

Reproduction and embryonic diapause in a marsupial: Insights from captive female Honey possums, *Tarsipes rostratus* (Tarsipedidae)

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Abstract

The reproductive physiology of the polyoestrous Honey possum (*Tarsipes rostratus*) is virtually unknown except that it shares with the kangaroos and wallabies the phenomenon of embryonic diapause. Its tiny size necessitates an alternate approach to study their reproductive cycle. We have accordingly utilised faecal steroid analysis. Baseline faecal cortisol levels in the Honey possum, at $4.1 \pm 0.3 \mu\text{g g}^{-1}$, are approximately 100-fold those of other mammals and are associated with adrenal glands that, on a mass-specific basis, are almost 10 times larger than the adrenals of other mammalian, including marsupial, species. Histological examination of the adrenal glands revealed no abnormalities, however, but their hypertrophy and the peaks recorded in faecal levels following disturbance suggest that the Honey possum is vulnerable to chronic stressors in the captive situation. Mean faecal progestagens ($124.4 \pm 107.3 \text{ ng g}^{-1}$) and oestradiol-17 β ($4.1 \pm 1.1 \text{ ng g}^{-1}$) in 4 non-pregnant females maintained long term were not different from those of 5 pregnant females ($101.4 \pm 61.0 \text{ ng g}^{-1}$ and $4.3 \pm 1.5 \text{ ng g}^{-1}$, respectively) and, on analysis, revealed a cyclicity of 24 ± 1.2 days. We would predict from this evidence that the gestation period, in the absence of lactation, is approximately 23 days. Four of the pregnant females, monitored from July to November under conditions of 10:14 L:D photoperiod, showed a fall in levels of progestagens from $175.9 \pm 10.8 \text{ ng g}^{-1}$ in July and August to $30.9 \pm 9.4 \text{ ng g}^{-1}$ in October, while mean faecal levels of oestradiol-17 β increased from $3.8 \pm 0.4 \text{ ng g}^{-1}$ in July to $5.7 \pm 0.3 \text{ ng g}^{-1}$ in October. September and October are months of peak reproductive activity in the wild and we suggest that these hormonal modulations may represent an entrained reproductive rhythm. Blastocysts appear to develop at varying rates, both within the one uterus, and between the two uteri of a single female. In addition, the time taken to reach the blastocyst stage may be longer than in any other marsupial studied to date. An association of the age of the pouch young with the stage reached by the developing blastocyst does not support the conclusion that blastocysts, once formed, grow slowly during lactation or diapause. Contrary to previous reports, we have documented what appears to be a lactational inhibition on blastocysts in diapause and have estimated the length of the 'delayed' reproductive cycle in two females as less than 2 weeks. Reactivation of blastocysts in *Tarsipes* has been shown to be stimulated by shortening day lengths after the summer solstice, a response similar to the annual breeding period of macropodid marsupials. Results from studying Honey possums in captive conditions suggest that the control of diapause in *Tarsipes* appears to be three-fold; lactational, photoperiodic and an entrained rhythm.

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1. Introduction

The Honey possum (*Tarsipes rostratus*) (Gervais and Verreaux, 1842) is a small, 7–10 g, exclusively nectarivorous marsupial, endemic to botanical regions in the south

west of Western Australia that are dominated by plants belonging to the Families Proteaceae, Epacridaceae and Myrtaceae. As the sole species in the Family Tarsipedidae (Kirsch, 1984), recent evidence indicates that its origin predates those of other extant Australian marsupial Families and that it may have existed as a separate lineage for at least 50 million years (Nilsson et al., 2004). The late Heather Vose drew attention to this unique marsupial after

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maintaining it in captivity for five months while recording its daily pattern of activity and feeding habits (Vose, 1972, 1973). She suggested that "...a colony bred in captivity would be of inestimable value..." to further the study of its "...life cycle, including oestrus, breeding season and life expectancy..." (Vose, 1972, pp. 66–67).

Today, all that we know of reproduction in the Honey possum is that their non-seasonal pattern of breeding, first proposed by Scarlett and Woolley (1980) and Woolley and Scarlett (1984), is linked to the abundance of nectar (Wooller et al., 1981; Wooller et al., 1993). Females experience multiple matings (Spencer and Bryant, 2000; Wooller et al., 2000) and sexual selection can effectively account for the extremely large testes in the male, which weigh more than four percent of the body mass, and the largest sperm of any mammal (Woolley and Scarlett, 1984). Observational recordings have described the length of lactation and pouch life (Russell, 1982, 1986), and anatomical studies have revealed the existence of embryonic diapause (Renfree, 1980a).

Embryonic diapause in marsupials was first described in monovular species, such as *Setonix brachyurus*, (Macropodidae) (Sharman, 1955a), and subsequently has been recorded in nearly all other macropodid species (see Renfree, 1978). In the tammar (*Macropus eugenii*), further development of the single embryo beyond the blastocyst stage is arrested, either by a seasonal influence (Berger and Sharman, 1969) mediated by the pineal gland (Renfree et al., 1981) or by the suckling action during lactation (Renfree, 1979). The latter, understood to be mediated by prolactin (Tyndale-Biscoe and Hawkins, 1977), maintains the *corpus luteum* of ovulation in a quiescent state and the blastocyst at approximately the 100 cell-stage (Smith, 1981). The discovery of embryonic diapause in several of the small, polytocous possums (Family Burramyidae) (Ward and Renfree, 1988; Ward, 1990) has highlighted some apparent differences between these small possums and the larger macropodid species. In the Honey possum, for example, the blastocysts are five to ten times larger than those of the macropodid species (Renfree, 1981); their size is variable within the one uterus, and they have been reported to have a continued slow growth during diapause (Renfree and Shaw, 2000).

The complete mechanism of control of diapause in the small possums remains unknown, except for a response shown by the Honey possum to shortening day length. In a laboratory trial, reproductive steroid levels in the faeces increased and blastocysts in diapause underwent hypertrophy and hyperplasia when females were exposed to an advancement of short days (Oates et al., 2004). Further evidence would suggest that Honey possums respond to the shortening day length after the southern summer solstice, as captive females, kept outdoors, all gave birth during the mid two weeks in February, regardless of the age of their blastocysts (Bradshaw et al., 2000). Also, a large proportion of females in their natural habitat gives birth during January to February (Wooller et al., 2000). Other

controls over diapause, however, are enigmatic, as Honey possums may breed several times a year under different photoperiods (Wooller et al., 2000).

It has been reported that lactation does not have an inhibitory influence over diapausing blastocysts of the Honey possum, as occurs for the macropodid marsupials. Blastocysts had not enlarged 50 days after removal of the pouch young (Renfree, 1981), nor were there new young in the pouch up to 93 days after the cessation of suckling (Renfree et al., 1984). Honey possums, similar to other small marsupials (Geiser, 1994) undergo daily bouts of torpor which are initiated by low environmental temperatures and a limited food supply (Collins et al., 1987; Withers et al., 1990). Periods of torpor may account for high individual variation in the field metabolic rate (FMR) of Honey possums measured in mid-winter (Nagy et al., 1995). These periods of torpor in some dasyurid marsupials have been reported to have a moderating influence on the gestation period (Dickman, 1985). Whether torpor may have been a factor inhibiting gestation and birth in the above manipulations by Renfree, is not known. The absence of young in the pouch for up to three months after suckling ceased, together with observations that blastocysts appear to grow slowly and continuously (Renfree, 1981) have been taken as evidence that the Honey possum features an embryonic diapause not under the primary control of lactation. As a result, it has been suggested that the gestation period in this species may be up to 60–80 days, longer than in any other marsupial and within this, a period of 21–28 days has been suggested as the 'active' gestation period (Renfree et al., 1984). Blastocysts in this species result from fertilisation during a *post-partum* oestrus, thus the oestrous cycle would be a little longer than the gestation period; at some 60–80 days, this would be the longest oestrous cycle known in any marsupial.

The diminutive size of the Honey possum makes it more than a challenge to investigate those physiological parameters, such as the length of the oestrous and reproductive cycles, which traditionally require methods of steroid hormone measurement in plasma. Also, the restraint of animals in captive conditions incurs an unknown degree of stress, particularly when they are confined in small cages (Bronson, 1998). Lack of exercise was proven to be the cause of atrophic ovaries in captive female opossums (*Didelphis virginiana*) (McCrary, 1938). In addition, many laboratory animals are housed in constant temperature rooms and are exposed to artificial light and temperature régimes, extraneous noise and handling. For the Honey possum, Vose (1972) comments (p. 66, lines 66–78) "... Sudden noise would bring an instant response, with evidence of fear in the cowering, hunched-up stance assumed. An increase in the noise level produced frenzied behaviour, with wild leaping from one side of the cage to another, and flinging of the body against the walls. On the single occasion this inadvertently occurred, it was 3 h and 45 min before the animal recovered sufficiently to approach the fresh food and blossom. Stress was also noticeable when the cage was periodically scrubbed out..."

Our long-term study has taken advantage of semi-natural conditions outdoors for maintaining Honey possums and reports on three main areas of investigation. (a) The confirmation of oestrous cyclicality, the length of the oestrous cycle and characterisation of reproductive steroid levels throughout gestation. (b) The length of diapause and factors influencing resumption of development in diapausing blastocysts. (c) The susceptibility of the Honey possum to stress.

2. Methods

2.1. Animals

2.1.1. Experimental holding conditions

All Honey possums were originally caught in pitfall traps in Scott National Park, (Lat 34°17'S, Long 115°13'E) 10 km east of Augusta in the south-west corner of Western Australia and transported to the School of Animal Biology, University of Western Australia in Perth.

Indoors, animals were housed in a constant temperature room (CTR), maintained at short daylength (10:14 L:D), as long daylength has been shown to inhibit ovarian activity (Bradshaw et al., 2004). They were kept either in small groups in a well-ventilated Perspex cage, approximately 1 m³ in volume, or singly in half the volume by the insertion of a central barrier. Natural vegetation, cuttings from Proteaceae species, was provided as a refuge and refreshed weekly.

Outdoors, each yard was 16 m² in area by 2 m in height and contained established species of flora endemic to *Banksia* woodland. Constructed of 1 cm² steel mesh, enclosures were overlaid with a fine-grade wire mesh that afforded maximum light penetration but prevented the escape of young and excluded predators. Yards were designed to interconnect such that 96 m³ could be made available. Each yard contained 10 pitfall traps (40 cm lengths of 15 cm diameter PVC piping set into the ground with the rim flush with ground level) fitted with lids when not in use. Artificial diet was provided daily for 6 days of the week.

2.1.2. Diet

The dietary components (see Table 1) were modified from those of Russell and Renfree (1989) in order to approximate as closely as possible the measured intakes of nectar and pollen in the field (Bradshaw and Bradshaw, 1999). *Banksia* honey, diluted to approximately 22% sugar

Table 1
Dietary components of a maintenance diet for Honey possums in captivity

Components	Volume (mL or g)	N (mg)	Digestible N (mg)	N % of total	% sugar
<i>Maintenance diet</i>					
Water	810				
Honey	300	75	75	1.8	ca. 80
Pollen	80	3200	2400	64	
Whey protein	10	1260	1260	34	
Total	1200	5095	3735		
Daily aliquot	10	42.5	31		ca. 2 g

Honey and pollen were obtained commercially from bees feeding in areas of sand-plain plant species, including *Banksia*, *Grevillea*, *Hakea*, *Adenanthos* and *Myrtaceae*. Pollen (4% N) quantity is adjusted for 75% digestibility (Bradshaw and Bradshaw, 2001). Whey protein (12.6% N) obtained commercially as Balance[®] ion exchange whey protein. Modified from Bradshaw et al. (2000).

(Brix reflectometer), was the substitute for nectar and a quantity of whey protein was added to compensate for a possible lack of essential amino acids. Allowing for a 75% digestibility of the pollen (Bradshaw and Bradshaw, 2001), the maintenance diet provided a daily intake of digestible nitrogen of 30–34 mg d⁻¹. Note that the minimum nitrogen requirement (MNR) for a 10 g Honey possum is 2.8 mg d⁻¹ (Bradshaw and Bradshaw, 2001); hence, the ration of 5.6 mg d⁻¹ in the Russell and Renfree (1989) diet would be close to minimal and probably would not have supported reproduction. For lactating females in our study, the daily allowance was increased to 15 mL daily, providing approximately 50 mg d⁻¹ digestible nitrogen.

2.1.3. Animal monitoring

2.1.3.1. *Pouch young*. Seventeen females in the outdoor conditions, and in the presence of males, were monitored during October 1997 to February 2002 for the presence of pouch young. No growth curve has yet been established for pouch young. Our estimation of their age and, indirectly, their time of birth, involved two methods:

- While in the pouch, and using an otoscope for very small young, an *in situ* measurement was based on a crown-rump dimension and the stage of pelage. The young are bright red at birth and approximately 4 mm in length. They gain about 4 mm in crown-rump length per week until the onset of furring, which occurs in the eighth week of the 9- to 10-week-pouch life. This method approximates the age to within one week.
- After weaning, the age is calculated from the equation, $y = 0.067x + 3.69$ ($r^2 = 0.84$), generated from the increase in body mass of young animals over time while raised in captive conditions (Bradshaw et al., 2000), where x = days from pouch emergence and y = body mass in grams.

2.1.3.2. *Non-pregnant females*. Faecal samples were collected daily from two females, #48 and #130, that were identified at the end of the monitoring period as non-pregnant by the presence of shelled ova, or eggs, in the reproductive tract. Female #48 was maintained in semi-natural conditions outdoors and surrounded by males, but confined singly in a wire-mesh cage of 0.72 m³ volume, for 34 weeks (November, 2002 to July, 2003). Female #130 was housed in the indoor conditions of the CTR, experiencing a photoperiod of 10:14 L:D and temperature regime of 22 °C day : 15 °C night for 43 weeks (April, 2003 to February, 2004) (see Table 2). Both received the artificial diet daily with occasional addition of Proteaceae blossom. Two other females, #35 and #133, maintained for 4 months in similar indoor conditions, identified as non-pregnant, were included in the analysis of the non-pregnant cycle.

2.1.3.3. *Pregnant females*. Pregnancy was also identified *a posteriori* by the presence of diapausing blastocysts or an embryo in the reproductive tract. One female, #64, was monitored for 12 months between February 2003 and February 2004 under a 10:14 L:D photoperiod, and, on sacrifice, contained a single late-term embryo in the uterus. This female had successfully weaned her young 3 months prior to the commencement of monitoring. Fertilisation was possible only during these three months; thus, depending on the time of fertilisation, blastocysts were in diapause for between 12 and 15 months (see Table 2). Analyses of faecal samples, collected over 7 months from 2 other pregnant females (#247 and #7) maintained indoors, are included in the mean and peak levels of reproductive hormones.

2.1.3.4. *Evidence of an entrained rhythm*. Four pregnant females, (#10, #33, #36 and #41), wild-caught during the winter solstice (15–25 June), were maintained indoors for 4 months under a short photoperiod (10:14 L:D). They were participants in an experiment to test the effect of an increase of nitrogen (as pollen) in the diet. Faecal sampling commenced on 8 July, 2003; 50 days later (26 August), one of the females, #33, received the high nitrogen (as pollen) diet and sampling continued until all animals were sacrificed 75 days later (9 November).

Table 2
Details of female Honey possums (*Tarsipes rostratus*) that underwent faecal monitoring and/or blastocyst investigation

Aspect studied	ID	Origin	Pre-monitoring conditions	Monitoring conditions ^a	Collection period	On dissection
Non-pregnancy	35	Wild	1 month CTR, NM, NPY	CTR, 10:14 L:D	Jul 2003–Nov 2003	O
	48	Colony	5 months outdoor, M, NPY	outdoor, natural, Photoperiod	Nov 2002–Jul 2003	O
	130	Wild	3 weeks outdoor, M,	CTR, 10:14 L:D	Apr 2003–Feb 2004	O
	133	Wild	3 months outdoor, M, NPY	CTR, 10:14 L:D	Jul 2003–Nov 2003	O
Pregnancy and diapause	64	Colony	5 months outdoor, M, weaned PY	CTR, 10:14 L:D	Feb 2003–Feb 2004	embryo
	7	Wild	3 months outdoor, NM, NPY	CTR, 15:9/10:14 L:D	Sep 2002–Apr 2003	B
	247	Wild	3 months outdoor, NM, NPY	CTR, 15:9/10:14 L:D	Sep 2002–Apr 2003	B
Entrained rhythm	10	Wild	2 months outdoor, M, NPY	CTR, 10:14 L:D	Jul 2003–Nov 2003	B
	33	Wild	2 months outdoor, M, NPY	CTR, 10:14 L:D	Jul 2003–Nov 2003	B
	36	Wild	2 months outdoor, M, NPY	CTR, 10:14 L:D	Jul 2003–Nov 2003	B
	41	Wild	2 months outdoor, M, NPY	CTR, 10:14 L:D	Jul 2003–Nov 2003	B
Blastocysts	40	Colony	20 months outdoor, M, 2 sPY		Sacrificed 26 Feb 2002	B
	118	Wild	Outdoor, M, NPY	CTR, 15:9 L:D	Sep 2002–Apr 2003	B
	37	Wild	Wild, 4 sPY		Feb 2005	B
	20	Wild	Wild, 4 sPY		Feb 2005	B
	22275	WA museum	Wild, 1 sPY		Mar 1984	B
	23235	WA museum	Wild, 3 PY		Oct 1985	B
	26985	WA museum	Wild, 2 PY		Aug 1986	B
	23254	WA museum	Wild, NPY		Feb 1985	B
	24550	WA museum	Wild, NPY		Oct 1982	B
	26926	WA museum	Wild, NPY		Nov 1986	B
	50992	WA museum	Wild, NPY		Mar 1998	B
	52095	WA museum	Wild, NPY		Mar 1998	B

^a All animals monitored for faecal steroids were housed individually. O, ova; B, blastocysts; M, with males; NM, absence of males; PY, pouch young; sPY, small pouch young; NPY, no pouch young.

2.1.3.5. *Evidence of stress.* Faecal samples were collected daily for periods up to two months from 3 females with no pouch young. They were chosen at random from the outdoor colony (together with 2 males as controls) and maintained in the small individual cages in the CTR. The samples were analysed for levels of cortisol and cortisol metabolites during periods of weekly weighing and pouch inspection of the females. This procedure involved capturing the animal, placing it in a small cloth bag, weighing, physical examination, and restraint of the animal during pouch inspection. While out of the cage, the vegetation was refreshed and the enclosure cleaned, which altered habitat and disturbed refuge areas.

2.1.4. Blastocysts

Blastocysts are attached closely to the uterine endometrium but can be removed by a careful 'rolling out' action. Together with eggs, when present, they were stained with haematoxylin, followed by Scott's solution, mounted in glycerol, and photographed. The early zygotes were distinguished from the unfertilised egg by the identification of dividing cells (blastomeres) and cleavage planes in the zygotes, where possible. The diameter of blastocysts is the mean of 6 measurements and includes the three embryo coats, the *zona pellucida*, and the mucoid and shell layers (Selwood, 2000). In the egg and early zygote, the thickness of the mucoid and shell coats is approximately 13% of the diameter, but thins to a uniform 3% of the diameter in the unilaminar blastocyst in diapause.

The diameters of 64 blastocysts were measured, from 8 preserved animals, kindly provided by the West Australian Museum, Perth, Western Australia, and from 10 captive animals (see Tables 2 and 4). There was no significant difference between the mean diameter of the preserved specimens ($1.83 \text{ mm} \pm 0.12$) and that of the fresh animals ($1.62 \text{ mm} \pm 0.21$) ($P > 0.05$) and they were treated as a single group. Six females (3 preserved animals and 3 captive animals) were carrying pouch young (PY). As these young had been born shortly before a *post partum* oestrus (Renfree, 1980a), the age of these pouch young provides an indication of the time of fertilisation, assuming that fertilisation occurs within a day or two after birth. If the pouch young, for example, are estimated to be 3 weeks-old when the female is sacrificed, then blastocysts will also be approximately 3 weeks-old, as they were conceived soon after the birth of the young.

The number of cells was counted in 17 blastocysts (see Table 4). Three of these were in the early stages of development (from #40 and #22275), 8 were diapausing blastocysts and 6 were blastocysts recovered from 3 females (#7, #33 and #247) maintained indoors, that were exposed to conditions designed to activate blastocysts. As a measure of cell number, the nuclei were counted either by using an Optimas programme (version 6.2) in which nuclei were counted in 3 measured surface areas (for the preserved blastocysts), or by using ImagePro software, in which the mean number of nuclei was obtained from ten prescribed areas (for the captive animals). From the mean number of nuclei and the mean of each area, the

total number of cells was estimated using the formula for the surface area of a sphere. The correlation between cell number and diameter of the blastocysts was analysed by linear regression and the differences between diapausing and activated blastocysts by ANOVA.

2.1.5. Adrenal glands

Adrenal glands, dissected from 4 captive females and 2 males and 2 wild-caught females, were weighed to 0.1 mg and fixed in 10% Buffered Formol Saline (BFS) before being transferred to 70% ethanol for storage. The specimens were processed through graded ethanols, chloroform and paraffin wax prior to embedding. The wax-embedded tissue was sectioned at 6 μm , mounted and stained with Harris' haematoxylin and eosin and the relative proportions of medulla and cortex measured.

2.2. Faecal steroid analysis

2.2.1. Cortisol and cortisol metabolites (C_M)

2.2.1.1. Time course of cortisol excretion and proportion of conjugated to un-conjugated steroid in faeces and urine. Tritiated cortisol (185 kBq of [1,2,6,7(N)- ^3H]-hydrocortisone, SA 2.88 TBq/mmol), dissolved in 0.13 mL sterile saline was injected i.p. into 2 female Honey possums that had been acclimatised for 2 days in a metabolism chamber (Bradshaw, 2003). Faeces and urine were collected quantitatively at 2-h intervals during the first day and at 4-h intervals for the next 2 days. For estimation of total radioactivity excreted, urine samples were counted directly; faecal samples were extracted in ethanol, dried, counted and corrected for extraction losses and residual radioactivity in the injection syringes. The proportions of unconjugated and conjugated steroid was assessed in urine and faeces by ether–water extraction (Bahr et al., 2000), as described in (Bradshaw et al., 2004).

2.2.1.2. Treatment of faeces. Faeces were collected daily and stored at -12°C . On microscopic examination (4 \times), sand and plant particles were removed from each sample. Thirty milligrams of well-mixed faecal powder was assayed from each sample, a volume that represents approximately 30% of the mean dry weight of faeces excreted per day by a Honey possum. The faecal sample was homogenised in twice-distilled water and then vortexed for 2 min with 3 mL di-ethyl ether (BDH, Poole, UK) with a recovery of [^3H]-cortisol of $87.8\% \pm 2.8$ (SE).

2.2.1.3. Radioimmunoassay. Two methods were utilised to measure cortisol and C_M ; for cortisol, the assay used an antibody with high specificity for cortisol (F3-314, Endocrine Sciences, USA) with the following cross-reactivities: cortisol 100%, prednisolone 52%, prednisone 26%, cortisone 30%, 21-desoxycortisol 6.8%, desoxycortisol 4.5%, corticosterone 2.9%, 17 α -hydroxyprogesterone 0.3%, tetrahydrocortisol 0.25%, tetrahydrocortisone 0.15%, dexamethasone 0.1%, and $<0.1\%$ with 20 α -hydroxyprogesterone, progesterone, pregnanetriol, aldosterone, oestradiol, oestriol, 20 β -hydroxyprogesterone, pregnanediol and testosterone. Briefly, extracted samples and 375 Bq [^3H]-cortisol, were incubated with cortisol antiserum diluted in 0.05 mol borate buffer, pH 8.0, containing 1.9% bovine γ -globulin and bovine serum albumin. Bound steroid was separated from free by incubation with Dextran-coated charcoal at $0-4^\circ\text{C}$ and an aliquot of the supernatant was counted for radioactivity to $<1\%$ error and standards data were transformed using a 4-parameter logistic equation.

The assay of C_M used a double antibody ^{125}I -labeled radioimmunoassay kit (Rats and Mice Corticosterone kit; Cat. No. 07-120102; ICN pharmaceuticals, Costa Mesa, USA), reported to cross-react only with cortisol metabolites (Wasser et al., 2000). Briefly, faecal extracts were incubated with the antibody diluted in phospho-saline gelatin buffer (pH 7) and [^{125}I]-corticosterone. Bound radioactivity was precipitated using a buffered (TRIS) solution of goat anti-rabbit gamma globulin with poly-ethylene glycol (PEG) and counted in a Prias Autogamma counter (Packard).

2.2.1.4. Validation. Validation of the assay utilised a series of pooled faecal samples containing increasing amounts of cold cortisol (125–5000 pg) that were extracted with diethyl-ether or 90% ethanol (Wasser et al., 1996) and assayed, either with, or without, pre-chromatographic treatment (500 mg

Silica Sep-pak Vac RC). Diethyl-ether extracts, without chromatography, gave the best estimates of the amount of cold cortisol added. In order to improve the accuracy of the assay, a dilution series from 1/8 to 1/3000 of both diethyl-ether and ethanol extracts was assayed (without chromatography) and compared. A more linear dilution series, indicating a 'cleaner' extract with less interference, was obtained with samples extracted in diethyl-ether. Cortisol standards (0–5000 pg) were added to a series of diluted ether extracts (1/8–1/3000). The dilution of 1/100 provided the most accurate estimation ($r^2 = 0.99$) of each cortisol amount added and all extracts were assayed at this concentration. All faecal samples were assayed using the two different antisera and there was agreement between estimates of faecal cortisol and those of C_M (both expressed in $\mu\text{g g}^{-1}$).

2.2.2. Progestagens (P_M) and oestradiol-17 β (E_2)

The treatment of faeces for these assays has been previously described and the principal metabolic products of progesterone in the faeces (progestagens) have been identified by chromatographic analysis (Bradshaw et al., 2004). The P_M radio-immunoassay employed a monoclonal antiserum chosen for its cross-reactivity with the metabolites, and this was kindly provided by Dr. Janet Roser, University of California, Davis, USA. With regard to E_2 , both the time course and nature of excretion *via* the faeces in the Honey possum have been established (Oates et al., 2004). Of the 65% oestradiol metabolites excreted in the faeces, only 20% are excreted in an unconjugated form (Bradshaw et al., 2004), thus oestradiol in the faeces represents approximately 13% of the total excretion. We chose an antiserum with high specificity for oestradiol (Bioquest, NSW, Australia), with the understanding that relative excretion levels across time would provide information sufficient to detect ovulation (Teskey-Gerstl et al., 2000). Both radioimmunoassays have been validated and are described in (Oates et al., 2004).

2.3. Analysis of hormonal profiles

2.3.1. Estimation of baseline levels and identification of peaks of progestagens (P_M), oestradiol-17 β (E_2), cortisol and cortisol metabolites (C_M)

An analysis of the long-term profiles of steroid excretion involved a statistical method of establishing a baseline level in order for significant peaks to be identified. For the reproductive steroids, this involved an iterative process (Graham et al., 2002) in which the mean concentration of all samples in each profile was calculated and values greater than 1.75 SD above the mean were removed from the series. The mean was re-calculated and the process repeated until no value was greater than 1.75 SD above the mean. The mean of the remaining values was considered to be the baseline level. A concentration of >4 SD above this baseline was accepted as a significant peak for both P_M and E_2 . For cortisol, 'spikes' in faecal cortisol concentrations are values 1 SD above the mean for a given animal. Baseline cortisol levels are derived from a re-calculation of the mean value after excluding the 'spike' concentrations.

2.3.2. Determining degree of correlation between P_M and E_2

Linear (Pearson) correlation analysis between P_M and E_2 suggested the possible source of their secretion. A positive correlation was interpreted as a single tissue origin, and a lack of any correlation was interpreted as a more complex or separate origin of P_M from that of E_2 , i.e., those associated with the sequential production of E_2 from follicular tissue and the subsequent production of progesterone from luteinised follicles, as occurs in the oestrous cycle.

2.3.3. Establishing an oestrous cycle

In those females containing eggs at the time of sacrifice, regular and significant peaks of E_2 were interpreted as the occurrence of ovulation (Curnow et al., 2001) and the number of days between them as the length of the oestrous cycle. The establishment of a composite cycle was obtained by normalising the number of days (ranging from 21 to 30) in 18 cycles from 4 females (#48, #130, #35 and #133) to the overall mean length of 24 days. Invariably associated with each proposed ovulatory peak of E_2

was a significant peak of P_M , occurring either concurrently or within 2 days afterwards. Raised P_M levels between these peaks were interpreted as originating from *corpora lutea*.

2.3.4. Analysis of pregnant profiles

The only Honey possum sampled long-term was #64, whose year-long profiles of E_2 and P_M were subjected to a 4-point moving averages analysis. This procedure was useful in smoothing daily variation and identifying overall trends. A composite of the 4-month profiles of #10, #36, #41 and #33, all of which were carrying blastocysts, is constructed from each mean data point of 4 animals (\pm SE), as all were sampled on the same day.

3. Results

3.1. Oestrous cycle

In the 4 females identified as non-pregnant, #s 35, 48, 130 and 133, levels of P_M and E_2 during the monitoring period were not significantly correlated (Spearman 2-tailed $P = 0.24, 0.96, 0.52$ and 0.07 respectively) and were a mean $124.4 \pm 107.3 \text{ ng g}^{-1}$ and $4.06 \pm 1.12 \text{ ng g}^{-1}$ respectively. The length of the cycles ranged between 21 and 30 days, providing a mean length for each female (with the number of cycles in brackets) as follows: $24.3 \pm 1.89 \text{ d}$ (6) in #130; $23.4 \pm 1.80 \text{ d}$ (5) in # 48; $22.2 \pm 1.97 \text{ d}$ (5) in #35 and 21.0 d (2) in #133, providing an overall mean of 18 cycles of 24.00 ± 1.15 days. No difference was observed in the mean cycle length between the female #48, housed outdoors, and #130 that was maintained indoors.

A composite of the P_M and E_2 levels during 24 days of 18 cycles, shows high levels of P_M concurrent with the presumed ovulatory peak of E_2 (Fig. 1a), and which reach a peak some 2 days later. Following this, levels fall and remain low until approximately the 15th day of the cycle, when they rise again and fall by the 24th day. The hormonal modulation of a single oestrous cycle is represented by female #133 (Fig. 1b), which occurred between days 95 and 116 of monitoring, just prior to the recovery of 5 eggs in the uteri on day 125.

3.2. Pregnancy

In contrast to non-pregnancy, the levels of excreted P_M and E_2 in the female (#64) maintained long-term were positively-correlated throughout the monitoring period ($r^2 = 0.153$; $p < 0.001$) and the pattern of steroid excretion shows a gradual rise throughout the year (Fig. 2), with three episodes of sustained excretion of P_M during days 210 to 218, days 225 to 242 and days 272 to 280 from the commencement of monitoring on 20 February, 2003. During January 2004, there was a marked rise in the level of E_2 on day 332, which persisted for 10 days until sampling was discontinued. During the following seventeen days, this female received an intra-peritoneal (IP) injection of 74 MBq [^3H]-progesterone, containing 7 ng progesterone, and followed 16 days later (3 days before sacrifice) with an IP injection of 74 MBq [^3H]-oestradiol, containing 5.8 ng oestradiol. The female was then sacrificed and an embryo of 2.7 mg was dissected from the uterus, measuring

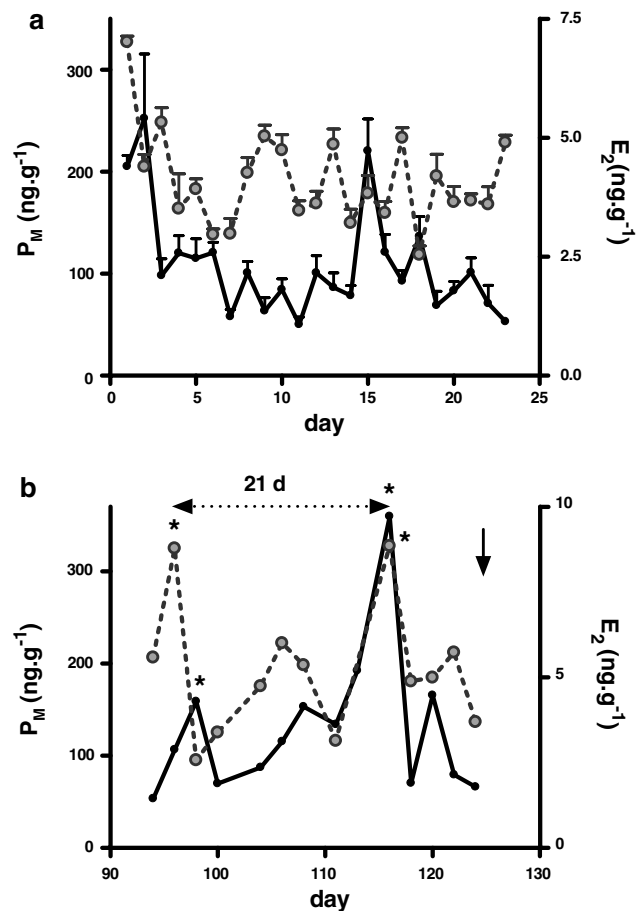


Fig. 1. Faecal concentrations (ng g^{-1}) of progesteragens (P_M) (solid line) and oestradiol-17 β (E_2) (dotted line) during (a) a composite of 24 days in 18 oestrous cycles in 4 Honey possums (*Tarsipes rostratus*), maintained in laboratory and semi-natural conditions outdoors (Mean \pm SEM) and (b) the final 21-day oestrous cycle of #133, containing 5 eggs on dissection (indicated by arrow). (*) marks values $>4\text{SD}$ above baseline levels.

4.01 mm in length (Fig. 3e). The developed claws in the fore-limb indicate that it was near full-term, as clawed digits do not appear until the day of birth in the opossum (McCready, 1938). This embryo resulted from a fertilisation that was possible only during the 3 months prior to February in the previous year, an indication that blastocysts can remain viable in diapause for at least a year.

3.3. Entrained rhythm

There was no significant difference in the mean concentrations of P_M and E_2 between the single female that received the increase in dietary nitrogen and the control females. A monthly analysis of hormone concentrations during July to November, however, revealed differences over time, both in P_M concentrations ($F_{3,12} = 9.194$, $P = 0.002$) and in E_2 levels ($F_{3,12} = 3.674$, $P = 0.04$, Kruskal–Wallis) (Table 3). A composite of the profile that was common to all 4 females (Fig. 4), regardless of the level of nitrogen in their diet, shows a substantial peak of excreted E_2 , lasting for approximately 15 days in September, followed by rising levels throughout October, which, by

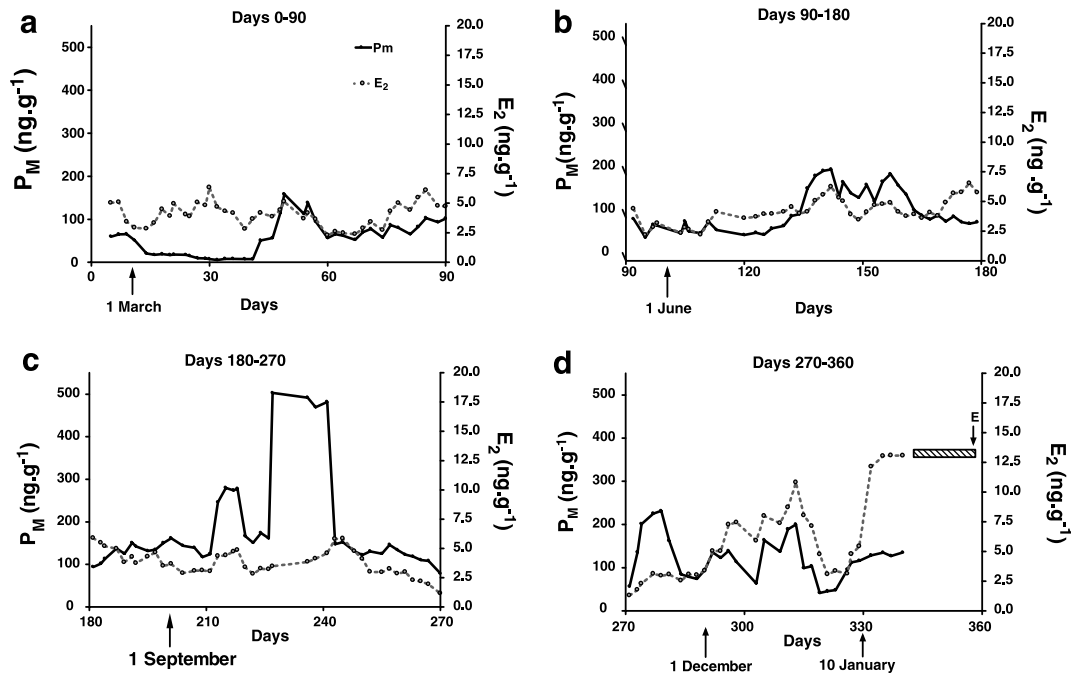


Fig. 2. Long-term profile of faecal concentrations (ng g^{-1}) of progestagens (P_M) (solid line) and oestradiol-17 β (E_2) (dotted line) in pregnant female #64 (*Tarsipes rostratus*) held under 10:14 L:D photoperiodic conditions during (a) March to June, (b) June to September, (c) September to December and (d) December to 18 January when sampling ceased. Female received hormone injections during subsequent 17 days (hatched bar) (see Section 3) and embryo (E) was dissected on 18th day. Concentrations were subjected to a 4-point moving averages analysis.

November, are approaching the high concentrations measured during the final stages of pregnancy in the female #64. In contrast, mean concentrations of P_M , in July and August fall during September ($P < 0.01$) and remain low during October.

3.4. Delayed pregnancy

Two females (Fig. 5), #16 and #131, lost pouch young during their period of monitoring and were observed to have smaller pouch young soon after. Female 16, housed outdoors, had very small young on 19 February, 1998 that were estimated at 3 days-old. When next examined on 18 March, the pouch young were estimated to be 4 weeks-old, confirming the birth of these young around 16 February. When next caught and examined on 8 April, the pouch young were estimated at 1 week-old. We interpret this as a loss of pouch young some time after 18 March, a resumption of growth of blastocysts and birth occurring between 29 March and 1 April. If pouch young were lost soon after 18 March, 12 days would be the maximum time for this 'delayed' pregnancy.

Closer monitoring of another female indicated that the 'delayed' pregnancy may be even shorter. Female #131 was housed indoors and, on 9 November, 1999, had small pouch young of estimated age 2 weeks. She was immediately placed in the outdoor yard with others but lost her young and significant body weight within the next three days. She was replaced indoors on 12 November, with no pouch young, and housed alone in order to recover. On 22 November, 10 to 12 days after losing the first young,

she had 2 very small pouch young of approximately 1 week-old, providing an estimated time of birth of 13–17 November. This approximation was confirmed by their emergence from the pouch by 1 February the following year, and their weight of 2.9 g. Taking into account the length of lactation, 9 to 10 weeks (Russell, 1986) and the size of the weaned young (routinely 2.5 g at emergence), their estimated time of birth, using this method, was between 12 and 20 November. Her pouch may have been empty from 10 November, thus, the length of the delayed pregnancy (from the time of loss of the suckling young to birth) is estimated to be between 3 and 7 days.

3.5. Pouch young (PY)

Forty-nine young were born to 17 female Honey possums maintained in captive conditions outdoors during 1997–2002 (see Fig. 6). Twenty-four litters (of 2 young in each and one with 3 young) were detected in the pouch but only 24 young survived to weaning. Births were confined to the months between July and the following March with no births recorded during January. A peak of births (27%) was recorded during February, with the greater proportion (60%) occurring from September to December. Pouch emergence for these young, thus, occurred from late spring through to early autumn.

3.6. Blastocysts

Between 1 and 7 blastocysts were found in any one female and there was a considerable variation in both size

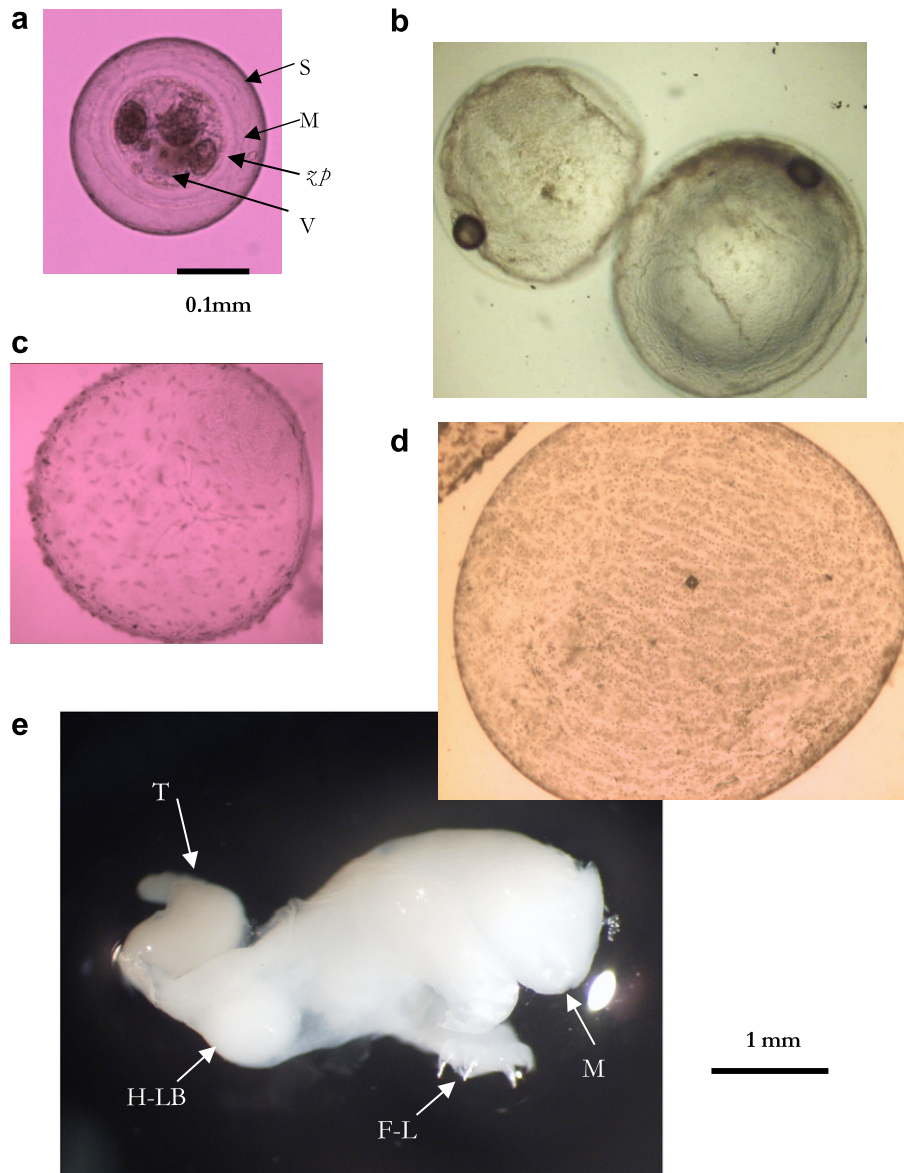


Fig. 3. (a) Unfertilised egg of the Honey possum (*Tarsipes rostratus*) with 3 egg coats, (S, shell layer; M, mucoid coat; zp, zona pellucida) surrounding the vitellus (V). (b) Unilaminar blastocysts containing ca. 750 cells. An unfertilised egg (brown) is caught up in the mucoid coat of each blastocyst (L. Selwood, *pers comm.*) (c) Diapausing unilaminar blastocyst within the shell coat. Note reduction of the mucoid layer. (d) Stimulated blastocyst, 3.8 mm diameter, containing 10,895 cells, extruded from the shell layer. (e) Lateral view of a near-term embryo from the right uterus of female #64, enclosed in extra-embryonic membranes, with a length of 4.01 mm and weight of 2.7 mg. The retinal pigment was detected as a faint ring at the edge of the lens. Tongue is protruding slightly from the mouth (M); hind limbs (H-LB) are present as buds and forelimbs (F-L) feature clawed digits. The tail (T) is flexed dorsally.

and number of cells, both within and between the individual uteri (see Fig. 7b). The diameter of 39 blastocysts presumed to be in diapause ranged between 0.42 mm and

2.90 mm, providing a mean of 1.80 ± 0.10 mm (Table 4) with 80% between 1 and 2.5 mm in diameter (Fig. 7a). The mean diameter of stimulated blastocysts, taken from

Table 3

Concentrations (ng g^{-1}) of faecal progestagens (P_M) and oestradiol-17 β (E_2) in 4 female Honey possums (n), maintained in short-day length (10 h light: 14 h dark) during July to November, 2003, (means \pm SE)

	July	August	September	October
Progestagens (P_M)	175.5 \pm 17.11 (4)	169.46 \pm 17.94 (4)	91.86 \pm 18.42 ^a (4)	87.43 \pm 6.77 ^a (4)
Oestradiol-17 β (E_2)	3.76 \pm 0.08 (4)	4.19 \pm 0.30 (4)	4.57 \pm 0.44 (4)	5.71 \pm 0.69 ^b (4)

^a Denotes significant difference ($P < 0.05$) in concentrations from those in July and August.

^b Denotes a significant difference ($P < 0.05$) in concentration from that in July and from those in July and August combined.

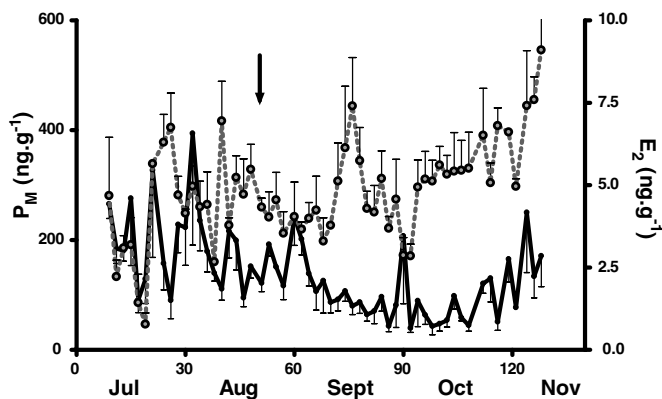


Fig. 4. Faecal concentrations of progestagens (P_M) (solid line) and E_2 (dotted line) in 4 female Honey possums (*Tarsipes rostratus*) carrying blastocysts in diapause and maintained indoors on short photoperiod (10:14 L:D). One female was changed to the increase in dietary nitrogen on 26 August (shown by arrow). Means \pm SEM.

females in experimental conditions that were designed to re-activate blastocysts, was 3.07 ± 0.31 mm and significantly larger than those in diapause ($P < 0.001$, $F_{2,61} = 24.97$). The number of cells in the stimulated blastocysts, 6798 ± 1066 , was also significantly greater than 2465 ± 473 in the unstimulated blastocysts ($P < 0.01$, $F_{2,14} = 14.32$) (Table 4).

Blastocysts recovered from females that had PY of estimated age less than 3 weeks-old had a mean diameter of 1.16 ± 0.13 mm, significantly smaller than that of blastocysts in diapause ($P < 0.01$). The difference in cell number between the two stages of blastocyst development was also significant (2-tailed $t_7 = 3.554$, $P = 0.009$) (unpaired t test with Welch correction). One female, (#40) with PY estimated at 7–10 days-old when sacrificed had 2 unilaminar blastocysts in the right uterus, 1.7 and 1.9 mm in diameter and containing 727 and 742 cells. The thickness of the mucoid coat in these blastocysts, 12.5% of the diameter (Fig. 3b) is close to the average 13% measured in the

unfertilised eggs (Fig. 3a). In the left uterus, one blastocyst of 0.48 mm in diameter had reached the unilaminar stage with approximately half the number of cells; the second blastocyst in the left uterus had collapsed and was classified as abnormal (Selwood, 1990). Seven eggs, 5 possibly unfertilised, and 2 in the early stages of cell division were also present in both uteri of this female. We interpret from this female that the rate of development of blastocysts is not uniform between the two uteri and at 7–10 days-old have clearly not yet reached the stage of entering diapause. In another female, #22275, in which the pouch young were estimated to be 2 weeks-old, although blastocysts had grown to diapausing size, again, one had fewer cells (see Table 4). It would appear that the fertilised egg of the Honey possum takes a minimum of 2 weeks to complete its autonomous development before it reaches the mature blastocyst stage entering diapause.

A regression analysis revealed a significant correlation between the number of cells and the diameter in those blastocysts considered to be in diapause ($P = 0.01$, Spearman $r = 0.73$), indicating that blastocysts enlarge due to the increase in the number of cells (Fig. 8). This was not the case with blastocysts that had been stimulated; in these, the increase in the number of cells was largely independent of blastocyst size ($P = 0.24$, Spearman $r = -0.6$), an indication that mitotic division results in smaller cells.

3.7. Cortisol and cortisol metabolites (C_M)

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

The total recovery of injected [3 H]-cortisol in faeces and urine averaged 79.8% after 56 h of collection. Of the recovered radioactivity, 65.8% of the steroid was excreted in the urine and 34.2% in the faeces. In the faecal samples, 65% of the steroid was excreted within 10 h after injection with the

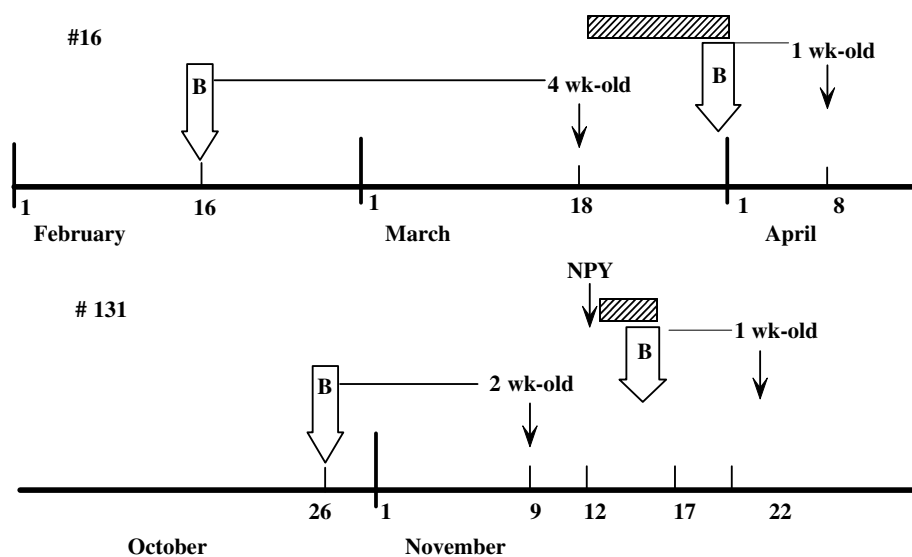


Fig. 5. Time-line of sequential events in the proposed delayed pregnancy (hashed bar) of female Honey possums (*Tarsipes rostratus*), #16 and #131, (see Section 3). B—Estimated time of birth. NPY—no pouch young.

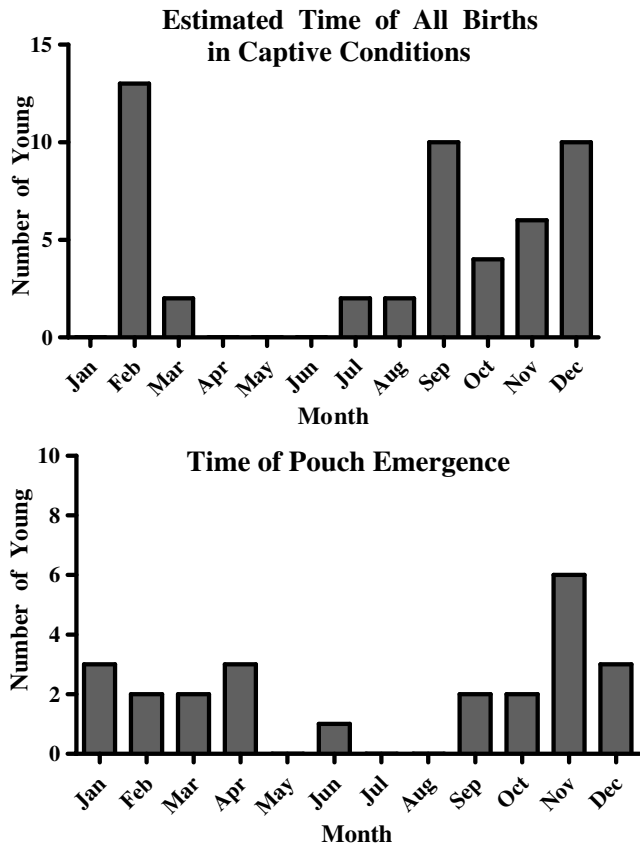


Fig. 6. Production of young from 1997 to 2002 of Honey possums (*Tarsipes rostratus*) in semi-natural captive conditions. Time of birth (above) is estimated from examination of the stage of development of pouch young. From a length of 4–5 mm after birth, and bright red in colour, crown-rump length increases at an approximate rate of 4 mm each week until the 7th–8th week of the 9-week pouch life, when furring occurs. Time of pouch emergence (below) is estimated after weaning, from the equation $y = 0.067x + 3.69$, (x = days from pouch emergence, y = body mass in g).

remainder excreted during the next 20 h. The metabolised cortisol in the faeces was excreted as 85% conjugated (aqueous phase) and 15% as unconjugated (organic phase). Of cortisol in the urine, 7.9% was excreted as glucuronides, 88.1% as sulphates and 4% as unconjugated.

3.7.2. Faecal cortisol and C_M levels

The mean level of cortisol and C_M (in parentheses) in the females was $6.1 \pm 0.19 \mu\text{g g}^{-1}$ ($9.2 \pm 1.9 \mu\text{g g}^{-1}$), comparable with the levels in the 2 males, which averaged $4.5 \mu\text{g g}^{-1}$ ($8.6 \mu\text{g g}^{-1}$). Following disturbance to the Honey possum, cortisol and C_M rose to peak at mean levels of $17.1 \pm 1.4 \mu\text{g g}^{-1}$ ($36.5 \pm 9.6 \mu\text{g g}^{-1}$) in the females and $12.1 \mu\text{g g}^{-1}$ ($34.6 \mu\text{g g}^{-1}$) in the males. An indication of the effect of disturbance on cortisol and C_M levels in 2 females (#17 and #126) is shown in Fig. 9.

3.8. Adrenal gland sizes and structure

Relative adrenal mass in mg kg^{-1} from a number of eutherian (Chester Jones, 1957) and marsupial species

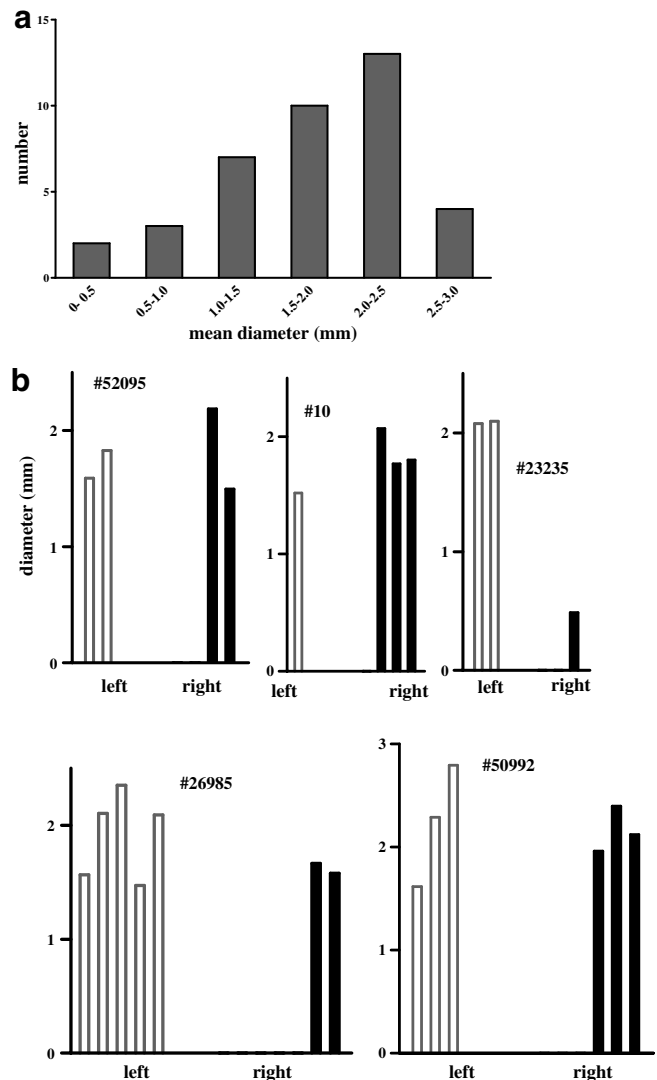


Fig. 7. Measurements of blastocysts from Honey possums (*Tarsipes rostratus*) showing (a) range of size in 39 blastocysts presumed to be in diapause, and (b) variation in size and number, both within the one uterus and between the two uteri of 4 preserved Honey possums (#52095, #23235, #26985 and #50992) and one fresh animal (#10).

(McDonald, 1977) is collated in Table 5. With the exception of the guinea pig, *Cavia porcellus*, it is evident that the size of the adrenals in both male and female Honey possums is of the order of 10 times larger than those of other mammals. Sections of the adrenal glands of a male and a female Honey possum from the colony revealed no evidence of abnormality in the gland, only hypertrophy when compared with the adrenals of other species. The cortex averaged 88% of the total area of the gland and the medulla 12%, figures that do not differ from the means of 83% and 17% respectively for 3 eutherian species ($P = 0.47$). The *zona glomerulosa* at 23.7% of the cortex compares with a figure of 10% for the rat (Wistar) and the area of the *zona fasciculata* at 52.3% of the cortex is lower than the 75% recorded for the rat. The relative size of the *zona reticularis*

Table 4

Size and number of cells in Honey possum (*Tarsipes rostratus*) unilaminar blastocysts from preserved Museum specimens and fresh captive animals in (a) early stages of development, (b) during diapause, and (c) recovered from Honey possums changed to a short photoperiod (*) or blastocysts recovered from a female with rising levels of reproductive steroids during October (**)

Animal ID	Details of blastocysts		
	Estimated age†	Diameter (n)	Cell count (n)
<i>(a) Developing</i>			
40	10 d	1.25 ± 0.27 (4)	735 (2)
37	1–2 wk	0.60 ± 0.08 (3)	
20	2 wk	0.99 ± 0.075 (4)	
M 22275	2 wk	1.68 ± 0.18 (4)	869 (1)
Means		1.16 ± 0.13 ^{a,c} (15)	779 ± 37 ^{d,f} (3)
<i>(b) In diapause</i>			
M23235	3 wk	1.56 ± 0.43 (3)	
M26985	3 wk	1.83 ± 0.12 (7)	2319 ± 541 (3)
10	5–7 mos	2.03 ± 0.06 (4)	1812 ± 191 (4)
36	“	1.03 (1)	
41	“	1.35 (1)	
118	7–9 mos	0.8 (1)	
M23254	n/a	0.53 ± 0.05 (3)	
M24550	n/a	2.52 ± 0.15 (5)	
M26926	n/a	1.73 ± 0.25 (4)	5515 (1)
M50992	n/a	2.20 ± 0.15 (6)	
M52095	n/a	1.78 ± 0.13 (4)	
Means		1.80 ± 0.10 ^{a,b} (39)	2465 ± 473 ^{d,e} (8)
<i>(c) Stimulated</i>			
7*	6–9 mos	3.38 ± 0.13 (4)	10,895 (1)
247*	6–9 mos	3.69 ± 0.17 (4)	5096 ± 473 (3)
33**	5 mos	1.22 (2)	7303 (2)
Means		3.07 ± 0.31 ^{b,c} (10)	6798 ± 974 ^{e,f} (6)

†—age is estimated from approximate age of pouch young (PY) (see Section 2). For female #s 10, 36, 41 and 118, with no PY, age is estimated from previous contact with males (see Table 2). (n)—number of blastocysts measured or counted. Means (± SEM). Significance noted by paired superscripts (^{a–a}, ^{b–b}, ^{d–d}, ^{e–e}), $P < 0.01$; paired superscripts (^{c–c}, ^{f–f}), $P < 0.001$.

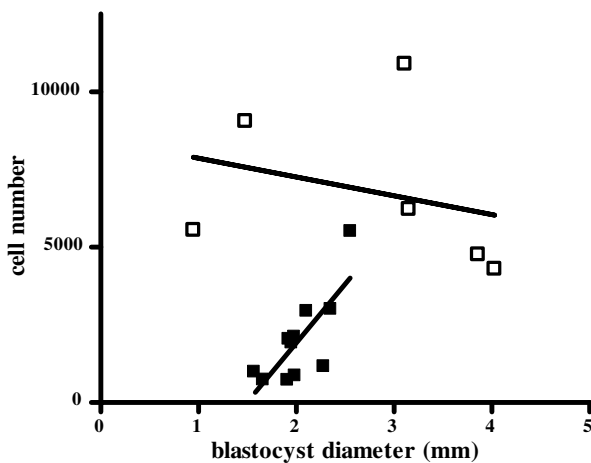


Fig. 8. Correlation between size and number of cells in: (■) unilaminar blastocysts ($y = -603.1x + 8465$; $r^2 = 0.58$; $P = 0.006$), and (□) stimulated blastocysts ($y = 3785x - 5656$; $r^2 = 0.085$; NS). Stimulated blastocysts are from female Honey possums (*Tarsipes rostratus*) maintained in photoperiod of 12 h light: 12 h dark changed to 9 h light: 15 h dark.

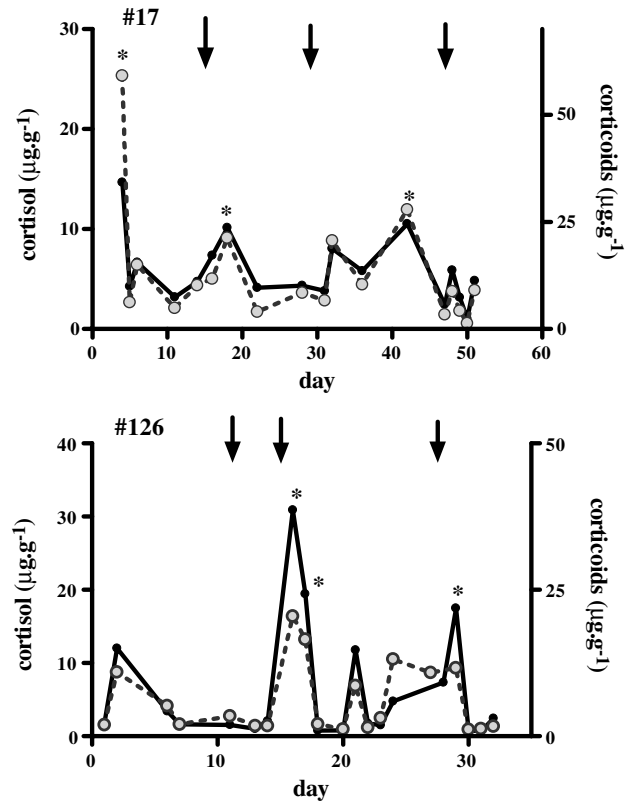


Fig. 9. Levels of faecal cortisol ($\mu\text{g g}^{-1}$) (solid line), and faecal corticoids ($\mu\text{g g}^{-1}$) (cortisol metabolites) (dashed line), in 2 female Honey possums (*Tarsipes rostratus*) housed indoors and monitored from 13 August 2001 (#17) and from 27 April 2000 (#126). Disturbance, which included capturing, weighing and pouch inspection, is marked by arrows. Values $>1\text{SD}$ above the mean are noted by an asterisk (*).

is comparable between the two species at approximately 25%.

4. Discussion

Details of the reproductive biology of the tiny marsupial Honey possum are, at best, sketchy, and it has been placed by Renfree (1981) in a separate category, together with the burramyid marsupials, because it features a diapause with certain aspects that appear to be quite different from those of macropodid marsupials. Blastocysts appear not to undergo the facultative delay of lactation (Renfree and Shaw, 2000) and have been reported to grow slowly during lactation and diapause (Ward, 1990). With the application of faecal steroid monitoring, and long-term monitoring of individuals, we have been able to provide more insight into the reproductive physiology of this unique marsupial.

4.1. Oestrous cycle

We estimate the length of the oestrous cycle of the Honey possum to be approximately 24 days, in agreement with a preliminary report by Bradshaw et al. (2004). This is shorter than the 28–32 day oestrous cycles of most macrop-

Table 5
Adrenal mass of 2 male and 4 female Honey possums maintained in the outdoor colony, and 2 field caught animals

Relative adrenal size in mammalian species		
Eutherian ^a	Adrenal mass (mg kg ⁻¹)	
<i>Homo sapiens</i>	90–100	
<i>Canis familiaris</i>	100	
<i>Ovis aries</i>	65–110	
<i>Rattus spp.</i>	100–200	
<i>Oryctolagus cuniculus</i>	100–200	
<i>Mus domesticus</i>	350	
<i>Mesocricetus auratus</i>	155–187	
<i>Cavia porcellus</i>	1175–1260	
Metatherian ^b	Male	Female
<i>Macropus giganteus</i>	40	107
<i>Macropus rufus</i>	57	108
<i>Trichosurus vulpecula</i>	60	108
<i>Vombatus hirsutus</i>	40	51
<i>Phascolarctos cinereus</i>	49	41
<i>Perameles nasuta</i>	115	110
<i>Sarcophilus harrisi</i>	171	163
<i>Dasyurus viverrinus</i>	140	
<i>Antechinus stuartii</i>	168	
<i>Didelphis virginiana</i>	300	
Mean mass:	114 ± 26	98 ± 12
<i>Tarsipes rostratus</i>		
Laboratory colony	1326	1640
	839	2420
		1569
		1308
Mean mass:		1734 ± 239
Field caught		1893
Field caught juvenile		793
Overall mean mass:	1082	1603 ± 223

Sizes are compared with those of 8 eutherian species and 10 other marsupials measured.

^a From Chester Jones (1957).

^b From McDonald (1977).

odid marsupials (Sharman, 1955b; Merchant, 1979; Merchant and Calaby, 1981; Rose and McCartney, 1982) but comparable with that of smaller marsupial species, such as 25.5 days in *Didelphis virginiana* (Hartman, 1923), 23.25 days in *Sminthopsis macroura* (Woolley, 1990) and 25.7 days in the brush-tailed possum, *Trichosurus vulpecula* (Pilton and Sharman, 1962). We have confirmed that oestrous cycling, although variable in length occurs in conditions featuring a short photoperiod, the absence of males, optimal diet, and a minimum of handling and, unlike that of the Virginian opossum, in confined conditions. Although faecal levels of hormones are only a guide to those in the plasma, the composite pattern of hormone secretion during the cycle (see Fig. 1) has a P_M profile that is not dissimilar from that of the macropodid marsupials (Cake et al., 1980; Hinds and Tyndale-Biscoe, 1982; Walker and Gemmell, 1983; Rose, 1989a, 1999). The excretion of P_M into the faeces has a biphasic profile with an early

peak that is, in this species, more closely associated with the presumed ovulatory peak of E₂. This is similar to that recently described in the squirrel glider, *Petaurus norfolcensis*, (Woodd, Czarny, Gunn and Sturrock, 2006) but its significance at this stage is unclear. We suggest that the later, and more sustained increase during the last 10 days of the cycle, represents the full functioning of the *corpora lutea*, as in other marsupials studied in this regard (Cake et al., 1980; Harder and Fleming, 1981; Tyndale-Biscoe and Hinds, 1981; Hinds and Tyndale-Biscoe, 1982; Walker and Gemmell, 1983; Gemmell et al., 1987; Fletcher, 1989; Hinds and Selwood, 1990; Jones and Rose, 1992). The lower levels prior to this may represent the output from slowly developing *corpora lutea*, or post-ovulatory follicles (Bradshaw and Bradshaw, 1992).

4.2. Pregnancy

Pregnancy, in our study, was complicated by the phenomenon of embryonic diapause. In the three pregnant females carrying blastocysts in diapause for varying lengths of time, both mean and peak levels of E₂ and P_M were not different from those of non-pregnancy. In the year-long pregnancy of #64, there is a marked increase in the excretion of E₂ during the period of shortening day length (18–28 January), that may be interpreted as a signal for terminating diapause (Weichert, 1942; Clark, 1968; McLaren, 1968; Psychoyos, 1973). We have shown that shortening day length is a stimulatory factor for cessation of diapause and blastocyst growth in the Honey possum (Bradshaw et al., 2000; Oates et al., 2004), but this pregnant female was maintained in a constant short-day length. It remains conjecture, in this case, whether the stimulus for the Honey possum is a shortening day length (as demonstrated in the tamar wallaby by Sadleir and Tyndale-Biscoe (1977), or a permissive effect of short days during this time of year. The near-term embryo found in the reproductive tract may also have resulted from the stimulatory action of oestradiol injected three days before sacrifice, rather than the raised physiological levels that the animal experienced during the previous 17–27 days. Birth did not occur, however, probably due to the stress of confinement, but we can conclude that diapause in this female, and thus pregnancy in the strict sense, lasted from 12 to 15 months.

A precise determination of the length of a gestation period that is uncomplicated by lactation, even in a captive female, may be problematic as mating may not be concurrent with fertilisation. As the Honey possum, however, is known to experience a *post-partum* oestrus (Renfree, 1980a), we may assume that a gestation period would be a little shorter than the oestrous cycle. If our estimate of the length of the oestrous cycle in the Honey possum is substantiated, we can posit a gestation period of approximately 23 days with a profile of P_M excretion largely resembling that of the oestrous cycle, as is the case for progesterone concentrations in all other marsupials studied to date (Cake et al., 1980; Harder and Fleming, 1981; Hinds and

Tyndale-Biscoe, 1982; Hinds, 1989). It is the fully functioning *corpus luteum* in the reproductive cycle of these species that supports implantation and organogenesis (Renfree, 1980b), while it is the lower levels of progesterone that are secreted after ovulation, punctuated by the early ‘spike’, that are associated with slow growth of the embryo, or even periods of embryonic stasis (Hinds and Selwood, 1990).

4.3. Embryonic development

Fewer blastocysts are produced by the Honey possum compared with other polytocous marsupials (Hill and O’Donoghue, 1913; Hartman, 1923; Godfrey, 1969) and they are considerably larger. At an average 1.8 mm in diameter, and containing approximately 2000 cells, the unilaminar blastocyst of the Honey possum is 3- to 18-fold larger than those of other polyovular species measured to date (Hill, 1910; Hartman, 1919; McCrady, 1938; Clark, 1967; Selwood, 1980; Selwood and Young, 1983; Ward, 1990), and contains between 20 and 50 times the number of cells. Diapausing blastocysts in Honey possums also range in size between females, with the largest blastocysts approximately 3.5 times the size of the smallest, which compares with 1.3 times in *Dasyurus viverrinus* (Hill, 1910) and 1.1 times in *Trichosurus vulpecula* (Selwood, 1986). Considerable variation may also be found within individual females, where the largest blastocyst in one uterus may be up to 1.8 times the size of the smallest. There is also variation in the rate of development of blastocysts within an individual, although this may not be uncommon in polytocous species (Hill and O’Donoghue, 1913).

We may gain some insight into the length of time required to reach the stage of the diapausing unilaminar blastocyst from the females whose blastocysts were not fully developed (#40 and M22275). From the age of the pouch young of these two females, the blastocysts were approximately 7–10 days-old in the one and approximately 2 weeks in the other. It would appear that the fertilised egg of the Honey possum takes a minimum of 2 weeks to complete its autonomous development before it reaches the mature blastocyst stage entering diapause. This is a few days longer than the 11 days required to reach the same stage in *Dasyurus viverrinus* (Hill and O’Donoghue, 1913). Thus, the evidence, although fragmentary, points to a slow and variable rate of growth of blastocysts even within the one animal, and once formed, a diversity in size and cell number appears to be the norm. Once stimulated, however, development involves an increase in mitotic division that is independent of the size already reached by the blastocyst. This situation is not unlike the later stages of development of the vesicular embryo of *M. eugenii*, where primitive streak formation occurs in vesicles of different sizes (Renfree, 1972; Renfree and Tyndale-Biscoe, 1973).

We found no evidence that blastocysts grow slowly once they enter diapause. Three females (#10, #36 and #41 in Table 3) containing blastocysts in diapause were maintained for 4 months; at the end of which, both size and cell

number of the blastocysts were within the diapausing range. Instead, we interpret from our data that the increasing size of blastocysts during the first 2–3 weeks of lactation is the result of their particularly slow rate of development on endogenous resources (Tyndale-Biscoe and Renfree, 1987) before they reach the final stage when they may enter diapause. This may also be the case in the small burramyid possum, *Cercartetus concinnus*, in which it has been reported that blastocysts grow during diapause (Clark, 1967; Ward, 1990). The evidence is based on an increase in diameter of blastocysts from 0.2 to 0.6 mm during the first 20 days of lactation, followed by an approximate period of 30–35 days when the “unilaminar blastocyst did not grow very much” (Ward, 1990). It is quite possible that blastocysts in this species also develop very slowly to the stage reached at diapause, considering that the gestation period may be quite long. In *Cercartetus concinnus*, the oestrous cycle length is 40.1 ± 2.2 days (Oates, 2005) and a gestation period necessarily a little shorter (like the Honey possum) could accommodate a period of 20 days or more for maturation of the blastocysts.

4.4. Organogenesis

Organogenesis in the Honey possum may be brief. New-born young measure approximately 4.5 mm in length and one litter of 3 young was reported to weigh 3, 4 and 5 mg at birth (Renfree et al., 1984). We recorded a stimulated blastocyst already at 4 mm in diameter that had only begun to differentiate pluriblast cells (L. Selwood, personal communication). The embryo recovered in our study measured a similar 4 mm, weighed 2.7 mg and was clearly late-term, as judged by the clawed digits in the fore-limb, a stage reached in two other marsupial species either on the day of birth (McCrady, 1938), or on the day prior (Selwood, 1980). Although the length of time for a bilaminar blastocyst to complete embryogenesis is variable between marsupial species, the time required for organogenesis is remarkably similar for species of similar weight (Renfree, 1980b) and, in the Honey possum, may be less than the 4–5 days in *Antechinus stuartii* (Selwood, 1980), the opossum *Didelphis virginiana* (McCrady, 1938) and the bandicoot *Isodon macrourus* (Lyne and Hollis, 1977) and equivalent to the 2–3 days in *Dasyurus viverrinus* (Hill and O’Donoghue, 1913).

4.5. Delayed gestation

The length of the delayed cycle is the time from reactivation of the unilaminar blastocyst to birth and is, therefore, shorter than the normal gestation period. We have suggested that the blastocyst reaches the diapausing stage at a minimum 2 weeks after fertilisation. A postulated 23-day gestation period, in this instance, would leave 7–10 days for embryogenesis and organogenesis to occur, the two stages that are completed during a delayed gestation. In captive conditions outdoors, two females in our colony

responded to a recorded loss of their pouch young with the appearance of a second set soon afterwards. From calculating the approximate day of birth of the second cohort, judged by their size, we estimate a maximum of 12 days in the one, and between 3 to 7 days in the other, as the length of the delayed gestation period in these two females. Some days may be required for the *corpora lutea* to recover from a lactational inhibition (Tyndale-Biscoe and Renfree, 1987) but these approximations of the short period required for embryogenesis, following on from an estimated 2 weeks for unilaminar blastocyst formation, indicate a difference in length between a normal and a 'delayed' gestation period of some 16 days. This is a little longer than the 13-day difference between the two cycles in the potoroo, *Potorous tridactylus*, (Shaw and Rose, 1979; Rose, 1989), which has been suggested as the time required for development of the blastocysts in this species.

4.6. Stress in confinement

The conditions of the captive females appear to be a critical factor for reproductive success. In addition to the pregnant female monitored long-term, our records of seven other females, maintained for lengthy periods in optimal dietary and photoperiodic conditions indoors, show that possibly one gave birth in these conditions, while the remainder only produced young when transferred to the outdoor yards. We would suggest that successful resumption of a reproductive cycle and birth, by the practice of removing the suckling young, is contingent upon the particular conditions that surround the animal. Faecal levels of corticoids, which are in micrograms per gram concentration in the Honey possum, are extremely high when compared to levels of 2–10 ng g⁻¹ in Gilbert's potoroo (*Potorous gilbertii*) (Stead-Richardson, 2005), the only other marsupial in which, so far as we are aware, faecal cortisol levels have been measured to date. These levels are also approximately a hundred-fold higher than those routinely reported in eutherian mammals (Wasser et al., 2000), except for the domestic cat (*Felis catus*), in which, baseline cortisol levels range from 30–160 µg g⁻¹ (Graham and Brown, 1996). These high glucocorticoid levels in the Honey possum, coupled with their extremely large adrenal glands, and increased excretion rates when disturbed, all indicate that this species may be stress-prone and highly susceptible to disturbances. Although oestrous cycling appears to be largely unimpeded by confined conditions, in contrast to the Virginian opossum (McCrary, 1938), we would suggest that such conditions, together with the degree of handling necessary for pouch monitoring, may well be a non-metabolic stress that inhibits some reproductive processes, such as birth (Bronson, 1998).

4.7. Diapause

The ability to retain blastocysts in diapause for varying periods of time (Bradshaw et al., 2000) is consistent with an opportunistic pattern of breeding (Wooller et al., 2000).

For the highly-specialised Honey possum, this pattern has been linked to the peak flowering periods of food plants; in natural populations, reproductive periods occur both during shortening (May to June) and lengthening days (September to October) (Wooller et al., 1984, 1999). There is evidence, however, that the first reproduction of the year is a response to the shortening day length after the southern summer solstice, operating as a *zeitgeber* (Oates et al., 2004), similar to the response seen in the macropodid marsupials (Sadleir and Tyndale-Biscoe, 1977; McConnell and Tyndale-Biscoe, 1985; Loudon and Curlewis, 1987) and the brush-tail possum, *Trichosurus vulpecula* (Gemmell, 1990; Gemmell and Sernia, 1992). The peak of births during February and March in the captive Honey possums held outdoors, provides further evidence that the first reproductive period of the year is a response to a stimulus other than an increase in food supply, as these animals were receiving a constant diet throughout. Subsequent reproductive periods, however, may be entrained to stimuli other than photoperiod.

We were unable to show a stimulatory effect of increased dietary protein during September and October, but may, instead, have uncovered an entrained reproductive rhythm. All four pregnant females had a sustained rise in E₂ during September, followed by a fall, which we interpret as an increase in ovarian activity, possibly an increase in follicular development. In the Honey possum, featuring a *post partum* oestrus, follicle maturation is associated with impending birth. That birth did not occur in these females is most likely the result of stress in confined conditions. This increase in reproductive activity is present in field populations, in which the lengthening days in September, the austral spring, herald one of the breeding periods for Honey possums in the wild and is also notable in our captive animals. In other small marsupials, similarly, it is the onset of lengthening days after the winter solstice that stimulate reproductive activity (Godfrey, 1968; McAllan and Dickman, 1986; Gemmell, 1987), although long days *per se* delay sexual maturity in the small dasyurid marsupial, *Antechinus flavipes* (McAllan and Geiser, 2006). Certainly, long days are inhibitory on ovarian steroid production in the Honey possum (Bradshaw et al., 2004). The four female Honey possums, brought in from the field during the winter solstice, would thus be expected to reproduce during the lengthening days. They were however, maintained in a short-day length. In female dasyurid marsupials when moved to a constant photoperiod (Dickman, 1985), some changes in reproductive activity were recorded. The hormonal response of the Honey possum, independent of the prevailing photoperiod, may represent an entrained reproductive rhythm.

4.8. Conclusion

It is evident that the control of reproduction displayed by the Honey possum presents something of a chimera of the varied patterns seen in other Families of marsupials, including the facultative inhibition of lactation. Breeding is

stimulated by shortening day lengths, as in the macropodids, but it also breeds during lengthening photoperiods, as do the dasyurids. Its opportunistic mode of reproduction in the face of varying food resources in nature is reminiscent of the red kangaroo, *Macropus rufus*, and the euro, *Macropus robustus*, that breed opportunistically in the arid areas of Australia (Frith and Sharman, 1964; Sadleir, 1965; Ealey, 1967; Newsome, 1975; Tyndale-Biscoe, 2005). There is also evidence that high cortisol levels associated with environmental stressors lead to the retention of diapausing blastocysts for very long periods of time and this mechanism may account for the link between food availability and breeding that has been observed in the wild. Periods of low plant flowering and food availability may be stressful for the Honey possum and elevated cortisol levels associated with this may result in a suppression of blastocyst development until such time as the animals receive a strong dietary signal.

Despite the fact that the Honey possum is one of the most specialised of any marsupial with its unique suite of dietary adaptations for a nectarivorous diet, it appears to have retained a pleisomorphic pattern of reproduction with both facultative and obligatory controls. Photoperiodic stimulation plays a rôle in its reproduction, but unlike species such as the tammar, it would not appear to be obligatory and other environmental cues allow it to breed, essentially, at any other time of the year. It is thus able to maximise its opportunities for reproduction that are only limited by the availability of the resources needed to fuel the reproductive effort.

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