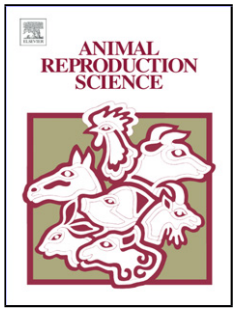

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Non-invasive monitoring of male and female numbat (*Myrmecobius fasciatus:* Myrmecobiidae*)* reproductive activity

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ABSTRACT

The reproductive endocrinology of the highly endangered numbat (*Myrmecobius fasciatus*) is described for the first time. Patterns of faecal steroid secretion (progesterone [PM], oestradiol-17β [E2] and testosterone [TM] metabolites) were examined within a captive numbat population over 1 year and revealed a highly synchronized seasonal pattern of reproduction. TM secretion increased progressively from September to November, peaked in December and then decreased in February. All females displayed luteal phases (1 to 3), between late-November to late-March, in association with pregnant (Pr, *n* = 4), non-productive mated oestrous cycles (NMEC, *n* = 8) and non-mated oestrous cycles (NEC, *n* = 6). The mean oestrous cycle length was 30.2 ± 1.1 d (*n* = 11) and was comprised of a mean follicular (*n* = 11) and luteal (*n* = 18) phase length of 16.2 ± 1.6 d and 14.0 ± 0.8 d, respectively. No variation in mean luteal phase length or PM concentration according to cycle type (Pr, NMEC, NEC) or cycle number (1st, 2nd or 3rd cycle) was detected. Longitudinal profiling of PM secretion confirmed that the female numbat is seasonally polyestrous and that the luteal phase occurs spontaneously. Changes in the secretion of E2 provided little instructive information on oestrous cycle activity. Mating success was 31%, with age and subject having no effect on mating success. Timing of introduction, of male to female, appeared to impact mating success, with paired animals introduced for a shorter time frame (≤ 14 d) prior to the first observed mating successfully producing young. Collectively, results of the present study confirm that PM and TM can be reliably used to index numbat reproductive activity.

Keywords: Enzyme-immunoassay; Faecal steroids; Oestrous cycle; Progesterone; Seasonality; Testosterone

1. Introduction

The numbat (*Myrmecobius fasciatus*) is a small (~500g) termitiverous marsupial, with a distribution currently limited to two naturally occurring remnant populations in Western Australia (WA) and to several smaller re-introduced populations in New South Wales, South Australia and WA (Friend and Thomas, 2003). With fewer than 1,000 numbats remaining in the wild, captive breeding is an important component of the conservation effort (Friend and Burbidge, 2008). At present, there is only one captive breeding colony of numbats in the world (Perth Zoo, WA) and the rate of reproductive failure in this species (18.5% to 55.3%: Lawrence et al., 2008; Power et al., 2009) is great compared to that (23% to 27%) of other captive marsupials (Fletcher 1989; Duckworth et al., 1998). The etiology of this failure is currently unknown, but a better understanding of numbat reproductive endocrinology should provide valuable insight into this problem.

Previous studies into numbat reproductive biology have identified the female to be polygynous, facultatively polyestrous and polyovular (Power and Monaghan, 2003; Power et al., 2009). Breeding is known to be highly seasonal, with most females coming into oestrus in January (Friend and Burbidge, 2008). Males also have a distinct reproductive cycle, undergoing seasonal changes in sperm production, gland secretion, testicular and accessory gland size (Power et al., 2009). Currently, there is no published information on the reproductive endocrinology of male or female numbats. As such information on the frequency, duration and ovulatory mechanisms of the oestrous cycle has largely been inferred from behavioural observations. Endocrinology is not only important for characterising the reproductive cycle and timing of reproductive events in the female numbat, but also for validating the practical value of using other oestrous detection procedures, e.g. urogenital cytology (Power et al., 2009). Documenting male

androgen concentrations will also lead to a better understanding of breeding synchrony between the sexes and help assess male fertility (Millis et al., 1999).

To date, progesterone concentration has been measured throughout the reproductive cycles of only 30 of the 210 extant species of marsupials (review: Bradshaw and Bradshaw, 2011), nine of whom belong to the family Dasyuridae (the taxa the numbat is most closely related to). Likewise, the annual pattern of testosterone secretion has only been reported for a small number of male dasyurids (McDonald et al., 1981; Bryant, 1986; Bradley, 1987; Millis et al., 1999; Hesterman and Jones, 2009). Whilst earlier research on marsupial reproductive endocrinology has mostly relied on measurement of plasma steroid hormones, recent attention has turned to the use of non-invasive faecal steroid analysis. This technology has been successfully applied to 14 marsupial species, including five dasyurids (Hogan, 2010; Bradshaw and Bradshaw, 2011). Longitudinal studies of faecal androgen secretion in male dasyurids were first described for the Tasmanian devil (*Sarcophilus harrisii*) and spotted-tailed quoll (*Dasyurus maculatus*) by Hesterman and Jones (2009). For female dasyurids, monitoring of oestrogen and/or progesterone faecal metabolites has been conducted in the chuditch (*Dasyurus geoffroii*: Stead-Richardson et al., 2001), Tasmanian devil (Hesterman et al., 2008a), red-tailed phascogale (*Phascogale calura*: Foster et al., 2008), spotted-tailed quoll (Hesterman et al., 2008b) and Julia-Creek dunnart (*Sminthopsis douglasi*: Pollock et al., 2010). The aims of this study were to validate the use of faecal steroid analysis in the numbat and to use this technology to (1) map the seasonal pattern of reproduction in this species, (2) characterise the female oestrous cycle, (3) determine whether pregnancy can be recognised endocrinologically, and (4) highlight factors associated with mating success.

2. Materials and methods

2.1. Animals and study area

The history of the numbats utilised for this study is presented in Table 1. Mean body weight (± SE) of the male and female numbats was 526.0 ± 18.0 g and 524.6 ± 16.9 g, respectively. No animals had any history of reproductive disease and all remained clinically healthy throughout the study period. Numbat captive husbandry has been described by Power and Monaghan (2003). Briefly, the animals were housed individually, in an outdoor breeding complex containing 24 enclosures. Each enclosure was of similar size (2.4 m [W] x 5.0 m [L] x 2.0 m [H]) and furnished with a sand/mulch substrate, logs, grass tussocks, branches, rocks and nest boxes. Animals were maintained on an artificial diet of 'termite custard', made up of low-lactose milk powder, egg, vitamins and baked termite mound, with live or thawed termites added just prior to feeding. The animals were fed twice daily at 09:00 h and 13:00 h. This study was approved by the Perth Zoological Parks Authority Animal Research and Ethics Committee (#S 8/2009-2010) and was conducted in parallel with Perth Zoo's captive numbat breeding program. Males were paired with females once signs of sexual interest were observed in both sexes, i.e. increased phonation and physical activity (pacing) in the female (signs of proestrous) and increased sternal gland secretion and spermatorrhoea in the male (Power et al., 2009). The female was considered to be in 'oestrus' when mating was observed (Power et al., 2009). All breeding information and behaviour was sourced from Perth Zoo keeper's daily reports, which were stored electronically on a central Animal Recording Keeping System (ARKS).

2.2. Faecal sample collection, storage and extraction

Faecal samples were collected for 12 months (April-2010 to March-2011). During the non-breeding season (March-November), faecal samples were collected every two days from each animal (n = 13), whilst during the breeding season (December-February)

samples were collected every day (if possible). To facilitate individual sampling when the numbats were paired (1♂:1♀) for breeding (6 to 8 weeks), a faecal marker in the form of food dye was orally administered to the male (Hogan et al., unpublished). Freshly defecated, faecal pellets were collected from the ground, placed into 1.2 mL sterile cryogenic vials (Corning Life Sciences; Perth Scientific, WA) and stored at minus 20°C until extracted. The faecal extraction protocol was adapted from a previously published method (Graham et al. 1993). Briefly, 1 to 2 g of thawed wet faeces was dried (90 min @ 60°C) using a Binder oven (Binder Inc.; RS Australia, Perth WA), sieved through a wire mesh screen (to remove sand) and then pulverized using a mortar and pestle. Aliquots of well-mixed powder (0.18 to 0.22 g) were mixed with 4.5 mL of 80% methanol, vortexed until homogenized, and then placed overnight on a rotating shaker. The following morning, samples were removed from the shaker, centrifuged (10 min @ 4500 rpm) and the supernatant decanted into glass storage vials (Livingstone Pty Ltd; Perth Scientific, WA). All extracts were stored at minus 20°C before and after analysis.

2.3. Enzyme-immunoassays (EIA): progesterone (PM), oestradiol-17β (E2) and testosterone (TM) metabolites

Faecal steroid concentrations were analyzed in duplicate using microtiter EIA plate procedures previously reported for these steroids (Munro and Stabenfeldt, 1984; Shideler et al., 1993). PM concentrations were measured using a monoclonal anti-P antiserum (CL425) diluted 1:10,000, with a 3CMO-horshradish-peroxidase (HRP)-P label diluted to 1:30,000 as well as P standards (0.78 to 200 pg/50µL) (UC Davis, California, USA). E2 concentrations were measured using a polyclonal anti-E2 antiserum (R4972) diluted 1:10,000, with a HRP-E2 label diluted to 1:50,000 as well as E2 standards (1.95 to 500 pg/20µL) (UC Davis, California, USA). TM concentrations were measured using a polyclonal anti-T antiserum (R156/7) diluted 1:10,000, with a HRP-T label diluted to

1:15,000 as well as T standards (4.7 to 1200 pg/50μL) (UC Davis, California, USA). Major cross-reactivities (> 5%) were: (a) progesterone (100%), 5α-Pregnan-3,20-dione (55%), 5β-Pregnan-3β-ol-20-one (12.5%) and 5β-Pregnan-3,20-one (8%) for the P antibody, (b) oestradiol (100%) for the E2 antibody, and (c) testosterone (100%) and 5α-dihydrotestosterone (57.4%) for the T antibody.

Laboratory assay validation was achieved by demonstrating parallelism between absorbance graphs of serial diluted standards and pooled samples (Fig. 1). Extract dilution rates were based on concentrations of pooled samples that resulted in 50% binding. PM samples were run at a 1:12 dilution during the non-breeding season and at a 1:128 dilution during the breeding season (Fig. 1). All E2 samples were run at a 1:5 dilution, whilst TM samples were run at a 1:10 dilution during the non-breeding season and at a 1:66 dilution during the breeding season (Fig. 1). PM, E2 and TM assay sensitivities were 0.82 ± 0.02 pg/50μL (n = 48), 0.67 ± 0.03 pg/20μL (n = 10) and 0.65 ± 0.01 pg/50μL (n = 37), respectively. Inter- and intra-assay CV for the PM, E2 and TM assays were 6.71 ± 0.37% and 6.64 ± 0.53 %, 5.99 ± 0.75% and 5.01 ± 0.99%, and 4.96 ± 0.32% and 5.26 ± 0.50%, respectively.

Biological validation of the PM assay was achieved by mapping the PM profiles of four known pregnancies. Using confirmed mating and parturition dates, a progressive elevation and peak in luteal PM concentrations post-mating, followed by a decline in PM concentrations just prior to parturition (1 to 2 d) was demonstrated in four females (F1, F7-F9). Biological validation of the TM assay was achieved by demonstrating a relationship between the timing of significant TM elevation (October-January) with enhanced male reproductive function. Using the results of Power et al. (2009; a separate study that also evaluated male numbat reproductive function at Perth Zoo) and observations made during this study, it was confirmed that initial TM elevation (October)

corresponded with the onset of male sternal gland activity, whilst peak TM elevation (December) corresponded with the onset of sperm production and mating behaviour.

2.4. Data processing and statistical analysis

2.4.1. Definitions and baseline calculation

Estimation of baseline hormone concentrations used an iterative process (Graham et al., 2001). The average concentration of all samples for each animal was calculated and values greater than 1.75_{SD} above the mean were 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 to be the baseline concentration. The luteal phase was defined as starting from the first significant sustained increase in PM concentration above baseline concentrations and concluded when concentrations returned again to basal (Finlayson et al., 2006). A significant sustained increase in PM concentration was defined as ≥ 3 consecutive samples greater than 2.0_{SD} above baseline concentrations (Oates et al., 2007). The time between the end of one luteal phase and the beginning of the next luteal phase was classified as either a follicular phase (characterised by behavioural signs of pro-oestrus and/or oestrus) or a period of inter-oestrus (characterised by a lack of reproductive behaviour) (Hogan et al., 2010). If separated by a follicular phase oestrous cycle length was defined as the time period between the start of one luteal phase and the start of the next (Finlayson et al., 2006). Anoestrus was defined as a prolonged period of reproductive inactivity between two successive reproductive cycles, characterised by the absence of luteal phases (Hogan et al., 2010). Each luteal phase was associated with a pregnancy (Pr), non-productive mated oestrous cycle (NMEC) or a non-mated oestrous cycle (NEC). A NMEC cycle involved a mating but no production of offspring, presumably due

to fertilisation failure, embryonic loss or dystocia. Gestation was defined as the interval between mating and birth (Power et al., 2009). Seasons were distinguished as autumn (March-May), winter (June-August), spring (September-November) and summer (December-February).

2.4.2. Hormonal data

Residual plots were used to test hormonal data sets for normal distribution. To meet assumptions of normality, hormone measurements were transformed using a natural logarithm (log_{10}_hormone in ng/g of dried faeces). Factorial ANOVA using a general linear model (GLM) was used to test for differences between females and males in mean baseline PM and TM concentrations, respectively. Variation of repeated measurements of TM concentrations were analyzed by repeated-measures ANOVA, using season or month as a within-subject factor and individual as a subject factor. Variation between females in mean PM concentrations during the luteal phase and anoestrus were assessed using one-way GLM ANOVA (subject effects fixed). Two-way GLM ANOVA with interaction (using anoestrus, luteal phase, follicular phase and inter-oestrus) was used to test for the degree of variation in mean PM concentration between females in different reproductive states. For comparison of mean luteal PM concentrations within different cycle types (Pr, NMEC or NEC) repeated-measures ANOVA was used, with cycle type as a within-subject factor and individual as a subject factor. Repeated-measures ANOVA with cycle status (i.e. anoestrus, luteal phase, post-parturition) as within-subject factor and individual as a subject factor was used to compare mean E2 concentrations over Pr cycles (n = 4). Tukey's HSD all-pair wise comparison tests were used in conjunction with ANOVA testing to find which means were significantly different from one another.

2.4.3. Phase length and reproductive success

For comparison of mean lengths (luteal phase, follicular phase and oestrous cycle) within different cycle types (Pr, NMEC or NEC) and different cycles (1 to 3 cycles per female per breeding season) repeated-measures ANOVA was used with subject, cycle number and cycle type effects being fixed. Pearson Chi-Squared goodness of fit test was used to examine the association between age and subject (i.e. previously proven sires/dams) on the probability of reproductive success. Statistical testing was completed using Minitab (Version 17, 2007) and SAS (SAS®/STAT, Version 8.2, 2001). Significance levels for all tests were set at $P \leq 0.05$ and means are given with standard errors (SE) unless otherwise noted.

3. Results

3.1. Baseline concentrations

There was a difference ($F_{8,783} = 33.93$, $P < 0.01$) between females in mean PM baseline concentrations. F3 (86.74 ± 1.84 ng/g) had a greater baseline mean than that of all other females, whilst F2 (47.91 ± 2.04 ng/g) and F9's (46.84 ± 2.83 ng/g) baseline means were lower than the seven other females. The baseline mean of F5 (73.45 ± 2.00 ng/g) was greater than F6 (62.66 ± 1.93 ng/g), but both F5 and F6 had baseline means not statistically different from those of the remaining four females. There was a difference ($F_{2,258} = 12.23$, $P < 0.01$) between males in mean TM baseline concentrations, with M1 (70.73 ± 3.20 ng/g) and M2 (68.70 ± 3.04 ng/g) having similar yet greater means than M3 (49.77 ± 2.72 ng/g) and M4 (54.97 ± 2.84 ng/g).

3.2. Male seasonality

Mean TM concentrations differed significantly between seasons ($F_{3,645} = 40.07$, $P < 0.01$) and months ($F_{11,613} = 34.63$, $P < 0.01$) (Fig. 2). Mean TM concentration was

256 greater in spring (618.16 ± 1.21 ng/g) and summer (614.19 ± 1.20 ng/g), than compared
257 to autumn (74.20 ± 1.20 ng/g) and winter (97.77 ± 1.19 ng/g). Mean October-January
258 TM concentrations were greater than those recorded for all other months, but similar to
259 each other (Fig. 2). Mean August (230.30 ± 1.23 ng/g) and September (198.15 ± 1.26
260 ng/g) TM concentrations were less than mean October-January concentrations, but
261 greater than mean March-June concentrations. There was no difference in monthly
262 mean TM concentrations from February to July (Fig. 2).
263
264 *3.3. Characterisation of the oestrous cycle and polyoestry*
265 All nine female numbats displayed periods of elevated PM secretion. Three
266 females (F1, F5, F6) displayed three luteal phases (Fig. 3A), three females (F2-F4)
267 displayed two luteal phases (Fig. 3B), and three females (F7-F9) displayed one luteal
268 phase over the 12-month period. Of the 18 observed luteal phases, four were associated
269 with a pregnancy (Pr), six were associated with a non-mated oestrous cycle (NEC) and
270 eight were associated a non-productive mated oestrous cycle (NMEC). Four females
271 (F1, F7-F9) became pregnant during their first luteal phase and gave birth to 1 to 4
272 pouch young (PY); F1 lost a single PY within days after parturition and proceeded to
273 display two additional luteal phases (Fig. 3A). The timing and length of each luteal
274 phase, with associated cycle type and follicular phase length (if present) is presented in
275 Figure 4. All females did not have their first luteal phase/oestrous cycle during the same
276 time period. Females with earlier first cycles were not synchronized with those females
277 that had three oestrous cycles over the observational period, e.g. F1, F5 and F6 were
278 ranked 2nd, 4th and 5th in terms of first cycle commencement.
279 A total of 11 oestrous cycles (1 x Pr (lost PY), 8 x NMEC, 2 x NEC) were
280 recorded, ranging in length from 28 to 35 d, with a mean length of 30.2 ± 1.1 d. Oestrous
281 cycle length did not vary between the females ($F_{5,10}$ = 2.79, P = 0.14), but luteal ($F_{8,18}$ =

3.45, P = 0.04) and follicular phase ($F_{5,10}$ = 9.07, P = 0.02) lengths did. Mean (n = 18) luteal phase length ranged from 10 to 18 d with a mean value of 14.0 ± 0.8 d. Mean luteal phase length did not vary significantly according to cycle number ($F_{2,18}$ = 1.58, P = 0.26; i.e. between 1st, 2nd or 3rd cycles) or cycle type ($F_{2,18}$ = 0.30, P = 0.75; i.e. Pr, NMEC or NEC cycles). Mean (n = 11) follicular phase length ranged from 11 to 21 d with a mean value of 16.2 ± 1.6 d. Data was insufficient to test follicular phase and oestrous cycle length variation according to cycle number and type.

Mean anoestrus, luteal and follicular phase PM concentrations for each female are shown in Table 2. Mean luteal ($F_{8,117}$ = 0.42, P = 0.91) and follicular ($F_{5,71}$ = 1.17, P = 0.46) phase PM concentrations did not vary between the females, but mean anoestrus PM concentrations did ($F_{8,901}$ = 17.57, P < 0.01) (Table 2). Mean luteal phase PM concentration did not vary according to oestrous cycle number or cycle type ($F_{2,117}$ ≤ 0.75, P ≥ 0.48), but varied between the different cycle phases ($F_{3,1156}$ = 435.17, P < 0.01). For instance, mean luteal phase PM concentration (435.51 ± 1.02 ng/g) was greater than that of the follicular phase (110.16 ± 1.07 ng/g), inter-oestrus (86.70 ± 1.07 ng/g) and anoestrus (72.44 ± 1.02 ng/g). There was no difference in mean follicular phase and inter-oestrus PM concentrations, with both being greater than mean anoestrus PM.

3.4. E2 concentrations

E2 profiles showed no relationship with oestrus and showed no evidence of oestrous cyclicity, ranging from 15.7 to 68.2 ng/g with a mean value of 33.07 ± 0.55 ng/g. Mean E2 concentrations did not vary significantly between females and no significant variation was observed across the sampling period ($F_{2,190}$ = 0.28, P = 0.77). Mean anoestrus (32.93 ± 1.07 ng/g), luteal (35.51 ± 1.08 ng/g) and post-parturitional

(33.82 ± 1.05 ng/g) E2 concentrations were not significantly different from each other. There were no peaks in E2 associated with oestrus, nor were there any troughs concurrent with elevated PM.

3.5. Mating and reproductive success

Due to the majority of mating activity occurring within nest boxes, it was not possible to quantify the length of female receptivity or length and frequency of mating bouts. Mating behaviour was directly observed (via video surveillance) in six out of the nine females, indirectly confirmed through the finding of a sperm plug in F3 and F5, and never observed in F2 (as this female was never paired with a male) (Table 3). Mating success was 31%, with 4/13 observed matings resulting in PY (Table 3). There was no association between age (χ^2 = 2.37, DF = 6, P = 0.88) and prior success (i.e. whether a proven sire/dam; χ^2 = 8.55, DF = 12, P > 0.10) with the probability of mating success. However, there did appear to be an association between timing of introduction and mating success. For instance, males introduced to females for a mean length of 9.8 ± 2.0 d (range 5 to 14 d) prior to the first observed mating resulted in mating success, whilst males introduced to females for a mean length of 19.9 ± 4.4 d (range 6 to 49 d) prior to the first observed mating resulted in no mating success.

Observed matings (n = 13) were always followed by a rise in PM concentrations 1 to 9 d later, with a mean interval of 3.5 ± 0.7 d (Table 3). When considering the successful matings only, increases in PM concentrations occurred 1 to 5 d later, with a mean interval of 3.3 ± 1.2 d. There were six occasions when elevated PM secretion was not preceded by an observed mating (Table 3). Following all observed matings (n = 13), no matings (n = 6) and successful matings (n = 4) the mean interval between the initial significant rise and peak in PM concentrations (during the luteal phase) was 7.6 ± 0.5 d,

7.3 ± 0.9 d and 7.5 ± 0.7 d, respectively (Table 3). Just prior to parturition (1 to 2 d) PM concentrations started to decrease. PM concentrations were no longer elevated (i.e. 2_{SD} above baseline) on the day of parturition and they returned to baseline concentrations 2 to 8 d (mean: 5.0 ± 2.1 d) after parturition. Gestation length varied from 11 to 16 d (F1: 11 d; F7: 16 d; F8: 16 d; F9: 14 d) with a mean value of 14.3 ± 1.2 d. Duration of elevated PM in pregnant females was 10 d for F1, 11 d for F7, 12 days for F8 and 16 days for F9 (mean 12.3 ± 1.5 d).

4. Discussion

Longitudinal profiling of faecal testosterone and progesterone metabolites has confirmed that (1) male numbats undergo an annual change in testosterone that is clearly linked to a seasonal pattern of reproduction, and (2) the female numbat is seasonally polyestrous with spontaneous ovulations; these reproductive patterns are similar to those observed in seasonally breeding dasyurid marsupials (Tyndale-Biscoe and Renfree, 1987; Taggart et al., 2003; Hesterman et al., 2008ab; Hesterman and Jones, 2009). The mating strategy of the numbat is polygynous, with males mating with more than one female (Power and Monaghan, 2003), and breeding is restricted to the summer months (Power et al., 2009). In this study, male TM secretion increased progressively from September to November, peaked in December (corresponding with the onset of the mating period), decreased in February, and remained baseline from February to August. A previous study examining the reproductive function of male numbats revealed that they undergo a seasonal change in reproductive condition. Specifically, testicular volume, bulbourethral gland size and sperm production is greater from November to February, with peaks occurring in December (Power et al., 2009). Therefore, the observed increase in TM several months prior to the breeding season was most likely associated with preparation of the testis and accessory glands for the

mating season. Results from Power et al. (2009) and the current study indicate that at the completion of the breeding season numbat testes regress, with corresponding decreases in sperm and testosterone production. A similar phenomenon has also been reported in other dasyurids that have a mating strategy similar to that of the numbat, e.g. the kowari (*Dasyuroides byrnei*) and eastern quoll (*Dasyurus viverrinus*) (Fletcher, 1983; 1985).

In the present study, evidence of female reproductive cyclicity was effectively monitored by changes in PM secretion. Mean oestrous cycle duration was 30.2 ± 1.1 d. Due to its close phylogenetic relationship with the Dasyuridae, the numbat was expected to have an oestrous cycle length roughly similar to that of other seasonally breeding dasyurids. The estimate in the present study was comparable to mean oestrous cycle lengths reported for the Tasmanian devil (~32 d; Hesterman et al., 2008a) and spotted-tailed quoll (~ 29 d; Hesterman et al., 2008b) using PM measurements and the red-cheeked (*Sminthopsis virginiae*, ~30 d) and fat-tailed (*S. crassicaudata,* ~31 d) dunnart using plasma progesterone measurements (Jackson, 2003). Despite having similar oestrous cycle lengths, the length of the luteal (~14 d) and follicular (~16 d) phases recorded for the numbats in the present study were dissimilar in duration and proportion to those previously reported for the Tasmanian devil (18 d LP, 14 d FP) and spotted-tailed quoll (21 d LP, 8 d FP). In the Tasmanian devil and spotted-tail quoll, the luteal phase occupies 56% and 72% of the oestrous cycle, whereas in the numbat the luteal phase only occupied 47%.

Longitudinal PM profiling confirmed that female numbats are seasonally polyestrous, exhibiting up to three oestrous cycles during the reproductive season. Interestingly, results of the present study indicate that the numbat breeding season is not confined to the summer months (Power et al., 2009), but ranged from late-November to early-April. According to Perth Zoo records, litters have only ever been produced

between January 7th and February 26th, which is reflective of the numbats only being paired for breeding from mid-December to mid-February (Lawrence et al., 2008). During this study's breeding season, four of the observed non-mated oestrous cycles occurred because the male was separated from the female prior to her last cycle of the season. This represents a significant loss in mating opportunities and Perth Zoo needs to lengthen their pairing schedule in the future.

As in most marsupials, there were no significant differences in the mean duration of the luteal phase or in the magnitude/duration of PM profiles between the mated and non-mated numbat oestrous cycles (Fletcher, 1985; Tyndale-Biscoe and Renfree, 1987; Hinds, 1989; Hinds and Selwood, 1990). These findings indicate that vaginal/cervical stimulation during coitus does not play a role in the timing of ovulation in this species (Tyndale-Biscoe and Renfree, 1987). In addition, luteal PM profiles (length and magnitude) were also similar in pregnant and non-pregnant cycles, suggesting that the presence of the numbat foetus has little or no endocrine control over maternal progesterone secretion: this phenomenon is also true for the eastern quoll (Hinds, 1989) and Tasmanian devil (Hesterman et al., 2008a).

Observed matings were always followed by a luteal phase and there were numerous luteal phases that were not preceded by an observed mating (further evidence of spontaneous ovulation). Dissimilar to spontaneous breeders in the Dasyuridae family, increased progesterone concentrations were not present during pro-oestrus or during the follicular phase of the cycle, suggesting that an alternative hormonal cue influences female receptivity in this species. In contrast, elevated progesterone has been linked with the induction of sexual receptivity in the eastern quoll (Hinds, 1989), kowari (Fletcher, 1989), brush-tailed phascogale (Millis et al., 1999) and spotted-tailed quoll (Hesterman *et al.*, 2008b). Luteal phase onset occurred 1 to 9 days following mating, with mean luteal PM peaking 7 days from luteal onset. Extended

ovulatory intervals (i.e. the interval between mating and a significant increase in progesterone) appears to be characteristic of dasyurids (Fletcher, 1985; Hinds 1989), with this interval varying between 2 to 12 days and 3 to 9 days in the spotted-tailed quoll and Tasmanian devil, respectively (Hesterman et al., 2008ab). This extended timeframe from copulation to ovulation may have a role in permitting mating with several different males, as supported by evidence of sperm storage in several dasyurid species (Taggart et al., 2003) and variations in reported gestation lengths of the same species (Hesterman et al., 2008a). In this study, mean gestation length was 14.3 ± 1.2 d; an estimate similar to that reported by Friend and Whitford (1993; ~14 days), but less than that reported by Power et al. (2009) (~17 days). In cases when ovulation does not occur at a fixed time in relation to mating, then the interval from luteal onset to birth (rather than from mating to birth) provides a more realistic gestation length, which for the numbats was 12.3 ± 1.5 d.

Mating success during this study was poor, with only 31% of observed matings resulting in pouch young. Females must be mated within 48 hours of oestrus for conception to occur (Friend and Whitford, 1993), with mating lasting anywhere from 1 to 60 minutes (Power and Monaghan, 2003). In the present study, there was no evidence of a 'male effect' on female numbat reproduction, with the commencement of oestrous cycling not being initiated by the introduction of the male, nor terminated following male removal. Optimal breeding age in the numbat appears to be 1 to 4 years, with an increase in pouch young loss and unsuccessful matings when at age's ≥ 5 years (Lawrence et al., 2008). Contrary to this previous result, there was no effect of age or subject (i.e. proven sire/dam status) on mating success of the numbats used in this study. However, timing of introduction (of male to female) seemed to have an impact on mating success, with animals paired for a shorter duration (≤ 14 days) prior to the first observed mating/oestrus being the pairs that successfully produced young. Obviously,

436 Perth Zoo's current methodology of using behavioural indicators to determine female
437 readiness required modifying (e.g. one male was introduced 49 days prior to the
438 female's first oestrus) as long-term pairing seems to decrease the likelihood of mating
439 success. Perhaps urogenital cytology, a methodology which has been previously
440 investigated in the numbat (Power et al., 2009), should be used in the future to more
441 accurately determine the timing of female oestrus and the appropriate time to introduce
442 the male. Other contributing factors to the poor reproductive performance of the captive
443 numbats might include (1) diet - numbats are known to feed exclusively on termites in
444 the wild and their artificial custard diet in captivity might lack essential nutrients (Power
445 and Monaghan, 2003), (2) pairing incompatibility, (3) male or female infertility, and (4)
446 mating failure – i.e. it is difficult to discern during mating bouts whether the male is
447 successfully introducing his penis and ejaculating into the female reproductive tract.
448 Whilst faecal oestrogen/oestradiol metabolite monitoring has been successfully
449 used to characterise the ovarian cycle (i.e. period of follicular development and oestrus)
450 of a range of marsupials (e.g. Tasmanian devil, spotted-tailed quoll and honey possum)
451 the E2 assay used in the current study proved ineffective as a method for assessing
452 female numbat reproductive activity (Oates et al., 2007; Hesterman et al., 2008ab). Brief
453 or small increases in circulating levels of oestrogens are likely to be masked by pooling
454 of metabolites in bile and faeces (Schwarzenberger, 2007) and elevations in oestradiol
455 have been undetectable in a variety of marsupial species (phascogale: Foster et al.,
456 2008; potoroo: Stead-Richardson et al., 2010; wombat: Hogan et al., 2010: dunnart:
457 Pollock et al., 2010). No measurement has yet been reported in any marsupial of the
458 partitioning of oestradiol excretion between urine and faeces. Therefore, the
459 comparatively lesser concentrations of E2 in the numbat may represent a fraction of the
460 total oestradiol excreted. Further investigation into characterisation of numbat ovarian
461 cycles by faecal oestrogen monitoring is needed. The next step would be to use HPLC

(high performance liquid chromatography) to detect the specific oestradiol metabolites excreted in the faeces or to use an alternative assay, which has a broader range of hormone cross-reactivity.

In conclusion, the present study has resulted in an improved understanding of numbat reproductive biology and further demonstrated the effectiveness of faecal hormone measurement as a non-invasive technique for monitoring reproductive physiology in marsupials. Longitudinal profiling of TM secretion revealed that male numbats have a distinct seasonal pattern of reproduction, which is highly synchronized with that of the female. While changes in the secretion of E2 provided little instructive information on oestrous cycle activity, longitudinal profiling of PM secretion confirmed that the female numbat has reproductive patterns/parameters similar to that observed in seasonally breeding dasyurid marsupials. For instance, the female numbat is seasonally polyestrous, a spontaneous ovulator, has a mean oestrous cycle length of ~30 days, a luteal phase that occupies ~47% of the oestrous cycle, a breeding season extending from late-November to early-April, a PM hormonal equivalence between pregnant and non-pregnant cycles, an ovulatory interval of 1 to 9 days and a gestation length of 12 to 14 days. Mating success during this study was poor (~31%) and success was not influenced by animal age or status (i.e. whether a proven sire/dam), but was influenced by the timing of introduction, with long-term pairing decreasing the likelihood of mating success. These results provide the basis of a more detailed understanding of the reproductive physiology that will underpin improved methods of numbat reproductive management and fertility assessment.

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Table legend

Table 1

History of the numbats (*M. fasciatus*) utilized from Perth Zoo

Table 2

Mean faecal progesterone metabolite concentrations (ng/g) recorded for nine captive female numbats (*M. fasciatus*), during anoestrus, the follicular phase and the luteal phase ($P \leq 0.05$)

Table 3

Reproductive parameters for nine captive female numbats (*M. fasciatus*) at Perth Zoo (2010-2011)

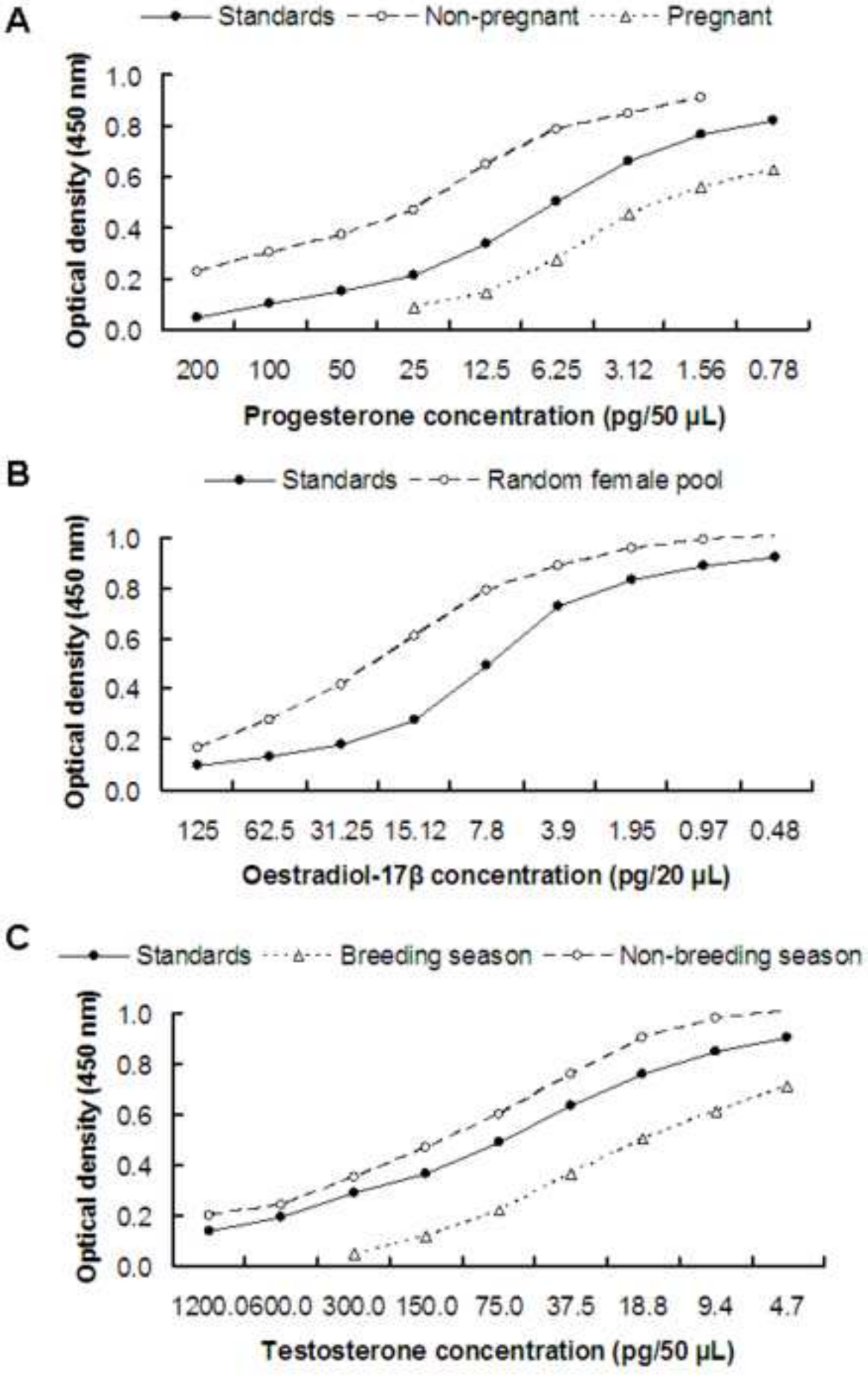
Figure Legend

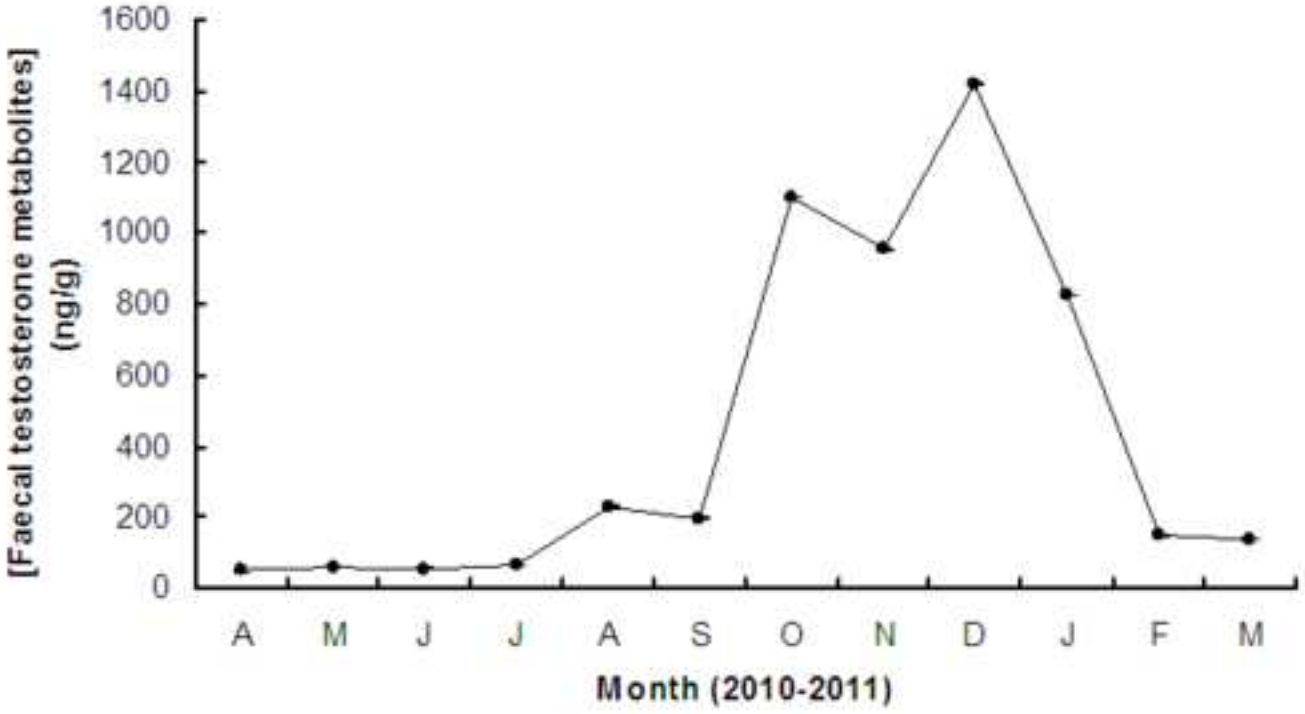
Fig. 1. Parallelism between a dilution of standard (A) progesterone (closed circle) and serial dilutions of non-pregnant (open circle) and pregnant (open triangle) female faecal extract pools, (B) oestradiol-17β (closed circle) and serial dilutions of a random female faecal extract pool (open circle), and (C) testosterone (closed circle) and serial dilutions of breeding (open triangle) and non-breeding (open circle) season male faecal extract pools

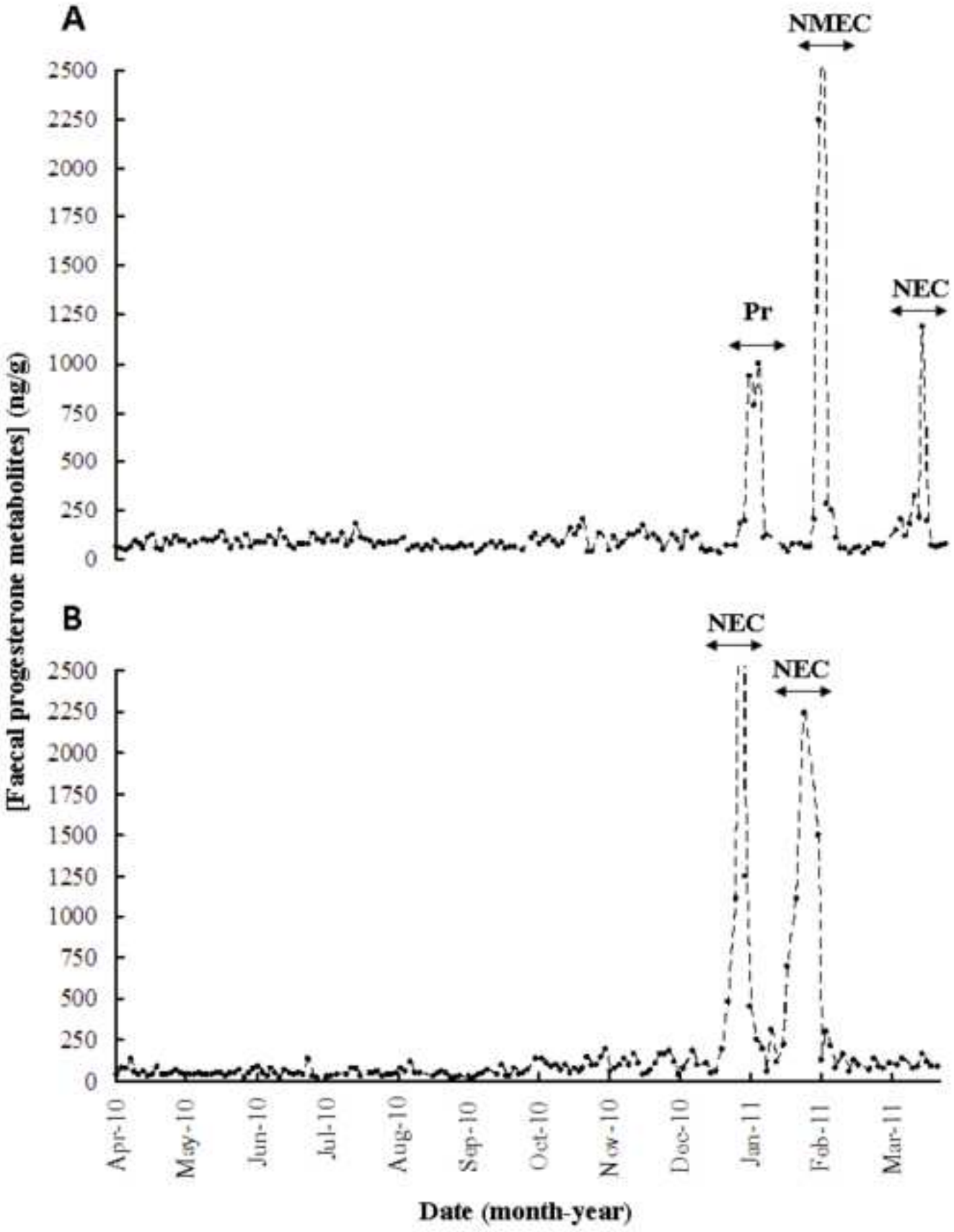
Fig. 2. Male (*n* = 4) numbat (*M. fasciatus*) mean (± SE) monthly faecal (ng/g of dried faeces) testosterone metabolite concentrations (April-10 to March-11)

Fig. 3. Faecal progesterone metabolite concentrations (ng/g dried faeces) recorded for two captive female numbats, over a 12-month period (April 2010–March 2011): (A) Numbat F1, showing three luteal phases and (B) Numbat F2, showing two luteal phases. Key: Pr = pregnancy; NMEC = non-productive mated oestrous cycle; NEC = non-mated oestrous cycle

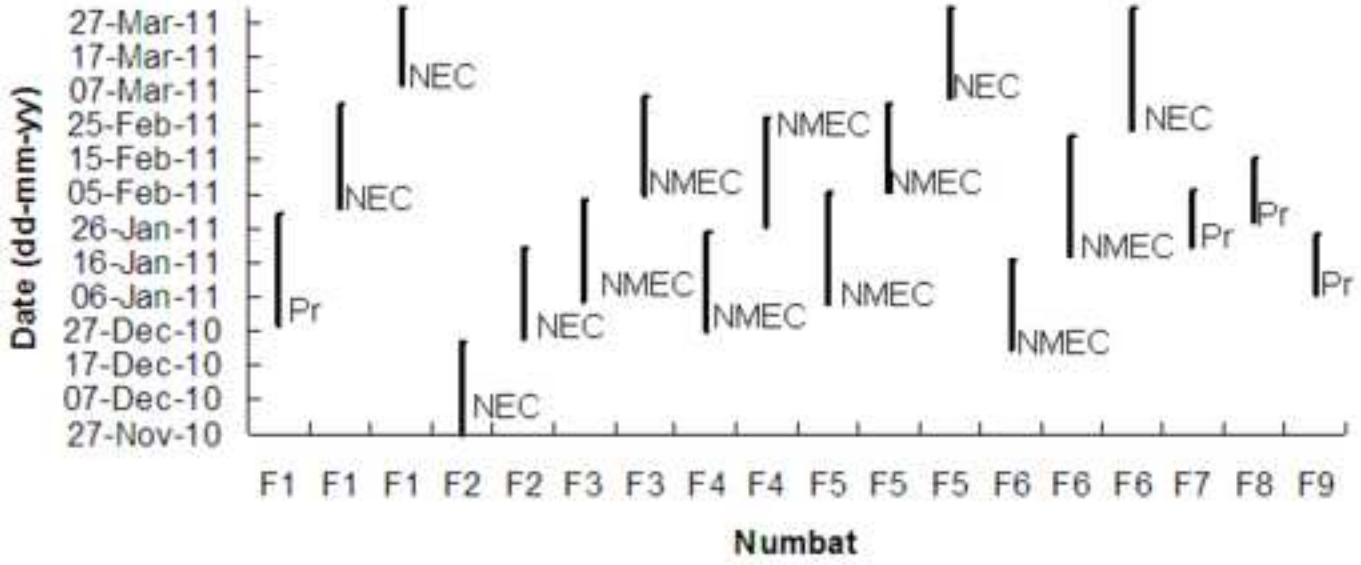
Fig. 4. Timing and length of observed luteal phases (*n* = 18)/oestrous cycles (*n* = 11) in nine female numbats during April 2010 to March 2011. Key: Pr = pregnancy; NMEC = non-productive mated oestrous cycle; NEC = non-mated oestrous cycle







Figure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Numbat

Table 1

History of the numbats (*M. fasciatus*) utilised from Perth Zoo

Numbat	Age (yr; 2011)	Born*	Arrival at zoo	Proven sire/dam
M1	4	Captivity	2007	2008: 0 PY; 2009: 3 PY; 2010: 0 PY; 2011: 0 PY
M2	≥ 6	Wild	2008	2009: 1 PY; 2010: 0 PY; 2011: 4 PY
M3	≥ 6	Wild	2008	2009: 4 PY; 2010: 4 PY; 2011: 0 PY
M4	≥ 3	Wild	2009	2010: 3 PY; 2011: 0 PY
F1	≥ 7	Wild	2004	2005: 4 PY; 2006: 4 PY; 2007: 4 PY; 2008:4 PY; 2009: 0 PY; 2010: 3 PY; 2011: 1 PY (lost)
F2	7	Captivity	2005	2006: 0 PY; 2007: 2 PY; 2008: 4 PY; 2009:4 PY; 2010: 4 PY; 2011: 0 PY
F3	≥ 7	Wild	2004	2005: 0 PY; 2006: 4 PY; 2007: 4 PY; 2008: 0 PY; 2009: 4 PY; 2010: 0 PY; 2011: 0 PY
F4	4	Captivity	2007	2008: 4 PY; 2009: 4 PY; 2010: 4 PY; 2011: 0 PY
F5	3	Captivity	2008	2009: 3 PY; 2010: 0 PY; 2011: 0 PY
F6	≥ 4	Wild	2009	2010: 3 PY; 2011: 0 PY
F7	1	Captivity	2010	2011: 4 PY
F8	1	Captivity	2010	2011: 2 PY
F9	≥ 2	Wild	2010	2011: 4 PY

*Captivity, animals were born at Perth Zoo (31°58'S, 115°51'E); wild, animals were caught from Dryandra Woodland (31°46'S, 117°1'E); PY = pouch young

Table 2

Mean faecal progesterone metabolite concentrations (ng/g) recorded for nine captive female numbats (*M. fasciatus*), during anoestrous, the follicular phase and the luteal phase ($P \leq 0.05$)

Animal ID	Anoestrus	Follicular phase	Luteal phase
F1	82.04 ± 1.04^{ABC}	108.64 ± 1.05	377.57 ± 1.26
F2	53.33 ± 1.04^{D}	80.72 ± 1.04	330.37 ± 1.35
F3	85.90 ± 1.03^{A}	123.88 ± 1.05	570.16 ± 1.41
F4	71.45 ± 1.04^{BC}	102.80 ± 1.05	504.66 ± 1.49
F5	83.95 ± 1.05^{AB}	121.90 ± 1.04	552.08 ± 1.33
F6	70.15 ± 1.04^{C}	101.39 ± 1.05	542.00 ± 1.33
F7	77.27 ± 1.06^{ABC}	112.46 ± 1.07	387.26 ± 1.65
F8	83.95 ± 1.06^{ABC}	122.46 ± 1.07	425.60 ± 1.60
F9	49.77 ± 1.06^{D}	78.89 ± 1.07	469.89 ± 1.60
Total	72.44 ± 1.02	110.16 ± 1.07	435.51 ± 1.05

Different superscript letters (^{a,b,c}) within same table column indicates significant mean differences

Table 3

Reproductive parameters for nine captive female numbats (*M. fasciatus*) at Perth Zoo (2010-2011)

Numbat	Oestrous cycle	Pairing dates	Days with male	Mating observations	Mating dates	Luteal PM increase	Luteal PM peak	Mating success
F1	1	20-Dec to 28-Dec	8	2	26 & 28-Dec	31-Dec	8-Jan	1
	2	19-Jan to 26-Jan	8	0	No mating	1-Feb	5-Feb	0
	3	No pairing	0	0	No mating	11-Mar	21-Mar	0
F2	1	No pairing	0	0	No mating	25-Dec	2-Jan	0
	2	No pairing	0	0	No mating	23-Jan	30-Jan	0
F3	1	21-Dec to 9-Feb	51	1	11-Jan	14-Jan	20-Jan	0
	2	21-Dec to 9-Feb	51	1	29-Jan	6-Feb	14-Feb	0
F4	1	20-Dec to 22-Feb	65	1	25-Dec	26-Dec	4-Jan	0
	2	20-Dec to 22-Feb	65	1	18-Jan	19-Jan	30-Jan	0
F5	1	21-Dec to 21-Feb	63	1	9-Jan	10-Jan	16-Jan	0
	2	21-Dec to 21-Feb	63	1	5-Feb	8-Feb	14-Feb	0
	3	No pairing	0	0	No mating	3-Mar	12-Mar	0
F6	1	21-Dec to 1-Feb	43	1	27-Dec	31-Dec	7-Jan	0
	2	21-Dec to 1-Feb	43	1	19-Jan	26-Jan	3-Feb	0
	3	No pairing	0	0	No mating	22-Feb	1-Mar	0
F7	1	6-Jan to 21-Jan	16	1	17-Jan	22-Jan	29-Jan	1
F8	1	6-Jan to 10-Feb	36	1	24-Jan	25-Jan	31-Jan	1
F9	1	30-Dec to 17-Jan	19	1	7-Jan	9-Jan	18-Jan	1

Key: PM = faecal progesterone metabolites; F = female