether and partitioned with 0.5 ml d. water. The ether fraction was passed through a mini-column containing 1 mg activated Neutral Alumina (Woelm TLC Alumina N, from Alltech, Illinois, USA) equilibrated in ether: absolute ethanol (98:2) and the eluate (2 ml) was dried down for derivatisation.

2.5. Preparation of reference steroids and derivatisation for GC/MS analysis

Most steroids were obtained commercially (chromatographically pure progesterone from E. Merck, Darmstadt; chromatographically pure 17 β -oestradiol, 5 α - and

5 β -pregnan-3,20-dione, 5 α - and 5 β -isomers of pregnan-3 α - and 3 β -ol-20-one, from Sigma Chemical, St Louis, MO), but the di-hydroxylated pregnane series were derived from these stocks by a reduction process (Kirk and Hartshorn, 1968), which invariably results in products rich in the 20 β -isomer. Briefly, approximately 1 mg of each mono-hydroxylated pregnane was dissolved in 0.5 ml ethanol and 1 mg sodium borohydride added. After stirring overnight, and mixing with 100 μ l acetone for 10 min to destroy the remainder of the reagent, the reaction mixture was dried down, re-dissolved in 200 μ l dichloromethane, and washed with 200 μ l water. The bottom layer was removed and dried over anhydrous sodium sulphate. Reduction of the 5 β -pregnan-3 β -ol-20-one gave two isomeric diols in a ratio of 4.17:1 (analysed by GC/MS). The major isomer was assigned 5 β -pregnan-3 β ,20 β -diol and the minor isomer as 5 β -pregnan-3 β ,20 α -diol. Reduction of 5 α -pregnan-3 β -ol-20-one gave two isomeric diols in a ratio of 6.81:1 (analysed by GC/MS). The major isomer was assigned 5 α -pregnan-3 β ,20 β -diol and the minor isomer as 5 α -pregnan-3 β ,20 α -diol. Further evidence was obtained by comparison of the latter result to a standard of 5 α -pregnan-3 β ,20 α -diol (Sigma Chemical, St Louis, MO), which confirmed the identity of the minor component of the reduction mixture. The 4 remaining isomeric diols, 5 β -pregnan-3 α ,20 α -diol, 5 β -pregnan-3 α ,20 β -diol, 5 α -pregnan-3 α ,20 β -diol, and 5 α -pregnan-3 α ,20 α -diol were obtained from 5 α - and 5 β -pregnan-3,20-dione using similar reduction processes.

All compounds and purified faecal extract were derivatised by trimethylsilylation as follows: Using silylated glassware throughout, 50 μ l of 10% pyridine (Sigma–Aldrich) solution of methoxylamine hydrochloride (Sigma–Aldrich) were added to the dried faecal eluate, 1 mg of each steroid, or its reduced metabolite, and heated to 60 °C for 1 h. After drying, the mixture was re-dissolved in 100 μ l *N*-trimethylsilylimidazole (TMSIM): *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Alltech Associates, Baulkham Hills, NSW, Australia) (3:1) and heated at 100 °C for 2 h. Using Pasteur pipettes containing 3 ml Lipidex-5000 (hydroxyalkoxypropyl Sephadex from Packard Bioscience, Groningen, Netherlands), the mixture was chromatographed, eluting with *n*-heptane. The initial 2 ml eluate was collected, dried down and reconstituted in 100 μ l *n*-heptane for chromatography.

2.6. Chromatographic analysis of faeces

GC/MS analysis utilised a Micromass AutoSpec equipped with a Hewlett–Packard 5890 series II gas chromatograph. The GC operating in the splitless mode was fitted with HP-1 column (0.25 \times 30 m; Film 0.25 μ Hewlett–Packard) using helium (75 kPa) as carrier gas. The injection port was heated to 250 °C and the oven programmed with an initial temperature of 40 °C held at 2 min followed by heating at 50 °C min⁻¹ to a tempera-

ture of 200 °C. This temperature was held for 1 min followed by heating at 2 °C min⁻¹ to 230 °C, then immediately heated at 10 °C min⁻¹ to the final temperature of 280 °C and held for 10 min. The transfer line from the GC to the mass spectrometer was set at 250 °C and the mass spectrometer operated in the EI mode at 70 eV with an ion source temperature of 200 °C. For the low-resolution work, the instrument was scanned from mass 750 to 50 at 1 s/decade with a resolution of 1000.

The low-resolution single-ion resonance (SIR) determination of progesterone, as the bis-*O*-methyloxime, detected the ions 372.3 and 341.3, and SIR determination of mono-hydroxylated pregnanes, as 3-trimethylsilyl-20-*O*-methyloxime, detected the ions 298.3 and 388.3. The interference of co-eluting faecal contaminants required a more sensitive method for the detection of pregnanediols. For the high-resolution SIR work, the instrument was tuned to a resolution of 10,000 using perfluorokerosene and the ions 269.230069, 118.0769, and 117.0736 were detected.

2.7. Radioimmunoassay

2.7.1. Extraction of reproductive steroids from faeces

Faecal samples were either freeze-dried or placed in a constant temperature oven (Memmert) at 45 °C for 24 h and dried to constant weight. Each sample was examined microscopically (4×) for sand or plant particles (which were discarded), then pulverised on a glass slide. Thirty milligrams of dried, mixed faeces weighed into a glass-stoppered extraction tube, were vortexed with 0.15 ml borate buffer (0.1 M, pH 8). After addition of 1.35 ml absolute ethanol, each sample was vortexed and placed in a water bath maintained at 79 °C and boiled for 20 min. The samples were centrifuged and the supernatant carefully removed to an assay tube. The extraction process was repeated with the addition of 1.5 ml 90% ethanol. The combined supernatants were dried down (at 37 °C under compressed air), re-dissolved in 0.5 ml assay buffer and stored at -15 °C for assay.

2.7.2. Antisera

Oestradiol-17β antiserum (E26-47, Endocrine Sciences Laboratories, CA, USA) has the following cross-reactivity (relative to 100% binding of 17β-oestradiol): 1.3% with oestrone, 0.6% with oestriol, 0.2% with 16-keto-oestriol, <0.1% with 16β-oestradiol, 17α-oestradiol and dehydroepiandrosterone, <0.05 % with 19-nortestosterone, testosterone, 17α-oestriol, estrone sulfate, cortisol, cortisone, progesterone, 17-hydroxyprogesterone, pregnanetriol, 5α- and 5β-pregnanedione, 11-desoxycortisol, androstenedione, and corticosterone.

Progesterone antiserum (Coat-A-Count Progesterone from Diagnostic Products, CA, USA) has the following cross-reactivity (relative to 100% binding with progesterone): 43.8% with 5α-pregnan-3β-ol-20-one, 31% with 5β-

pregnan-3α-ol-20-one, 23.3% with 5β-pregnan-3β-ol-20-one, 16% with 5α-pregnan-3α-ol-20-one, 9% with 5α-pregnan-3,20-dione, 3.5% with 5β-pregnan-3β,20α-diol, 3.4% with 17α-hydroxy-progesterone, 3.2% with 5β-pregnan-3,20-dione, 0.2% with 20α-dihydroprogesterone, and <1% with 5α-pregnan-3α,20α-diol, 5α-pregnan-3β,20α-diol, 17α-hydroxyprogesterone, oestra diol-17β, and testosterone (kit literature) (Wasser et al., 1996).

2.7.3. Oestradiol-17β (E₂)

Aliquots (100 μl) of buffered faecal extracts, together with a series of dried ethanolic standards (chromatographically pure E₂ from Sigma–Aldrich, St Louis, USA), were incubated overnight at room temperature (22 °C) with 1:200 dilution of antiserum in a 0.05 mol borate buffer (pH 8.0) containing 167 Bq [2,4,6,7-³H]oestradiol-17β (Amersham, UK), 0.05 % bovine γ-globulin (Cohn Fraction II and III, Sigma–Aldrich, MO, USA), and 0.2% bovine albumin (Fraction V, Sigma–Aldrich). Samples and standards were then incubated at 0 °C for 10 min with a suspension containing 5 mg acid-washed Norit A charcoal (ICN Pharmaceuticals, Ohio, US) and 0.5 mg dextran T500 (Pharmacia Chemicals, Uppsala, Sweden) in 1 ml buffer. After centrifugation for 10 min at 4000 rpm and 0 °C, an aliquot of the supernatant was counted for radioactivity to less than 1% error and standards data were transformed using a 4-parameter logistic equation.

2.7.4. Progestagens

Progesterone and progesterone metabolites (PM) were measured using the kit, Coat-A-Count Progesterone (Diagnostic Products, CA, USA), that utilises a solid-phase ¹²⁵I radioimmunoassay with antibody-coated tubes. Briefly, 100 μl faecal extract, together with 100 μl each of a series of standards, were incubated with 1 kBq ¹²⁵I for 3 h at room temperature. Tubes were decanted and measured for γ-radiation in a Prias gamma counter (PGD Auto-Gamma, Packard Instruments, Meriden, CT, USA). Radioactivity bound was analysed using a logit-log plot.

2.8. Validation

For PM assays, all samples of individual animals were measured in a single assay, and the inter-assay variation averaged 9.3 ± 2.3% (n = 5). Serial dilutions of an ethanolic extract of Honey possum faeces showed parallelism with the standard curve for PM, using progesterone as a standard (Fig. 1A). For E₂ assays, intra-assay variation was 9.8%, and inter-assay variation was 12.6% (n = 4). Buffered extracts of Honey possum faeces were assayed for E₂ over a wide range of dilutions from 1/1 to 1/64 and a linear relationship was observed with dilutions increasing from 1/8 to 1/64. Serial additions of E₂ at a 1/8 dilution of the extract were parallel with the standard curve (Fig. 1B).

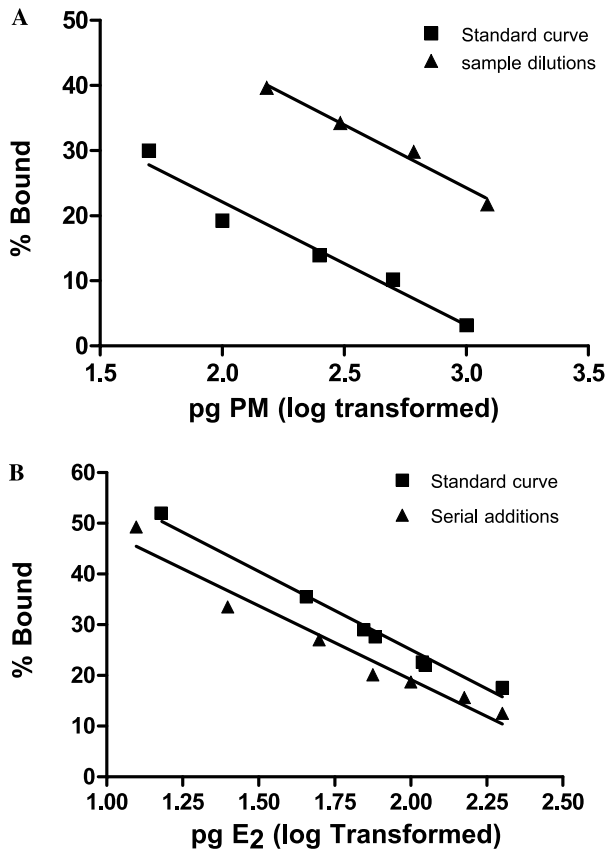


Fig. 1. (A) Serial dilutions of an ethanolic extract of Honey possum faeces showing parallelism with the standard curve for PM. (B) Parallel response with serial additions of E₂ to a 1/8 dilution of faecal extract.

2.9. Analysis of hormonal profiles

Individual hormonal profiles were analysed according to the following criteria.

(a) *Comparison with mean hormone levels in male faeces.* One male was confined to a cage of similar dimensions to those of the females, but located in semi-natural conditions (as above) and daily samples were collected during 60 days and assayed together with the female samples (Table 2). We assume that both oestrogen and progesterone have constitutive roles in both

males and females, but the additional progestational role of P in females would be reflected in differing levels and patterns of secretion of this hormone to that seen in the male. Differences between male and female faeces were analysed using the Kruskal–Wallis non-parametric analysis of variance (ANOVA) and an unpaired two-tailed *t* test with Welch correction, as SDs differed significantly.

(b) *The degree of correlation between PM and E₂.* This was assessed by linear regression analysis and provided an indication of the secretory nature of the ovary. If the levels are highly correlated, we would interpret this as a single tissue being the source of their secretion; whereas a lack of correlation would indicate a more complex, or separate origin of P from that of E₂.

(c) *Estimation of baseline levels and identification of peaks of faecal PM and E₂.* This estimation used an iterative process (Graham et al., 2002), in which the average concentration of all samples in each profile was calculated and values that were greater than 1.75 SD above the mean were considered significant and removed from the series. The process was repeated until no values were greater than 1.75 SD above the mean, and the mean of the remaining values was recorded as baseline (Table 3). Peaks that were >5 SD above baseline were considered significant for E₂, and >4 SD above baseline as significant for PM.

3. Results

3.1. Time-course of steroid excretion in faeces and urine

The total recovery of injected [¹⁴C]oestradiol (in both faeces and urine) averaged 88.4% after 56 h of collection. Of the recovered radioactivity, 63% of the steroid was excreted in the faeces and the remaining 37% was eliminated in the urine.

Maximum levels of radioactivity in the faeces of both animals were reached by 6 h after injection and a mean 95% of the total was eliminated within the first 12 h post-injection (see Fig. 2). The passage of radioactivity in the urine was less rapid, with peak levels not occurring until 8 h after injection, and only 85.2% of the radioactivity

Table 2 Comparison of progestagens (PM) (ng g⁻¹) and oestradiol-17β (E₂) (ng g⁻¹) in the faeces of four female Honey possums with one male

	Male	Female				
		3	20	21 (2001)	21 (2002)	48
	23 September 2002– 19 November 2002	6 May 2001– 31 October 2001	28 April 2000– 20 November 2000	13 August 2001– 31 December 2001	3 May 2002– 31 October 2002	13 August 2001– 9 April 2001
E ₂	14.43 ± 0.79 (9)	14.62 ± 0.51 (65)	14.52 ± 0.66 (63)	23.38 ± 0.71 (80)	20.3 ± 1.0 (65)	14.6 ± 0.4 (76)
Comparison with male	—	NS	NS	<i>P</i> < 0.0001	<i>P</i> < 0.01	NS
PM	146.96 ± 14.57 (23)	188.29 ± 12.70 (65)	195.06 ± 11.64 (63)	332.54 ± 20.64 (80)	310.86 ± 16.11 (65)	142.64 ± 12.53 (76)
Comparison with male	—	<i>P</i> < 0.05	<i>P</i> = 0.01	<i>P</i> < 0.0001	<i>P</i> < 0.0001	NS

Data, expressed are mean values ± SE, (*n*) is the number of values. *P* is the degree of significance with *P* > 0.05; NS, not significant.

Table 3

Baseline concentrations and degree of correlation between faecal progestagens (PM) and oestradiol-17 β (E₂) in four female Honey possums throughout their period of faecal monitoring

Animal ID	Coefficient of determination	Tissue source	Baseline level of PM	Baseline level of E ₂	Reproductive activity
20	$r^2 = 0.05$ NS	Complex	164.84 \pm 7.25 (43)	11.76 \pm 0.14 (54)	Commencing oestrous cycles
21 (2001)	$r_2 = 0.08$ $P = 0.012$	Single	232.91 \pm 16.30 (25)	21.56 \pm 0.61 (27)	Declining
21 (2002)	$r_2 = 0.53$ $P < 0.0001$	Single	296.55 \pm 15.34(52)	16.95 \pm 0.54 (49)	Declining
3	$r_2 = 0.47$ $P < 0.0001$	Single	156.16 \pm 9.57 (31)	11.48 \pm 0.33 (29)	Declining
48	$r_2 = 0.34$ $P < 0.0001$	Single	102.64 \pm 3.74 (49)	13.62 \pm 0.39 (49)	Suppressed

Data are expressed as mean values \pm SE, (n) is the number of values.

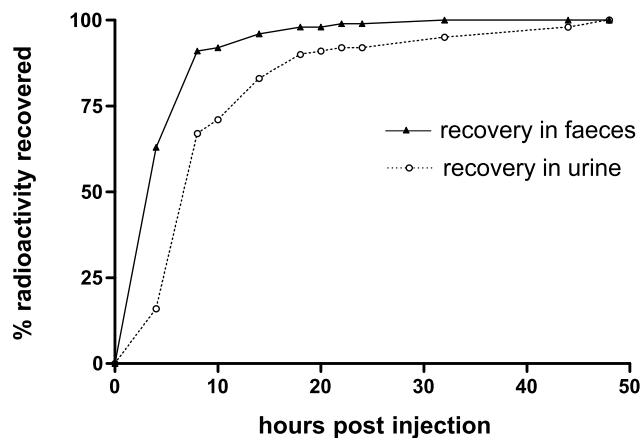


Fig. 2. Time-course of metabolism and excretion of administered [¹⁴C]oestradiol in the faeces of the Honey possum (*Tarsipes rostratus*).

was recovered by 12 h after injection. There was also greater variation between the two animals regarding rate of excretion in the urine. Ninety percent of the total radioactivity was excreted within 12 h in Animal number 1, but 20 h elapsed in Animal number 2 before a similar level of radioactivity was excreted.

3.2. Proportion of conjugated to unconjugated radiolabelled steroid excreted in faeces and urine

Based on the partitioning of radioactivity following differential ether extraction, and deconjugation of the aqueous phase, 39.7 \pm 7.1% of E₂ was excreted into the urine as conjugated glucuronides, 51.1 \pm 13.7% in a sulphated or non-hydrolysable form and 2.6 \pm 0.2% was unconjugated. Differential extraction of faeces with diethyl ether indicated that 80.3% of injected radiolabelled E₂ was in a conjugated form, and only 19.7% was unconjugated.

3.2.1. GC/MS identification of faecal steroids

A significant amount of pollen products remained after purification of the faecal extract, which made the detection of metabolites difficult. The results represent the combined findings from three different faecal pools, not all of which had the same compounds. Progesterone was present in trace amounts only, and the metabolites,

5 α - and 5 β -pregnan-3,20-dione, were not detected at all. There were significant quantities, however, of the mono-hydroxylated metabolites, 5 β -pregnan-3 β -ol-20-one as well as its 5 α -isomer. The investigation of all 8 isomers of the di-hydroxylated metabolites identified the presence of only trace amounts of 5 β -pregnan-3 β ,20 α -diol and its 20 β -diol isomer. E₂ could not be identified at the lowest level of detection.

3.3. Profiles of faecal steroids and analysis

Levels of both PM and E₂ excreted by female number 20 (Fig. 3) were not correlated, and during the first 90 days in the CTR, production was irregular and episodic, with one significant spike of E₂ on Day 27 and 3 peaks of PM on Days 10, 16, and 64. During the first week in August, however, three spikes of E₂ were measured (on Days 94, 98, and 103) that were > 10 SD above baseline levels. These were followed by peaks > 5 SD above the baseline on Days 113, 121, 136, 161, and 184, with the last 3 peaks occurring 25 and 23 days apart. Associated with these last three peaks in E₂ were three significant peaks of PM excreted on Days 133, 161, and 184, with the intervals between them being 28 and 23 days. A mean of these four intervals is 24.75 \pm 1.02 days which we have taken as the best estimate of the oestrous cycle in this species.

In contrast, levels of PM and E₂ in the faeces of the other three females were highly correlated (Table 3). Female number 3 (Fig. 4) maintained the fluctuating levels of E₂ throughout the 6 months of monitoring, with significant peaks on Days 14, 17, 35, 37, 108, 114, and 160, showing no regular interval between peaks. Peak levels of PM however, were reached only on Days 14 and 17, with the peak on Day 37 reaching 3.7 SD above baseline, and from then on, levels gradually declined. Female number 48 (Fig. 5) also maintained constant, but fluctuating, levels of E₂ throughout 8 months, with only one significant peak on Day 210. PM levels in this female were the lowest measured for all animals, but there were significant peaks on Days 21, 27, 47, 60, 70, 74, 154, 172, and 210. Female number 21 showed two distinct patterns of secretion in her two years of monitoring. In 2001 (Fig. 6), levels of both PM and E₂ were not only the highest of all the females, but also fluctuated more

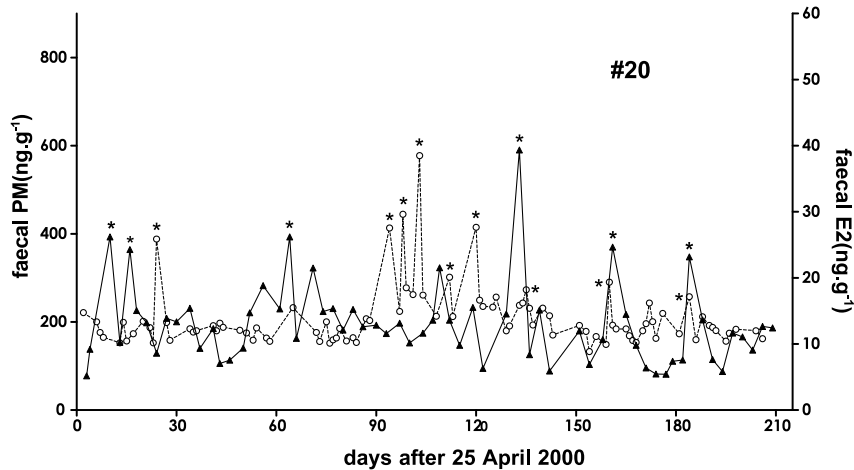


Fig. 3. Concentrations of progestagens (PM) (\blacktriangle) and oestradiol-17 β (E_2) (\circ) in ng g^{-1} excreted in the faeces of the female Honey possum number 20. * Denotes level of significance >4 SD above baseline levels for PM and >5 SD above baseline for E_2 .

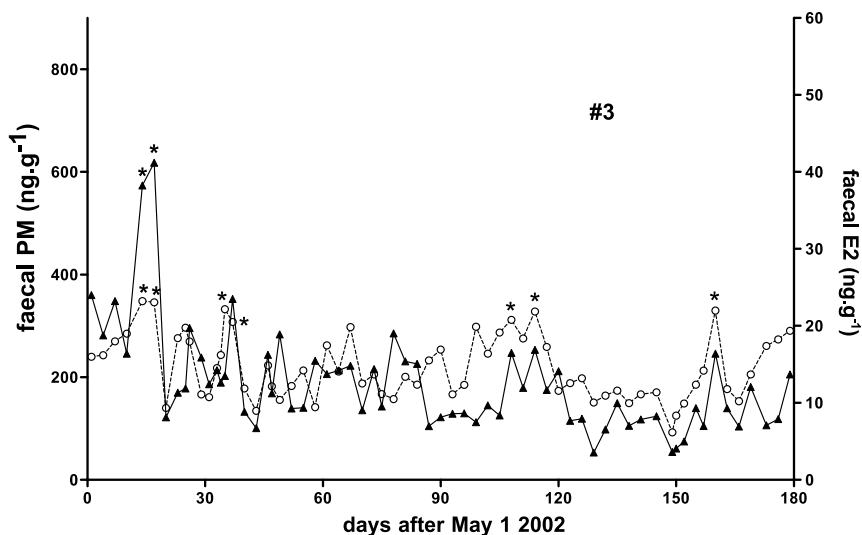


Fig. 4. Faecal levels of progestagens (PM) (\blacktriangle) and oestradiol-17 β (E_2) (\circ) in ng g^{-1} in the female Honey possum number 3. * Denotes level of significance >4 SD above baseline levels for PM and >5 SD above baseline for E_2 .

widely. Only one peak level of E_2 was recorded on Day 39, but significant PM levels occurred on Days 26, 29, 51, 63, 78, 105, 116, and 125. On re-entering the CTR in 2002, this female again exhibited similar wide fluctuations in hormone excretion (Fig. 7) with significant peaks of both PM and E_2 occurring simultaneously on Days 5 and 25. From then onwards, fluctuations diminished, and only two randomly spaced significant peaks of E_2 were recorded on Days 76 and 111.

4. Discussion

4.1. Characterisation and excretion of progesterone metabolites

The chemical characterisation, using GC/MS, of PM excreted in the faeces of the Honey possum gives us

some confidence in interpreting the profiles recorded from the different females. The aim of this part of the study was to confirm the presence of 5α - or 5β -reduced pregnanes that are the principal metabolites of progesterone excreted in mammalian faeces (Schwarzenberger et al., 1997). In common with most other mammals investigated, progesterone is excreted in trace amounts only (Hindle and Hodges, 1990; Thompson et al., 1998; Wasser et al., 1994) and its metabolism appears to have involved reduction at the C-3 position, with further reduction of the 5β -monohydroxylated pregnane to the 20α - and 20β -diols. E_2 was not identified in the chromatographic analysis. This probably reflects the fact that the radio-infusion study showed less than 20% of this steroid is excreted unconjugated in the faeces, thus levels of E_2 in the extract may have been below the limit of detection of the GC/MS. It is not unusual for mammals to excrete significant amounts of E_2 in a

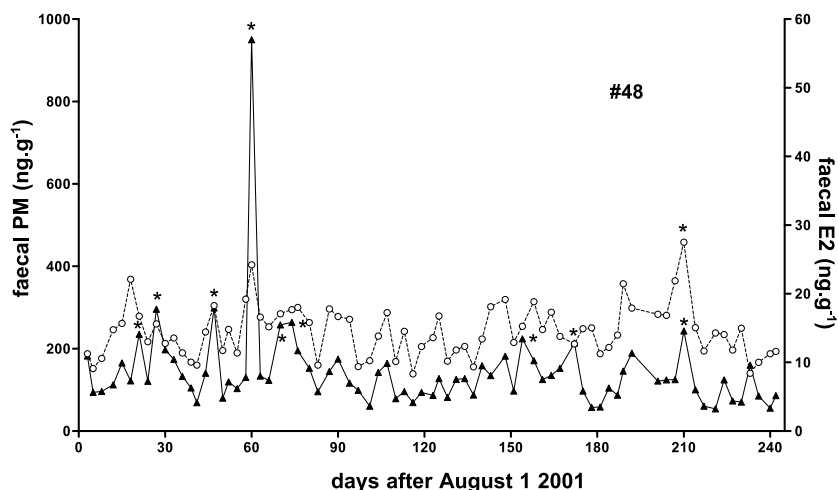


Fig. 5. Faecal levels of progesterone (PM) (\blacktriangle) and oestradiol-17 β (E_2) (\circ) in ng g^{-1} in the female Honey possum number 48. * Denotes level of significance > 4 SD above baseline levels for PM and > 5 SD above baseline for E_2 .

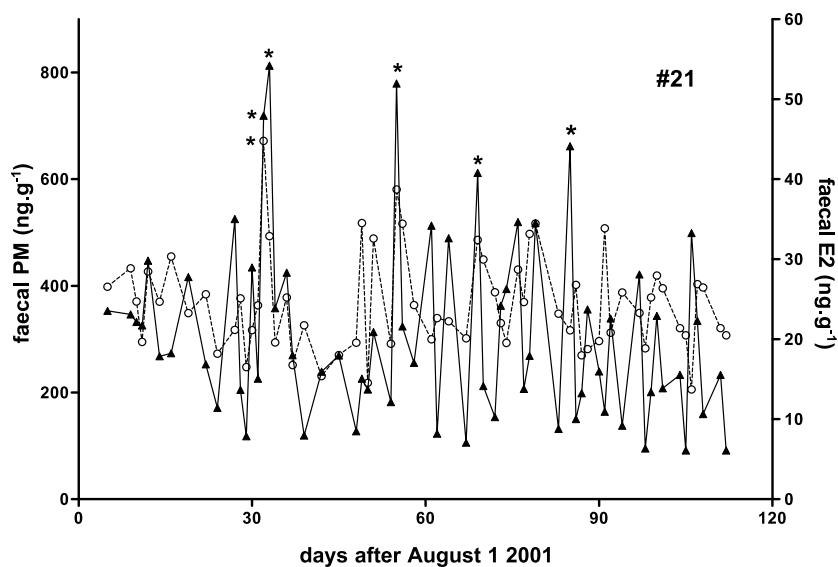


Fig. 6. Faecal levels of progesterone (PM) (\blacktriangle) and oestradiol-17 β (E_2) (\circ) in ng g^{-1} in the female Honey possum number 21 during 2001. * Denotes level of significance > 4 SD above baseline levels for PM and > 5 SD above baseline for E_2 .

conjugated form (Brown et al., 1994; Shille et al., 1990; Wasser et al., 1996; Ziegler et al., 1989) and the cotton-top tamarin excretes only 11% of E_2 as unconjugated (Ziegler et al., 1989). In the case of the Honey possum, the low proportion of E_2 excreted in the unconjugated form may be the result of a comparatively rapid transit through the intestine. Maximum levels of radioactivity appeared in the faeces of both animals 6 h after injection and 95% of the total was eliminated between 12 and 20 h post-injection. Richardson et al. (1986) demonstrated that the passage of pollen grains through the gut of the Honey possum was maximal at 6 h after ingestion, mostly voided after 12 h, and totally voided after 18 h. The results of the radio-infusion study are a close match with these pollen data and suggest that the faecal hormones reflect circulating levels from the previous day.

4.2. Steroid profiles

Stead-Richardson et al. (2001) first presented data on E_2 profiles in a dasyurid marsupial, the chuditch (*Dasyurus geoffroii*). Apart from a preliminary study of progesterone metabolites in the Greater bilby (*Macrotis lagotis*) (Curnow et al., 2001) and longer-term monitoring of PM in the southern hairy-nosed wombat (*Lasiornhinus latifrons*) (Paris et al., 2002), the current paper is the first to document longitudinal profiles of both PM and E_2 excretion in a marsupial.

The profiles differ in a fundamental way between individuals as demonstrated by the regression analysis. In the majority of cases (e.g., numbers 3, 21, and 48), the profiles for E_2 and PM are highly correlated, suggesting that the secretion of the two steroids is from a common source. We consider this to be of ovarian origin, which

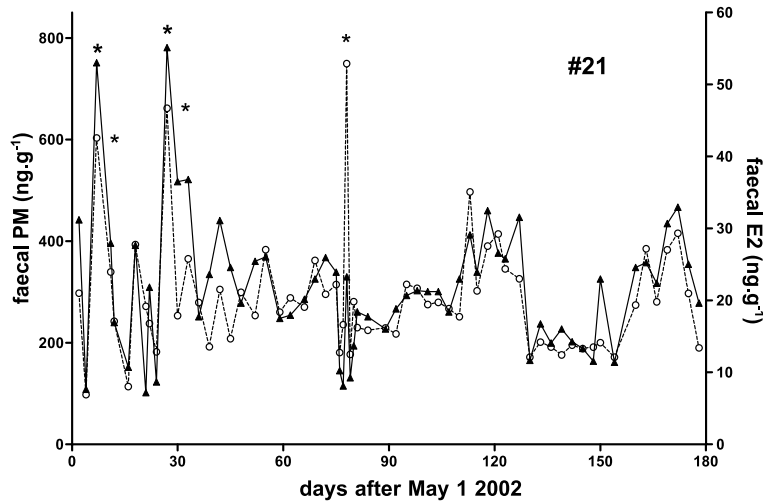


Fig. 7. Faecal levels of progesterone (PM) (\blacktriangle) and oestradiol-17 β (E_2) (\circ) in $ng\ g^{-1}$ in the female Honey possum number 21 during 2002. * Denotes level of significance >4 SD above baseline levels for PM and >5 SD above baseline for E_2 .

would include the *corpora lutea* of diapause, demonstrated to be capable of secreting E_2 in the quokka, *Setonix brachyurus* (Bradshaw and Bradshaw, 1992). From the faecal steroid profiles of these animals, secretion is episodic and constitutes what appears to be the basal constitutive secretion needed for the slowly expanding blastocysts.

The statistical independence of E_2 from PM secretion in female number 20 clearly indicates a different reproductive condition in this animal. This female gave birth to young in our colony when it had access to males (Table 1). It would have experienced a post-partum oestrus and mated again, resulting in blastocysts that remained in diapause during lactation. Her young were weaned by the beginning of March 2000, and the blastocysts would have been 5 months old at the commencement of faecal collection. Although we have evidence that blastocysts remain viable for 5 months in females in the outdoor colony (Bradshaw et al., 2000), the lack of correlation between E_2 and PM levels during the first four months in the CTR may have some significance with regard to the viability of the blastocysts. We suggest that blastocysts, most probably conceived at birth, may not have survived during these early months in the CTR when the secretion of E_2 and PM were not correlated. After 3 months in the CTR, female number 20 experienced a series of three bursts of E_2 secretion, which appear to have heralded the resumption of oestrous cyclicity. The three significant peaks in PM that follow, are 28 and 23 days apart and are of a mean 5.5 days in duration. With the understanding that the Honey possum experiences a post-partum oestrus, we would expect an ovulation to occur approximately 25 days prior to the end of each peak of PM. There are significant peaks in E_2 , 25 and 23 days apart, that are associated with each peak of PM, together with a peak on Day 113 that may be associated with the first ovulation. A significant peak

of E_2 also occurs on Day 121 that is only 15 days before the next proposed ovulation. A short first cycle in seasonally reproducing mammals is not uncommon (Legan et al., 1985; Morrow et al., 1999) and the length of the first reproductive cycle in this animal remains unclear. During the last three months in the CTR, however, this female appears to have experienced three oestrous cycles with an approximate interval of 25 days. The secretion of progesterone from the *corpora lutea* appears to be low for the first 18 days after their formation, and then rises to peak levels during the final 5–6 days of their life span. This profile of progesterone secretion is common to several marsupial species (Cake et al., 1980; Fletcher, 1989; Hinds and Selwood, 1990; Hinds and Tyndale-Biscoe, 1982; Jones and Rose, 1992; Walker and Gemmill, 1983) and it is the principal output of progesterone during the last 10–12 days of the cycle, in these species, that coincides with implantation and organogenesis (Renfree, 1980b). If the profiles of faecal PM in the Honey possum represent functioning *corpora lutea*, then their maximum secretion of progesterone for only 5–6 days suggests that organogenesis would be brief in this species.

With regard to the other three females (numbers 3, 21, and 48) that were captured during known mating periods in the field, they were assumed to be carrying blastocysts. This may not have been the case, however, with female number 21 in 2001, as the degree of correlation between PM and E_2 was very low ($r^2=0.08$), although the correlation was statistically significant ($P=0.012$). Nevertheless, there was a significant decline in the levels of faecal PM during the months of confinement in all three females, which suggests that the conditions in the CTR were unsupportive for normal reproductive activity.

The physiological processes that effect and maintain reproductive cyclicity in mammals are complex and may be difficult to interpret from laboratory data (Bronson, 1998); as well, the effect of stress is not well defined. In

the case of the Honey possum, there is a stress associated with confinement. Not all females give birth in semi-natural conditions outdoors (Bradshaw et al., 2000) and no female has given birth in the confines of the indoor cages. Also, Stead-Richardson et al., (unpublished) have demonstrated rises in faecal cortisol levels following weighing and pouch inspection, suggesting that there is a stress associated with periodic capture and handling. In an endeavour to minimise such stress, we limited the pouch inspection to monthly, and could not detect any apparent change in the levels of either PM or E₂ at the times of each weighing.

A factor that was not uniform during the course of the investigation was the different light régimes under which the animals were held. During 1999, female number 20, which appeared to resume oestrous cycles, was maintained in conditions that included a short day length (Table 1). The conditions experienced by all the other females during 2000 and 2001 involved a long-day length, and these females had levels of reproductive steroids that declined over the long term. This suggests that a lengthening day may be inhibitory on reproductive activity in the female Honey possum, although, in the field, females breed several times a year under different photoperiodic conditions. Here, their periods of reproduction appear to be more linked to an increase in the abundance of food (Wooller et al., 2000). There may, however, also be an influence of photoperiod, as all females in our colony, during 1998, gave birth eight weeks after the southern summer solstice, when day length is declining (Bradshaw et al., 2000). If this were to be the case, the Honey possum would resemble the macropodid wallaby, *Macropus eugenii*, which resumes ovarian activity after the southern summer solstice when the females are experiencing shortening days (Hinds and den-Ottolander, 1983; Sadlier and Tyndale-Biscoe, 1977). This is in contrast to the small marsupials species, *Antechinus stuartii* (McAllan and Dickman, 1986) and *Sminthopsis crassicaudata* (Godfrey, 1969) in which the onset of breeding is stimulated when the females encounter lengthening days.

This preliminary study highlights the utility of the faecal steroid approach in clarifying hormonal changes in species that are impossible to study using conventional endocrine methodology. The approach is particularly useful in the case of very small and fragile species, such as the Honey possum, and also in the case of rare and endangered species where ethical issues limit the use of invasive sampling techniques.

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