Plasma concentrations of progesterone and testosterone in captive woolly opossums (*Caluromys philander*)

M. Perret and M. Atramentowicz

CNRS UA 1183, M.N.H.N. Laboratoire d’Ecologie Générale, 4 avenue du Petit Chateau, F 91800 Brunoy, France

Summary. Plasma testosterone and progesterone concentrations were measured in captive woolly opossums, a didelphid marsupial originating from neotropical forests in French Guiana. Although not exposed to cyclic environmental conditions as in the field, both sexes exhibited spontaneous circannual changes in sexual hormones. Males showed synchronous variations in plasma testosterone characterized by significant elevated concentrations during April and September (8.6 ± 1 ng/ml, N = 5) and lower levels from May to July (3.6 ± 0.4 ng/ml). In females, synchronous periods of 2–3 successive oestrous cycles occurred. Between these periods, females remained acyclic. The oestrous cycle, determined by urogenital smears, lasted 28–45 days (n = 14) and included a 20-day spontaneous luteal phase in which progesterone concentrations reached 30–40 ng/ml plasma. Even though testosterone concentrations in paired males increased significantly in response to oestrous periods of the paired females, spontaneous circannual rhythms of sexual activity were not well synchronized between the sexes in captivity. When compared to field data, sexual activity of captive animals followed a pattern similar to that in wild animals, without any changes in males but with a delay of 3 months in females.

Keywords: reproduction; circannual cycles; progesterone; testosterone; woolly opossum

Introduction

Within marsupials, the Family Didelphidae is presently considered as ancestral, with numerous primitive characters being retained (MacKenna, 1969). Reproduction in didelphid marsupials has been reviewed by Tyndale-Biscoe & Renfree (1987), but of the 70 living didelphid species which are the predominant American marsupials, the reproductive function of only few has been well investigated: *Didelphis virginiana* (Hartman, 1928; Reynolds, 1952; Feldman & Ross, 1975; Harder & Fleming, 1981; Fleming & Harder, 1981), *Monodelphis domestica* (Fadem et al., 1982; Fadem, 1985, 1987; Baggott et al., 1987) and *Marmosa robinsoni* (Godfrey, 1975). For the other didelphids, and more especially for the South American ones, only scattered information about birth seasons, oestrous cycles or general eco-ethology is available (Davis, 1945; Biggers, 1966; Fleming, 1973; Tyndale-Biscoe & MacKenzie, 1976; Bucher & Fritz, 1977; Hunsaker, 1977; O’Connell, 1979; Charles-Dominique, 1983).

The woolly opossum, *Caluromys philander*, is an arboreal didelphid widely distributed in the northern and eastern regions of South America and this species may be an exception among the didelphids because it has a higher encephalization quotient, longevity and metabolic rate (Eisenberg & Wilson, 1981). This nocturnal species, weighing about 300 g, lives in the upper strata of tropical forests and has an opportunistic frugivorous diet supplemented with insects (Atramentowicz, 1988). The woolly opossum was studied in French Guiana by systematic mark–recapture methods and, from data on population densities and reproductive rate, it has been
described as a polyoestrous species which may have two litters a year, with a mean litter size of about 4 (Atramentowicz, 1986). However, although the first litter born in October/November was raised to weaning, the young of the second litter born in April/May mostly died in the pouch. One of the environmental cues that determines such 'pouch abortions' might be the period of relative food scarcity extending from May to August in French Guyana (Atramentowicz, 1982).

No information is presently available on the reproductive hormones in this species, and so variations in sexual hormones have been investigated in a captive population of woolly opossums exposed to constant environmental conditions.

Materials and Methods

Animals

Nine woolly opossums, Caluromys philander, were used in this study: they had been caught 3 years earlier at Cabassou (French Guyana, 53°W, 5°N) or born in captivity at the Laboratory of Ecology (Brunoy, France). The 5 males and 4 females were adult according to dental characteristics (Atramentowicz, 1986) and weighed 200–400 g. Three animals (2 ½ 2 and 1 ½) were kept isolated and 6 were paired. All animals lived in large cages (1 × 1·9 × 2·7 m) visually separated each other by wooden partitions. Animals were exposed to constant artificial daylight of 12 h light/12 h dark (lights on at 13:00 h) assuming there is little daylength variation in French Guyana throughout the year. Because of the nocturnal habits of woolly opossums, red lights were used during the dark phase to allow behavioural observations.

Animals were fed ad libitum with fresh fruit and a milky mixture including primate biscuits (ExtraLabo, Provins, France). The conditions of captivity were standard with respect to temperature (28–30°C) and relative humidity (~50%).

All animals were routinely examined for body weight and blood samples were collected 3 times a month throughout a year. Samples were taken more frequently (6 times a month) from 4 females to measure progesterone concentrations during the oestrous cycle. Blood samples (250 μl) were collected without prior anaesthesia, by puncture of the saphenous vein within 5 min of removing the animal from its cage during the daily sleeping period. After centrifugation, plasma was stored at −20°C until assayed.

To identify accurately the oestrous cycle in this species, urogenital smears were taken from 4 females every day for 2 months. Smears were then taken every week to detect the occurrence of oestrus throughout the year and daily when females entered cytological oestrus until leucocytic infiltration.

Radioimmunoassay procedures

Testosterone. Concentrations were measured in samples of 50 μl plasma using the radioimmunoassay procedure described by Schilling et al. (1984). Samples were diluted with 450 μl saline–phosphate buffer (0·05 mol/l, pH 7·4, 1% gelatin). After dichloromethane extraction, determinations were performed on two extracted aliquants corresponding to 3·2 and 16 μl plasma. The antiserum (diluted 1/120,000) was raised from testosterone 3-carboxymethylisoxime coupled to bovine serum albumin (from Dr M. G. Forest, INSERM, Lyon, France), and cross-reactivities were: testosterone, 100%, 5α-dihydrotestosterone, 62·5%, 5α-androstan-3a,17β-diol, <3%, other steroids, <1%. The mean intra-assay coefficient of variation based on all samples tested in duplicate was 6·3% (s.d. = 4·2) and the mean inter-assay coefficient was 8·5% (s.d. = 5·9) based on 25 samples with testosterone concentrations averaging 5 ng/ml.

To estimate the assay blank, testosterone concentration was measured in charcoal-stripped plasma from juvenile animals. Recovery of tritiated testosterone added to blank plasma was 92·2% (s.d. = 2·3, n = 10). The assay blank averaged 0·51 ± 0·04 ng/ml (n = 8). The sensitivity of the assay was calculated as twice the standard error for the assay blank, and was <10 pg/tube. The buffer blank was always lower than the sensitivity of the assay. To assess quality of the assay, 10 ng testosterone were added to 1 ml blank plasma: the value measured was 9·60 ± 0·7 ng/ml (n = 8) after correction for the blank. Serial dilution of pools of plasma containing ~10 ng testosterone/ml gave a displacement curve parallel to that obtained with testosterone standards (no significant difference tested using polynomial coefficients in variance analysis).

Lastly, testosterone concentrations were measured with or without chromatography on celite ethylene glycol columns. After elution with iso-octane:ethylacetate (80:20, v/v), chromatographed and non-chromatographed samples were significantly correlated (linear regression analysis, n = 15, P < 0·001).

Progesterone. Concentrations were measured in 60–80 μl samples of plasma using the radioimmunoassay procedure described by Perret (1986). Before double extraction by 5 ml diethyl ether, each sample was diluted to 1 ml with saline–phosphate buffer and tracer amounts of tritiated progesterone were added. After extraction, radioimmunoassay was performed on two extracted aliquants corresponding to 6 and 12 μl plasma. Recovery of progesterone after extraction was 86·7% (s.d. 9·6%). The antiserum was raised in a rabbit from Δ4-pregnen-3,20-dione-11-succinate coupled to bovine serum albumin (purchased from Pasteur Institut, Paris, France).
cross-reactivities were: progesterone, 100%; deoxycorticosterone, 3%; 6β-hydroxyprogesterone, 18%; 5a-pregnane-3,20-dione, 16%; other C21 steroids, <1% and C19 steroids, <0.03%. The intra-assay coefficient of variation based on all samples tested in duplicate was 9.2% (s.d. = 5.6) and the inter assay coefficient was 9.9% (s.d. = 4.7) based on 15 samples with a progesterone concentration of 8–20 ng/ml. A pool of plasma adjusted to 5 ng/ml measured 5.8 ± 0.3 ng/ml (n = 10) after correction for the blank and for recovery. As for the testosterone assay, blank plasma values and parallelism between the standard curve and serial dilution of plasma samples were verified. The blank plasma averaged 0.90 ± 0.09 ng/ml (n = 8) and the sensitivity of the assay was 6 pg/tube. Buffer blanks were always below the sensitivity of the assay. No chromatographic separation was made but the antiserum used has a very low cross-reactivity with a wide range of steroids and progesterone has been reported to be the major plasma ovarian steroid in numerous marsupials (Tyndale-Biscoe & Renfree, 1987).

Radioactive steroids (testosterone: sp.act. 94 Ci/mm; progesterone: sp.act. 85 Ci/mm) were purchased from Amersham International plc (Bucks, UK) and non-radioactive steroids from Sigma (St Louis, MO, USA).

Field data

Field observations were conducted during 1 year in a secondary forest of French Guyana, using mark–recapture methods previously described and breeding seasons were determined through trapping data (Atramentowicz, 1982). It is not possible to detect gestation in woolly opossums since no morphological changes occur in the pregnant female because of the reduced size of marsupial newborn (length 10 mm, body weight, 0.2 g) which is 1/1500th of the adult weight. However, multiple captures of the same individual allowed us to determine accurately the age of a litter and consequently birth time, using the snout–rump length measurements of young in the pouch (Atramentowicz, 1982). Assuming a gestation length of at least 3 weeks (see below) we estimated the occurrence of oestrous periods through 1 year from pouch young of 141 wild lactating females. Estimation of seasonal changes in fruit production was based on changes in the number of fruiting trees, recorded weekly through a 1200 m transect at Cabassou (Atramentowicz, 1982).

Statistical analysis

All values are means ± s.e.m. and statistical differences were tested using one-way analysis of variance. To compare different populations or to evaluate correlations between specific parameters we used Mann–Whitney and Spearman tests respectively.

Results

Reproductive characteristics of captive females

Determination of the oestrous period. In urogenital smears, taken every day for 2 months, different elements were recognizable: small ovoid parabasal cells, large nucleated or non-nucleated epithelial cells with a polygonal form, and polymorphonuclear leucocytes. In some cases, additional elements were present such as erythrocytes or large vacuolar granules of lipoidal secretion from paracloacal glands.

The start of the oestrous period was indicated by increasing numbers of epithelial cells in association with parabasal cells. The oestrous period lasted 6–10 days before a characteristic leucocytic infiltration which indicated the end of this period. Nevertheless, more or less numerous leucocytes were found throughout the entire cycle even before the oestrous period and epithelial cells may persist many days after leucocyte infiltration. Therefore, only a large proliferation of epithelial cells for several days followed by a clear heavy leucocytic infiltration has been considered as one oestrous period. The end of the cytological oestrus marked by leucocyte infiltration was designated as Day 0 of the oestrous cycle.

The oestrous cycle. Within the 4 captive females studied, variations of plasma progesterone concentrations and the occurrence of oestrous periods detected by urogenital smears (n = 26) are represented in Fig. 1. Each female entered oestrous 5–7 times a year and there was no difference between paired or isolated females. All but 4 oestrous periods were followed by a high increase in plasma progesterone concentrations and thus can be considered as true oestrous cycles whereas the 4 remaining would be anovulatory cycles.

No clear restricted breeding season was apparent for captive females, but in a 3-month period from the end of August to the end of November there were few oestrous periods (3 out of 26),
mostly anovulatory. By contrast, in December and January, the females cycled with a high degree of synchrony and the occurrence of oestrous periods throughout a year was statistically different from a randomized distribution ($P < 0.05$).

The cycle length, determined as the interval between two cytological oestrous periods, varied from 28 to 115 days. However, the interval between 2 to 3 successive oestrous cycles was much shorter, varying from 28 to 45 days with a mean of 38.6 ± 1.4 ($n = 14$). These sequences of ovarian activity were separated by a spontaneous period of decreased reproductive activity. During these periods, about 100 days long and present in February/March and September/November, females did not exhibit urogenital oestrus or, if they did, no increase in plasma progesterone concentrations was observed.

**Progesterone profile during the oestrous cycle.** To establish variations in plasma progesterone concentrations during an oestrous cycle, we have only considered the changes in cycles lasting no more than 45 days ($n = 14$). Means of progesterone concentrations were calculated for females sampled every 10 days (10 cycles) or every 5 days (4 cycles). For each cycle, progesterone concentrations were dated from the time of leucocyte infiltration.

Large and significant variations in progesterone concentrations were found (Fig. 2). They were characterized by a clear and significant increase from 5 to 25 days after leucocyte infiltration. This would indicate that the luteal phase lasted about 20 days and was followed by a follicular phase of the same duration. During the luteal phase, the teats became conspicuous and were frequently surrounded by a red–brown pigment while the marginal ridges of the pouch skin developed in this species which does not possess a permanent marsupium.

Females did not exhibit overt signs of oestrus. The only behavioural sign of the occurrence of oestrus consisted of non-aggressive contacts between the sexes whereas all encounters outside the
Fig. 2. Plasma progesterone concentrations (mean ± s.e.m.) during the oestrous cycle of captive females (14 cycles). The presence of epithelial cells and leucocytes in smears, and the development of the pouch were noted in relation to the oestrous cycle. Hormonal values were dated from the leucocyte infiltration (arrow) and the grey area indicates the estimated luteal phase. \*P < 0.05; \**P < 0.01.

Oestrous period were aggressive (e.g. hissing, chasing, jaw-gaping). During this experiment, none of the paired females became pregnant although sexual behaviours such as courtship and mounts were observed. Consequently, we have no accurate information on gestation length in the woolly opossum; for 3 gestations in captive females without a precise date of copulation, the duration was estimated between 20 and 28 days. A female trapped in the field and then kept isolated gave birth 21 days after capture. The gestation length in the woolly opossum would therefore be at least of the same duration as the interval between leucocyte infiltration and the fall in plasma progesterone measured during non-fertile cycles.

Reproductive function in captive males

Although the environmental conditions in captivity were constant, a single-factor analysis of variance for repeated measures revealed significant (P < 0.01) testosterone variations at monthly intervals for captive males tested during 1 year.

Testosterone changes in isolated males followed a pattern similar to those observed in paired males (P < 0.01) but at a lower level (testosterone annual means: 3.9 ± 0.1 and 6.3 ± 0.8 ng/ml for isolated and paired males respectively, \( P < 0.01 \)). When the changes in the monthly mean testosterone concentrations are expressed as a percentage of the individual annual mean, changes were characterized by two peaks located in April (\( P < 0.001 \)) and in September (\( P < 0.01 \); Fig. 3). A 3-month period with low testosterone concentrations (May–July) separated these two maxima.

A significant (\( P < 0.05 \)) but brief testosterone increase was found in paired males in response to an oestrous period of the paired females (mean testosterone value: 9.3 ± 0.7 ng/ml, \( n = 18 \) oestrous periods). The rise in testosterone concentrations in April could be linked to the resumption of oestrus in females (3/4), but not in September since few females experienced oestrus at this time.
Fig. 3. Circannual changes in plasma testosterone concentrations in captive males (N = 5). The monthly means (± s.e.m.) were expressed as the percentage of the individual annual mean. The broken line represents the annual mean (5.3 ± 0.7 ng/ml). Testosterone changes were not correlated with the sexual activity of captive females, indicated by the number of females in oestrus. **P < 0.01; ***P < 0.001.

Discussion

The regulation of reproductive activity by environmental factors and the changes in sexual hormones remain unknown for most marsupials of South America. Seasonal variations in food resources have been considered as the main factor that regulates reproductive patterns of woolly opossums in French Guyana (Atramentowicz, 1982). However, male and female woolly opossums exhibited variations in their sexual activity under constant conditions.

Within males, although there were no apparent variations in testicular size, as in several didelphids (Biggers, 1966; Fleming, 1973), plasma testosterone concentrations varied throughout the year from 1 to 20 ng/ml in captive males. These values compared well with testosterone measurements reported for Australian species (Lincoln, 1978; Tyndale-Biscoe & Renfree, 1987).

With respect to captive females, they cycled 5–7 times a year as in captive Virginia opossums (Didelphis virginiana) (Jurgelski & Porter, 1974). The length of the woolly opossum oestrous cycle, on average 38 days, was one of the longest measured for didelphids since duration of the oestrous cycle in this marsupial family ranges from 15 to 40 days according to species (Hartman, 1923; Godfrey, 1975; Bucher & Fritz, 1977; Fleming & Harder, 1981; Fadem & Rayve, 1985).

Except for the Virginia opossum (Harder & Fleming, 1981) concentrations of plasma progesterone during the oestrous cycle have not been studied in American marsupials. In woolly
Fig. 4. Relationships between (a) seasonal variations in rainfall and fruit production, (b) body weight of wild woolly opossums (mean ± s.e.m. for 15–26 animals per month) and (c) the percentage of the population of lactating females and of females entering oestrus within 1 month (10–14 females per month). Data for rainfall and fruiting trees were recorded at Cabassou, French Guyana (Atramentowicz, 1982). *P < 0.05; **P < 0.01.
opossums, progesterone concentrations increased rapidly from the end of the oestrous period, peaking by Day 10 of the cycle. The decline was equally rapid, beginning on Day 25 and reaching a basal level of 1–5 ng/ml. Consequently, the luteal phase would last about 20 days, i.e. no more than 60% of the cycle length, as in most marsupials (Tyndale-Biscoe & Renfree, 1987). The relatively high progesterone values during the luteal phase (~30–40 ng/ml) would indicate the presence of a large luteal mass as suggested by Harder & Fleming (1981) for the Virginia opossum. Indeed, in didelphids and also several dasyurids, the number of eggs shed at one ovulation was very high from 20 to 40 (Tyndale-Biscoe & Renfree, 1987). Nevertheless, this high progesterone concentration might be also attributable to high affinity binding for gonadal hormones by plasma proteins (Sernia et al., 1979).

Spontaneous rhythms of captive males and females appeared to be asynchronous which would perhaps explain the poor reproductive success in captivity. Indeed, testosterone concentrations in males were highest in April and September whereas females experienced synchronous periods of 2–3 successive oestrous cycles which began in April and December. However, despite this asynchrony, a stimulating influence of females upon testosterone concentrations existed in the woolly opossum: males kept with females showed higher testosterone concentrations than did isolated males and their hormonal concentrations increased again in response to oestrous females. Such testosterone rises in response to female stimuli are well established for marsupial males (Lincoln, 1978; Catling & Sutherland, 1980; Curlewis & Stone, 1985; Fletcher, 1985; Bryant, 1986). Conversely, a stimulating effect of males upon females has been described for the grey short-tailed opossum (Monodelphis domestica) using short-test pairing (Fadem, 1987; Baggott et al., 1987). Such an effect does exist in the woolly opossum (unpublished) but was not apparent in this study because the pairs were unchanged. Lastly, synchrony between females could be attributed to social stimuli especially for animals born in captivity as reported for Antechinus stuartii (Scott, 1986).

Under natural conditions, the body weight and the reproductive activity of woolly opossums undergo seasonal changes which have been correlated to the seasonal decrease in food resources of the natural environment (Fig. 4). Both sexes significantly lost weight in July, a period during which fruit production was decreasing. Associated with the body weight changes, the percentage of lactating females within each month varied significantly (P < 0.001: Fig. 4) with a maximum recorded when food availability was high. However, more than 50% of estimated oestrous periods occurred during the dry season, before fruit production had increased (Fig. 4). Wild females seemed to cycle with synchrony and to ‘anticipate’ the onset of the rainy season in November. Oestrous periods from March to June corresponded to the resumption of oestrus in females which had previously raised their first litter to weaning. That second ‘wave’ of oestrous periods might be underestimated since only fertile oestrous periods have been taken in account.

For wild males, they are generally considered as sexually potent throughout the year but assays of blood samples from 28 wild mature males showed that plasma testosterone concentrations of males sampled in July (beginning of the dry season: 2.9 ± 0.2 ng/ml, N = 7) significantly (P < 0.01) differed from values obtained in August (4.9 ± 0.3 ng/ml, N = 12) and October (end of the dry season: 5.8 ± 0.7 ng/ml, N = 9). Thus, testosterone concentrations decreased when environmental resources were limited in the field and increased precisely when most of the wild females entered oestrus.

In a constant environment, captive animals exhibited sexual cycles although their body weight remained high and constant, and these spontaneous rhythms followed a pattern similar to that of wild animals without any changes in males and with a delay of several months in females. Indeed, if the onset of sexual activity of captive females is considered as being delayed by 3 months, a significant (P < 0.05) correlation appeared between the distribution of oestrous periods in wild and captive females. That desynchronized period may represent the time accumulated by females free-running in captivity over 3 years. Changes in testosterone concentrations of captive males were surprisingly correlated (P < 0.05) to the distribution of oestrous periods for wild females except
in January–April, a period during which wild females were lactating while captive females experienced oestrus.

In numerous seasonal species including some marsupials circannual cycles of body weight, internal temperature or reproduction can persist in the absence of variations of the external environment (Canguilhem, 1985; Dickman, 1985; Tyndale-Biscoe & Renfree, 1987). These endogenous cycles are synchronized in the wild by an environmental cue of which the best studied is daylength. Photoperiodic changes determine the breeding season in numerous Australian species (Godfrey, 1969; Sadleir & Tyndale-Biscoe, 1977; Smith et al., 1978; Woolley & Watson, 1984; Gemmell et al., 1985, 1986; Gemmell, 1987), and probably in the Virginia opossum, the restricted breeding season of which depends upon the latitude in North America (Reynolds, 1952; Fleming, 1973; Hunsaker, 1977). However, this cannot be the case for the woolly opossum which lives at a latitude where daylength varied by less than 30 min a year.

In fact, the breeding season of a species may be considered to be the result of ecological constraints (food availability) and 'maternal investment', i.e. time and energy required for a female to rear young until weaning (Russell, 1982). In small marsupial species with a short gestation period and a rapid emergence of the young at the end of lactation, reproduction is very responsive to the occurrence of favourable conditions: temperature, quality and abundance of food (Newsome, 1965, 1973; Heinsohn, 1966; Gordon, 1971; Stoddart & Braithwaite, 1979; Barnes & Gemmell, 1984).

When environmental resources vary in a predictable fashion, species experience a restricted breeding season during the favourable period, but when the maternal investment is long, they have to anticipate the onset of high food availability (as in several dasyurids, Calaby & Taylor, 1981; Tyndale-Biscoe & Renfree, 1987). Didelphids living in neotropical rain forest distribute their maternal investment over about 3–4 months (Atramentowicz, 1986) and thus start to breed at the time when the dry season occurs and when environmental resources are still limited (Atramentowicz, 1982). As suggested by Fleming (1973) and O'Connell (1979) the cessation of high rainfall would be the determining signal for the breeding season to take place. However, in the woolly opossum, since the reduction in fruit production was correlated to decreased reproductive activity in both sexes, the presence of a sexual resting period could be a factor required for the next breeding season to begin.

We thank Pr J. Roffi for permission to use the technical facilities of the laboratory of Endocrinology (University Paris IX, France) and P. Belbenoit for blood samples of wild animals.

References

Bryant, S.L. (1986) Seasonal variation of plasma testosterone in a wild population of male eastern quoll Dasyurus viverrinus (Marsupialia, Dasyuridae) from Tasmania. Gen. comp. Endocr. 64, 75–79.
Cattling, P.C. & Sutherland, R.L. (1980) Effect of gonadectomy, season and the presence of female tammar wallabies (Macropus eugeni) on concentrations of
testosterone, LH and FSH in the plasma of male tammar wallabies. J. Endocr. 86, 25–33.


Sexual cycles in the woolly opossum


Received 3 March 1988