

The influence of photoperiod on the reproductive activity of female Honey possums, *Tarsipes rostratus* (Marsupialia: Tarsipedidae): assessed by faecal progesteragens and oestradiol-17 β

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Abstract

Six female Honey possums were kept on a 15L:9D light regime to simulate a long daylength over summer. After seven weeks, three females (Group 2) were changed to a shorter daylength of 10L:14D, while the other three females (Group 1) were maintained on the long daylength. Faecal oestradiol-17 β and progesteragen levels were measured during the experiment to detect any changes in reproductive rhythm, such as resumption of blastocyst development. Group 2 females were found to have very large and greatly expanded blastocysts with significantly higher levels of progesteragens after the change to short photoperiod ($p < 0.05$). In contrast, the Group 1 females had very small diapausing blastocysts and progesteragen levels did not change throughout the study. Overall oestradiol levels also increased significantly in Group 2 females ($p < 0.05$) but not in Group 1 females. These results demonstrate that a change from long to short days stimulates increased progesteragen output (and oestradiol-17 β to a lesser extent) that supports the growth and expansion of the blastocysts. Photoperiod, in particular its change to a shortening daylength, appears to be a stimulus for terminating diapause in the Honey possum during its first reproduction of the year. However, as subsequent breeding later in the year occurs when daylength is increasing, a similar role for photoperiod cannot be attributed and females may be entrained to other factors such as food resources.

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1. Introduction

The tiny Honey possum, *Tarsipes rostratus* (Gervais and Verreaux, 1842), inhabits the *Banksia* heathlands of the south-west of Western Australia where it feeds exclusively on nectar and pollen (Wooller et al., 1984). Its reproductive physiology is little known except for the occurrence of embryonic diapause (Renfree, 1980). Similar to macropods, Honey possums exhibit a *post partum* oestrus, when mating occurs around the time of birth (Renfree, 1980). Unlike macropods, however, the cessation of suckling does not appear to terminate

diapause (Renfree et al., 1984). An unknown factor(s) causes the resumption of development of the blastocysts, which results in the Honey possum having a variable gestation length (Bradshaw et al., 2000; Russell and Renfree, 1989).

Young have been found in the pouch of females in every month of the year and therefore Honey possums have been classified as continuous breeders (Wooller et al., 1981). Late January to February has been recorded as the principal breeding period of the year in southern regions (Russell and Renfree, 1989), and depending on the location of the population, this is followed by either one or two less-defined peaks of breeding, that have been linked with a period of maximum flowering (Wooller et al., 1984, 2000). The synchrony of the peak of

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births during late January to February has also been observed in a captive population (Bradshaw et al., 2000) and would suggest that photoperiod may play a role in the regulation of embryonic diapause in the Honey possum.

Most species of marsupial are seasonal breeders and photoperiod seems to be an important factor determining the time of reproduction. The effect of changing daylength and administration of melatonin (Hinds and Loudon, 1997) on reproductive activity has been investigated in several species of marsupial. *Isoodon macrourus* (Gemmell, 1987b), *Sminthopsis crassicaudata* (Godfrey, 1969; Smith et al., 1978) and *Antechinus stuartii* (McAllan et al., 1991) are all long-day breeders, and ovulation and pregnancy was inhibited by the simulation of shortening daylength. *Trichosurus vulpecula* is a short-day breeder and exogenous melatonin was found to stimulate ovulation and hence the start of the breeding season (Gemmell, 1987a).

Two species of wallaby, the tammar wallaby (*Macropus eugenii*) and Bennett's wallaby (*Macropus rufogriseus rufogriseus*), exhibit embryonic diapause and are highly seasonal breeders. For a time after the winter solstice the *corpus luteum* and embryo remain in quiescence, regardless of the presence of a pouch young (Curlewis et al., 1987). Several weeks after the summer solstice, there is a precise start to the breeding season with the reactivation of the embryo (Renfree and Tyndale-Biscoe, 1973). Only in mid- to late seasonal quiescence does treatment with exogenous melatonin, or a change from long to short photoperiod, result in termination of diapause in both the tammar (Hinds and den Ottolander, 1983; McConnell and Tyndale-Biscoe, 1985) and Bennett's wallaby (Loudon and Curlewis, 1987; Loudon et al., 1985). A longer daylength appears to initiate quiescence and a long period of increasing photoperiod may be required before shortening days will initiate reactivation of the embryo (Loudon and Curlewis, 1989). The Honey possum would appear to be similar to the tammar and Bennett's wallaby, with its synchronous resumption of breeding during February.

The present study investigated whether a change from a long to a short photoperiod may terminate embryonic

diapause in the Honey possum. To monitor any changes in reproductive activity during the experiment, use was made of faecal steroid analysis. This technique is widely-used for the detection of reproductive stages in endangered animals (Schwarzenberger et al., 1996, 1997), and is ideal for a small animal such as the Honey possum for which traditional methods of sampling are not possible. If photoperiod does indeed influence reproductive activity in the Honey possum, females changed from the long to short photoperiod may terminate diapause, resulting in birth and the appearance of young in the pouch. Modulation of the hormones excreted in the faeces, oestradiol-17 β , progesterone and its metabolites, should provide evidence of any changes in reproductive rhythm during the experiment such as a resumption of development in blastocysts.

2. Materials and methods

2.1. Animals and experimental design

Six female Honey possums were wild-caught from Scott National Park, (Lat. 34°17'S, Long. 115°13'E) Western Australia during June and July (see Table 1). Honey possums are a threatened species and only small numbers are allowed for experimentation due to ethical constraints. We have understood June and July to be a mating period and the likelihood was high that all females were carrying blastocysts. No pouch young were evident in any female at capture. All females were housed in outside animal yards in semi-natural conditions (Bradshaw et al., 2000) at the University of Western Australia in the presence of males until entering the experiment on the 20 September 2002, the approximate time of the spring equinox. Again, no pouched young were evident and we have assumed that any young that may have been born were lost during confinement. Animals were placed in a controlled temperature room (CTR) regulated at 22 °C during the day and 15 °C during the night. All six females were maintained on a long photoperiod of 15 h light and 9 h dark. The experiment was designed to advance the time by one

Table 1

Time of collection, time of entry to CTR, light regime experienced and reproductive condition for the six female Honey possums involved in this study

Animal No.	Body mass (g)	Date of collection	Date of entry to CTR	Group No.	Light cycle		Reproductive condition
					Period 1	Period 2	
118	9.0	June 2002	20 September 2002	1	15L:9D	15L:9D	Small diapausing blastocysts
115	8.6	June 2002	23 October 2002	1	15L:9D	15L:9D	8 unfertilised ova
6	10.9	July 2001	20 September 2002	1	15L:9D	15L:9D	Reproductive tract decomposed
247	15.5	June 2002	20 September 2002	2	15L:9D	10L:14D	4 large expanded blastocysts
7	10.0	June 2002	20 September 2002	2	15L:9D	10L:14D	4 large expanded blastocysts
2	8.2	June 2002	20 September 2002	2	15L:9D	10L:14D	Reproductive tract decomposed

month that the animals in their natural environment would be exposed to the summer solstice (December 23). Therefore, on 17 November 2003, approximately one month before the summer solstice, three of the six animals (Group 2) were placed in another CTR at the same temperature, but on a short photoperiod of 10 h light and 14 h dark. The remaining three animals (Group 1) were kept on the long photoperiod for the remaining 4 months of the experiment. Female #115 was included in the experiment approximately one month later (23 October 2003) than the other animals.

Animals were housed in small Perspex cages (60 cm × 40 cm × 30 cm) with vegetation and sawdust for shelter and were fed daily with 10 ml of diet comprising *Banksia* honey, sandplain pollen, a protein supplement and water. The diet contained 22% sugar and approximately 30 mg N/day, shown to be reflective of the nectar and pollen intakes in field animals (Bradshaw and Bradshaw, 1999). Fresh blossoms were placed in the cages each week to supplement their diet. Cages were cleaned and animals weighed fortnightly.

Faeces (10–50 mg) were collected daily from Monday to Friday at the same time as the animals were fed. Only freshly voided faeces were selected and stored individually in Eppendorf containers without preservative at –15 °C until processing.

2.2. Antisera

The oestradiol-17 β antiserum (C-9757 from Bioquest, NSW, Australia) was produced in sheep by immunising with 17 β -oestradiol-6-CMO-BSA and cross-reacted 100% with 17 β -oestradiol, 1% with oestrone, oestradiol-17 α and oestriol, and <0.1% with testosterone, androstenedione, progesterone, 17-hydroxyprogesterone, and cortisol. The monoclonal progesterone antiserum (provided courtesy of Dr. Val Lance, CRES, San Diego Zoo, USA) was raised against 4-pregnane-11-ol-3, 20-dione hemisuccinate: BSA and cross-reacted 100% with progesterone, 96% with 5 α -pregnane-3 β -ol-20-one, 36% with 5 α -pregnane-3 α -ol-20-one, 15% with 5 β -pregnane-3 β -ol-one and 17 β -hydroxyprogesterone, 13% with pregnenolone, 7% with 5 β -pregnane-3 α -ol-20-one, 5% with 5 β -pregnane-3 α , 17 α -diol, 20 α -one and <1% with pregnanediol-3-glucuronide, androstenedione, testosterone, oestradiol-17 β , oestrone, oestriol, 21-hydroxyprogesterone, 20 α -hydroxyprogesterone, and cortisol (Grieger et al., 1990; Wasser et al., 1994).

2.3. Faecal extraction

Faeces were dried overnight at 45 °C. Any foreign material, such as sawdust or plant matter, was removed and the samples crushed to a powder. The dried faeces (ranging from >10 to 30 mg) were weighed into a glass test tube and 300 μ l of distilled water was added and

vortexed. Samples less than 10 mg were not included and all samples were individually assayed. Di-ethyl ether (3 ml) (AR grade, Merck) was added to each sample and vortexed for 1 min. The ether layer of the supernatant was pipetted to a clean glass tube. The pellet was re-suspended in 2 ml of di-ethyl ether, and the process repeated, combining the two supernatants. Extraction efficiency averaged 95 \pm 2.0% and 95 \pm 2.3% for oestradiol-17 β and progesterone, respectively. The supernatants were dried under compressed air at 37 °C and taken up in 2 ml of 100% ethanol (AR grade, Merck). Eight hundred microliters (1:2.5 dilution) and 20 μ l (1:100 dilution) of the extract were dried under compressed air for the oestradiol-17 β and progesterone assays, respectively.

2.4. Radioimmunoassay

The purified extracts, together with dried ethanolic standards of oestradiol-17 β (Sigma Chemicals), were incubated overnight at 4 °C in 0.01 M PBS–BSA buffer (pH 7.0) containing oestradiol-17 β antiserum (1:35,000 final dilution) and 167 Bq [2,4,6,7-³H]oestradiol-17 β (Amersham (Buckinghamshire, UK)). The progesterone extracts and standards were incubated for 4 h at 4 °C in PBS–BSA buffer (pH 7.0) containing progesterone antiserum (1:12,500 final dilution) and 167 Bq [1,2,6,7-³H]progesterone (Amersham). Samples and standards were placed in an ice-bath for 15 min, after which, 1 ml of cold (0–2 °C) dextran-coated charcoal suspension (500 mg acid-washed charcoal, Norit A (ICN Pharmaceuticals, Ohio, USA) and 50 mg dextran T500 (Pharmacia Chemicals, Uppsala, Sweden) in 100 ml PBS–BSA buffer) was added and briefly vortexed. All tubes were incubated at 0 °C for exactly 10 min, before being centrifuged at 4000 rpm and 0 °C for 10 min. An aliquot of the supernatant (600 μ l) was added to 3 ml scintillant (Ultima Gold, Packard Technologies) and counted three times for 10 min each in a Packard Tri-Carb 2300TR Liquid Scintillation Analyser to less than 1% error with automatic quench correction. Standard data were transformed using a 4-parameter logistic equation and oestradiol-17 β and progesterone concentrations were expressed as nanograms/gram of dried faeces (ng g⁻¹).

In view of the high specificity of the oestradiol-17 β antibody we can be confident we were indeed measuring oestradiol-17 β (E₂) in the faecal samples. The progesterone antibody, on the other hand, was chosen for its cross-reactivity with progesterone metabolites (PM) that have been identified in the faeces of the Honey possum (Bradshaw et al., 2004) and referred to here as progestagens (Schwarzenberger et al., 1996). GCMS analysis of faecal samples by Bradshaw et al. (2004) failed to find any evidence of oestradiol metabolites that may have cross-reacted with the antibody.

2.5. Validation of faecal steroid measurements

Using the method of Frankel et al. (1967), sensitivity of the oestradiol-17 β assay was 0.29 ng g⁻¹ with 95% probability. The progesterone assay sensitivity was 0.41 ng g⁻¹ with 95% probability. Samples of a pooled collection of female faeces were assayed to determine intra- and inter-assay variation. The intra- and inter-assay variation was 10.3 and 17.1%, respectively, for the oestradiol-17 β assay. The intra- and inter-assay variation for the progesterone radioimmunoassay was 4.1 and 7.5%, respectively. Serial dilutions of an ethanolic extract of Honey possum faeces showed parallelism with the standard curve for both the progesterone ($p = 0.91$) and oestradiol-17 β ($p = 0.10$) assay. Dose–response studies were performed for each assay by adding 50, 100, 250 and 500 pg of unlabelled steroid to aliquots of a faecal extract. The oestradiol-17 β dose–response study generated a line with a slope of 1.1 ($r^2 = 0.997$) and the progesterone dose–response study produced a line with a slope of 0.99 ($r^2 = 0.998$).

2.6. Histology and reproductive condition of females

During this study there were no visible pouch young either in, or discarded from, the pouch. It was therefore necessary to determine the reproductive condition of each animal by dissection of the reproductive tracts. Females were euthanased with a lethal injection of Nembutal (Boehringer Ingelheim, NSW, Australia) at the completion of the study and their reproductive tracts were immediately removed. A small incision was made at the base of each uterus and scissors were used to gently produce a longitudinal incision. The uteri were opened and any blastocysts or ova were removed and immediately photographed. The blastocysts and ova were fixed in buffered formal saline (BFS) and stained with haematoxylin and Scott's solution and mounted in glycerol. The diameter and number of nuclei for each blastocyst were measured using ImagePro software.

2.7. Characterisation of profiles of faecal hormones and their statistical analyses

A detailed description of each hormone profile is presented in four of the six animals (Figs. 1 and 2), as two of the females (Nos. 6 and 2) died before the completion of the study, and consequently their profile was incomplete. Of the four animals, each profile was divided into two periods: for Group 1 animals, both periods consisted of long days, and for Group 2 animals, the first period consisted of long days and the second period of short days.

The results from all six animals, however, were included in calculating the mean overall levels of PM

and E₂ in the faeces (Figs. 3 and 4). Again, each profile was divided into two periods: for Group 1 animals, both periods consisted of long days, and for Group 2 animals, the first period consisted of long days and the second period of short days. Estimation of baseline and identification of peaks in PM and E₂ used an iterative process (Graham et al., 2002). The average concentration of all samples in each profile was calculated and values greater than 1.75 SD above the mean were considered significant and removed from the series. This process was repeated until no value was greater than 1.75 SD above the mean. The average of the remaining values was considered a baseline level. Peaks that were >4 SD above baseline levels were considered significant for both PM and E₂. The degree of correlation between PM and E₂ was assessed by correlation analysis and is an indication of the secretory nature of the ovary. If the levels are highly correlated, we interpret this as single tissue being the source of their secretion and such positive correlation may be the case during pregnancy (Curtis et al., 2001). On the other hand, a lack of correlation between the two hormones suggests separate origins for progesterone and oestradiol, indicating the events associated with follicular and luteal phases of the reproductive cycle.

Four-point moving averages were performed on the profiles to reduce noise in the data and identify any cyclical patterns that may be present. Two-way analysis of variance (ANOVA) was used to compare baseline, peak and overall means of faecal PM and E₂ concentrations between the two treatment groups and time periods.

3. Results

The results are presented below, first with descriptions of the hormone profiles for the four individuals that completed the study (Figs. 1 and 2). Second, a comparison of PM and E₂ levels in each light regime between the two treatment groups is described (Figs. 3 and 4) and this includes data from all six females. Slight decomposition of the reproductive tract in the two individuals (Nos. 6 and 2) that died during the course of the investigation prevented a positive identification of blastocysts or ova. Examination of their steroid profiles, however, revealed no obvious changes in levels prior to their death, and they were thus not excluded in the calculation of overall means.

3.1. Group 1 females

3.1.1. #118

As can be seen from Fig. 1A, both PM and E₂ levels did not change greatly while #118 was maintained continuously on a long photoperiod. Mean overall PM lev-

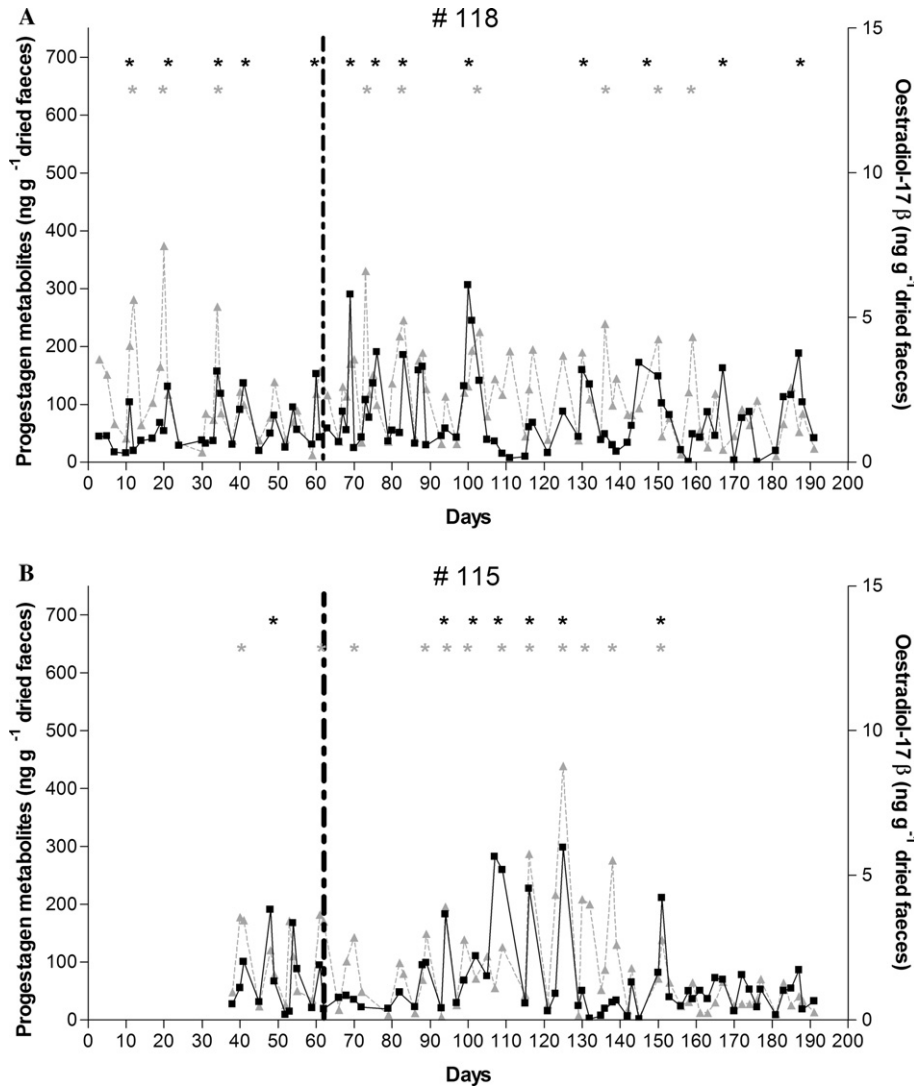


Fig. 1. Profile of faecal progesteragens and oestradiol-17β in Group 1 females (A) #118 and (B) #115. Levels are expressed in ng g^{-1} of dried faeces for each day sampled. Black broken line indicates the two time periods. Black squares = progesteragen levels, grey triangles = oestradiol-17β levels. Black * and grey * indicate significant peaks in PM levels and E_2 levels, respectively.

els did not differ significantly between the two assigned periods, increasing slightly from 62 ± 8 to $85 \pm 9 \text{ ng g}^{-1}$, and baseline levels remained constant at $36 \pm 3 \text{ ng g}^{-1}$. After day 60, #118 exhibited fairly regular peaks in PM and these cycles in secretion averaged 21.6 ± 2.2 days in length.

In contrast to the pattern of PM, there were no obvious cyclic changes in E_2 levels. The overall E_2 concentration averaged $2.3 \pm 0.3 \text{ ng g}^{-1}$ for the entire period. Mean baseline levels of E_2 were similar between the two periods with concentrations of 1.8 ± 0.2 and $1.6 \pm 0.1 \text{ ng g}^{-1}$. Peak E_2 levels averaged $6.2 \pm 0.7 \text{ ng g}^{-1}$ during the first period and actually decreased to $4.8 \pm 0.3 \text{ ng g}^{-1}$ later in the study.

There was a significant positive correlation between the levels of PM and E_2 ($p < 0.01$), suggesting that both hormones are secreted from a similar source. Removal

of the reproductive tract of female #118 revealed a small blastocyst with a diameter of 0.73 mm. Unfortunately, other blastocysts were lost during dissection.

3.1.2. #115

Overall PM levels remained similar in #115 throughout the sampling time, averaging levels of 68 ± 16 and $65 \pm 10 \text{ ng g}^{-1}$ for the two periods. However, peak levels of PM increased from 179 ± 11 to $196 \pm 32 \text{ ng g}^{-1}$ between the two periods. This was due to a series of peaks in PM starting at day 94, before levels decreased dramatically after 129 days (Fig. 1B). Levels then remained low for the rest of the period except for a brief peak of 211 ng g^{-1} at day 151. Baseline PM levels were shown to decrease over the two periods from 48 ± 10 to $30 \pm 3 \text{ ng g}^{-1}$, mainly due to the very low concentrations towards the end of the study.

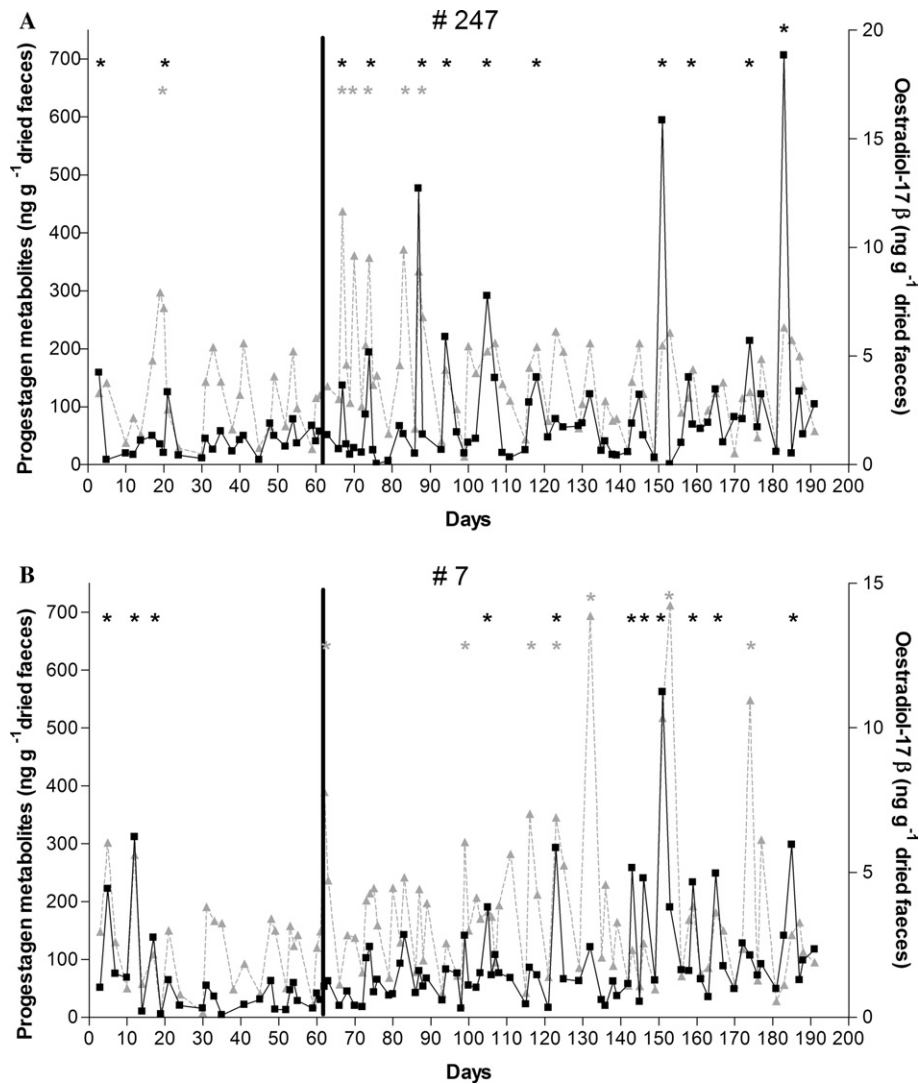


Fig. 2. Profile of faecal progesteragens and oestradiol-17 β in Group 2 females (A) #247 and (B) #7. Levels are expressed in ng g^{-1} of dried faeces for each day sampled. Black solid line indicates the two photoperiods. Black squares = progesteragen levels, grey triangles = oestradiol-17 β levels. Black * and grey * indicate significant peaks in PM levels and E₂ levels, respectively.

The pattern of E₂ secretion was very similar to that of PM. There was little change in the overall levels, averaging $1.7 \pm 0.2 \text{ ng g}^{-1}$ for the entire period. Peak levels of E₂ were higher in the second period rising from 3.6 ± 0.1 to $3.9 \pm 0.5 \text{ ng g}^{-1}$. This was due to several peaks of E₂ in succession between days 116 and 142, similar to PM. After this succession of peaks, E₂ levels decreased and remained at low concentrations.

The cyclical pattern of PM secretion present in all other females studied was not evident in #115. Although the levels of PM and E₂ were significantly correlated overall ($p < 0.05$), levels were not positively associated during the period of spiking in both hormones. Progesteragen levels were first to increase on day 94 followed by spikes in E₂ concentrations beginning on day 116. Progesteragen levels dropped to very low concentrations at day 129, while oestradiol-17 β remained elevated until

it returned to basal levels 7 days later. These peaks in E₂ preceded by a drop in PM levels may indicate ovulation in this female. Dissection of the reproductive tract of #115 revealed eight degenerating unfertilised eggs with an average diameter of $0.28 \pm 0.002 \text{ mm}$. The occurrence of ovulation is also supported by the E₂: PM ratio where there is little change until a large peak value of 1.8 at day 132. It appears this female has ovulated and then hormone levels have remained low for the remainder of the study.

3.2. Group 2 females

3.2.1. #247

While #247 was maintained on long photoperiod the overall PM levels ($x = 48 \pm 7 \text{ ng g}^{-1}$) were low and increased significantly to $95 \pm 16 \text{ ng g}^{-1}$ under short day-

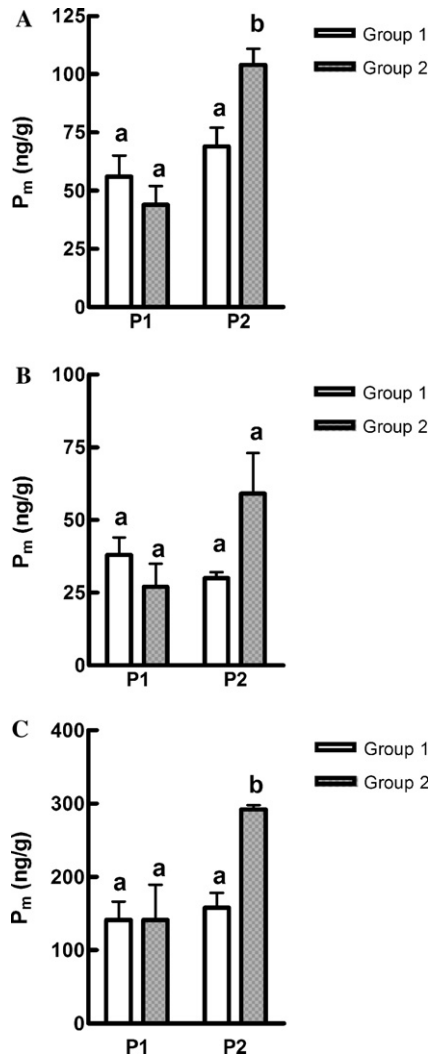


Fig. 3. Mean (A) overall, (B) baseline and (C) peak progesterone levels in Group 1 (white) and Group 2 (grey) female Honey possums. P1 (Period 1) and P2 (Period 2) on the x-axis denotes the two periods of photoperiod. Differing lowercase letters within treatments (P1 and P2) indicate statistical significance with $p < 0.05$.

length ($p < 0.05$). Fig. 2A demonstrates PM levels were very low until the change in photoperiod and then exhibited cycles in PM output, approximately 21.8 ± 2.2 days in length. Four-point moving averages revealed a trend of increasing levels in PM in these successive cycles after the photoperiod change. Further evidence of elevated PM concentrations after the photoperiod change was the significantly greater peak levels under short days ($x = 299 \pm 67 \text{ ng g}^{-1}$), compared to only $142 \pm 17 \text{ ng g}^{-1}$ under long days ($p < 0.05$).

The pattern of E₂ secretion was strongly correlated with that of PM ($p < 0.001$) as can be seen from the profile (Fig. 2A). The overall E₂ levels were the highest of any animal, with a mean of $3.1 \pm 1.8 \text{ ng g}^{-1}$. After the change to short photoperiod there was a series of large peaks in E₂ in a small amount of time. Following these peaks, the E₂ output appears to be slightly elevated.

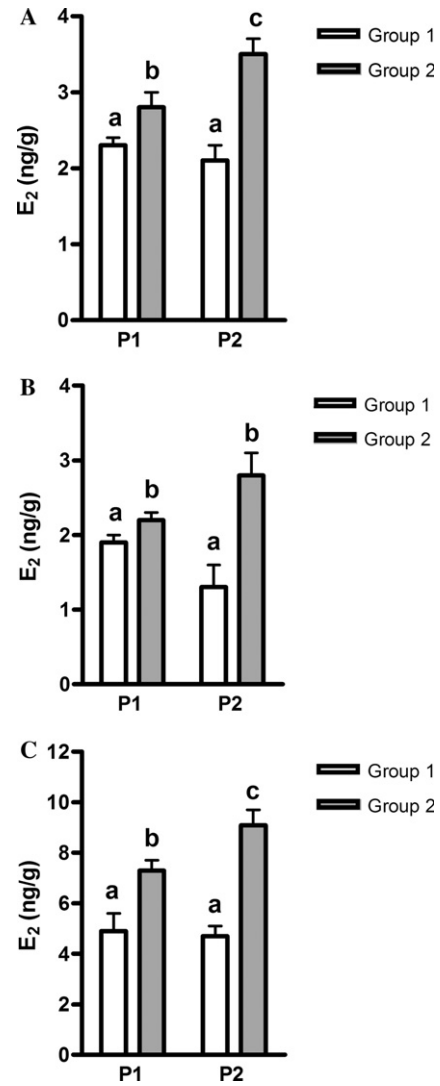


Fig. 4. Mean (A) overall, (B) baseline and (C) peak oestradiol-17β levels in Group 1 (white) and Group 2 (grey) female Honey possums. P1 (Period 1) and P2 (Period 2) on the x-axis denotes the two periods of photoperiod. Differing lowercase letters within treatments (P1 and P2) indicate statistical significance with $p < 0.05$.

Baseline levels significantly increased from 2.4 ± 0.3 to $3.4 \pm 0.2 \text{ ng g}^{-1}$ ($p < 0.01$) and peak levels rose from 7.6 ± 0.4 to $9.9 \pm 0.5 \text{ ng g}^{-1}$ ($p < 0.05$). Female #247 was discovered to have four greatly expanded blastocysts, two in each uteri. All the blastocysts were similar in size with a mean diameter of $3.53 \pm 0.15 \text{ mm}$ and contained an average 8686 ± 290 nuclei. This suggests that the elevated levels of the two hormones, PM in particular, have supported the growth and expansion of the blastocysts.

3.2.2. #7

Progesterone levels, overall, were low in #7 while maintained on the long photoperiod ($x = 58 \pm 13 \text{ ng g}^{-1}$), before gradually increasing during the short photoperiod ($x = 96 \pm 11 \text{ ng g}^{-1}$). Moving averages illustrated a cyclic

secretion pattern of 21.8 ± 2.3 days in length, with peak levels increasing steadily over the course of the experiment. The mean peak PM level increased from $224 \pm 50 \text{ ng g}^{-1}$ under long days to $280 \pm 38 \text{ ng g}^{-1}$ during short days. There was also a significant increase in baseline PM from 37 ± 5 to $55 \pm 4 \text{ ng g}^{-1}$ ($p < 0.01$).

Changes in E_2 concentrations were closely associated with the changes in PM ($p < 0.0001$). Overall E_2 levels were elevated from $2.6 \pm 0.4 \text{ ng g}^{-1}$ under long days to $3.5 \pm 0.3 \text{ ng g}^{-1}$ under short days. Peak levels of E_2 were also higher during short days ($x = 9.5 \pm 1.6 \text{ ng g}^{-1}$) compared to $7.8 \pm 0.1 \text{ ng g}^{-1}$ during long days and this is clearly illustrated in Fig. 2B. Dissection of the reproductive tract revealed two largely expanded blastocysts in each of the uteri. Several of the blastocysts had collapsed, but one was successfully retrieved and contained approximately 10,895 nuclei with a diameter of 3.82 mm. The elevated PM levels, and to a lesser extent, high E_2 levels are similar to those in #247 and appear to have supported the growth and development of the blastocysts.

3.3. The effect of changing photoperiod: differences between Group 1 and Group 2 females

Two-way analysis of variance revealed that mean overall PM levels in Group 2 females increased significantly from $44 \pm 8 \text{ ng g}^{-1}$ during long days to $104 \pm 7 \text{ ng g}^{-1}$ during short days ($p < 0.05$), whereas the overall levels for Group 1 females increased only slightly from 56 ± 9 to $69 \pm 8 \text{ ng g}^{-1}$ while continually maintained on long days (Fig. 3A). Similarly, peak PM concentrations increased significantly from 141 ± 48 to $292 \pm 6 \text{ ng g}^{-1}$ ($p < 0.05$) in females from Group 2, with Group 1 females showing a much smaller increase from 141 ± 25 to $158 \pm 20 \text{ ng g}^{-1}$ (Fig. 3C). There were no significant differences in mean baseline concentrations ($p = 0.15$), although Group 2 females exhibited an increase, while levels of Group 1 females decreased (Fig. 3B).

The overall E_2 levels were found to increase significantly in Group 2 animals, increasing from $2.8 \pm 0.2 \text{ ng g}^{-1}$ during long days to $3.5 \pm 0.2 \text{ ng g}^{-1}$ ($p < 0.05$) under short days (Fig. 4A). In Group 1 animals overall levels did not vary during the experiment averaging 2.3 ± 0.1 in the first period and $2.1 \pm 0.2 \text{ ng g}^{-1}$ in the second period. Similarly, baseline E_2 levels also decreased in Group 1 from 1.9 ± 0.1 to $1.3 \pm 0.3 \text{ ng g}^{-1}$ and increased slightly from 2.2 ± 0.1 to $2.8 \pm 0.3 \text{ ng g}^{-1}$ in Group 2 (Fig. 4B). Mean peak levels did not vary in Group 1 females averaging 4.9 ± 0.7 and $4.7 \pm 0.4 \text{ ng g}^{-1}$, but increased significantly from 7.3 ± 0.4 to $9.1 \pm 0.6 \text{ ng g}^{-1}$ ($p < 0.05$) in Group 2 females (Fig. 4C). Within the same time period mean baseline, peak and overall E_2 levels were always significantly higher in Group 2 females compared to females in Group 1 ($p < 0.05$).

4. Discussion

The experiment was designed to advance by one month the change in photoperiod that Honey possums experience after the summer solstice. The photoperiod change induced in this study was more exaggerated than that which the Honey possum naturally experiences, in order to magnify any differences between the groups of animals. It was evident from the results that photoperiod does play a role in the control of embryonic diapause in this species. The three females in which short days were advanced (Group 2) exhibited higher levels of E_2 and PM in particular, than the Group 1 females kept on long days. In addition, growth and development of the blastocysts in the Group 1 females were significantly stimulated by the premature decrease in daylength and were much larger than the blastocysts of Group 2 females.

There is virtually no information regarding plasma levels of reproductive hormones, or their pattern of secretion in the female Honey possum. A preliminary investigation into long-term faecal hormone excretion in the Honey possum (Bradshaw et al., 2004) suggests an oestrous cycle for this species of approximately 25 days that was estimated from cyclical changes of both oestradiol and progesterone. It is well established in marsupials that the *corpus luteum* is the major source of progesterone (Gemmell, 1995; Tyndale-Biscoe, 1986; Tyndale-Biscoe and Renfree, 1987) and in the macropodid wallaby, *Setonix brachyurus*, it has also been shown to secrete oestradiol (Bradshaw and Bradshaw, 1992). In the tammar, *M. eugenii*, Shaw and Renfree (1984) found an increase in plasma oestradiol coinciding with a peak in progesterone, suggesting a luteal origin for oestrogens at this time. We have interpreted a positive correlation between the levels of faecal PM and E_2 over time as secretion principally from a single tissue. In all the females in this study, while blastocysts were present, the secretion of E_2 and PM was highly correlated and may indicate the *corpora lutea* as the principal source of hormonal support for the diapausing blastocysts. Interestingly, during this correlated phase of secretion, there appeared to be cycles in production of PM that were consistently 21 or 22 days in length except for one female in which it was 17 days.

In the non-pregnant female (#115), PM and E_2 were also correlated except during the time of suspected ovulation. This period was characterised as ovulatory because the E_2 : PM ratio peaked dramatically before quickly falling to baseline levels. Peak E_2 : PM ratios have been used as a hormonal indicator of ovulation in similar studies (Curnow et al., 2001; Curtis et al., 2001). After ovulation both steroid levels were low, perhaps due to being held on an inhibitory light regime or due to the stress of captivity.

Generally, it appears that overall levels of PM above 90 ng g^{-1} and E_2 levels above 3 ng g^{-1} are the minimum

requirement for stimulating growth and development of the blastocysts. The small diapausing blastocysts found in #118 were slightly smaller in size (0.73 mm in diameter) than the 1.2–2.0 mm reported by Renfree (1980) and Renfree and Calaby (1981). The expanded blastocysts in this study were up to 3.82 mm in diameter, and this increase appears to have been supported by higher levels of PM, stimulated by the change in daylength.

Manipulative experiments of this type are difficult due to the size and fragile nature of the Honey possum. Also, reproductive cycles cannot be initiated by the removal of pouch young, as occurs in most macropodids. It is, therefore, virtually impossible to identify the reproductive condition of the animal at the start of the experiment, except in the case of lactation. It was important for this study that all females should be in the same reproductive condition and by taking the animals from the field at the same time this should have ensured that the blastocysts were of a similar age. It was evident after dissecting the reproductive tract at the completion of the study that all females had blastocysts except #115, which had ova. This female was put into the experiment at a later date and although it had been in the presence of males, it may not have had blastocysts at this time.

Variation among individual hormone profiles was apparent, but the change in photoperiod was shown to have a significant effect on the reproductive activity of female Honey possums. Group 2 females, when changed from a long to a short daylength, had very large blastocysts that had expanded to 3.5–3.8 mm in diameter and correspondingly high levels of PM. Overall E_2 levels also increased significantly in Group 2 females after the change to a shorter daylength. These data suggest that a change from long to short days stimulates increased output of PM (and E_2 to a lesser extent), which is associated with the growth and expansion of the blastocyst.

In contrast, the Group 1 females showed very small diapausing blastocysts or ova, with correspondingly lower levels of PM. The reproductive activity of Group 1 females appeared to be inhibited by long days, with low levels of faecal E_2 and PM. The non-pregnant female (#115) appeared to have ovulated while maintained on long days, suggesting that long daylength is not necessarily inhibitory. The period following the suspected ovulation, however, is characterised by low hormone levels that were suppressed for the remainder of the study, together with degenerating ova.

The effect of photoperiod in this study has highlighted a distinction amongst the small marsupials in which the Honey possum differs from the small dasyurid marsupials studied to date, where it is the onset of lengthening, rather than short days that stimulates reproductive activity (Godfrey, 1969; McAllan et al., 1991; Smith et al., 1978). Instead, the situation in the Honey possum resembles that of the much larger macropodids, in that a change to short daylength is responsi-

ble for initiating the first reproductive cycle of the year (Loudon and Curlewis, 1987; McConnell and Tyndale-Biscoe, 1985; Sadlier and Tyndale-Biscoe, 1977). As with macropodid marsupials, the results from this experiment suggest that the change from longer to shorter days may function as a *Zeitgeber*, synchronising endocrine changes needed for the first reproductive period of the year. As subsequent breeding later in the year occurs when daylength is increasing, a similar role for photoperiod cannot be attributed and females may be entrained to other factors, such as food resources related to their nectarivorous diet.

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