Mating-associated peak in plasma testosterone concentration in wild male grey-headed flying foxes
(Pteropus poliocephalus)

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Summary. Plasma testosterone (T) concentrations, measured in wild bats of P. poliocephalus in Queensland in 1983–87, showed a peak during the mating season in March. Plasma androstenedione (A) concentrations changed less dramatically with season. Mean testicular concentration and total content of T and A was substantially greater in March than in regressed testes in July–October. Paired adrenal glands were heavier during February to April than during September to November. In the same wild population, throughout a single breeding season (1987), plasma T concentrations were significantly higher in mid-March than 3 weeks previously or 3 weeks later. Testicular T content rose as the breeding season progressed, being greatest during March, coinciding with the large rise in plasma T concentrations. Testicular T concentration and content were correlated significantly with plasma T concentrations. Adrenal glands contained T, but the absolute concentrations were much lower than in the testis. No significant changes in plasma, testicular or adrenal A concentrations were found as the breeding season progressed. The large increase in plasma T during the mating season appears to be due to increased testicular production.

Keywords: testosterone; androstenedione; testis; flying fox; mating

Introduction

Adult male grey-headed flying foxes (Pteropus poliocephalus) show well-defined seasonal changes in reproductive parameters in the wild (McGuckin & Blackshaw, 1987) and in captivity (McGuckin & Blackshaw, 1991). During the autumn breeding season, plasma concentrations of testosterone rise dramatically and peak towards the end of the period of greatest testicular size. Leydig cells also appear active, histologically, during testicular recrudescence, well before the peak in peripheral testosterone concentration. The increase in plasma concentrations could be due to increased testicular production, an extragonadal source, a change in metabolic clearance, or a combination of these influences. This study sampled testes and adrenal glands from wild bats of P. poliocephalus in Queensland during the mating season to compare their contributions to increases in testosterone production.

Materials and Methods

1983–87 study

Data were collected from adult males from various camps in south-east Queensland in 1983–87. Some of the data on testicular weight and plasma testosterone concentration from material collected before 1986 have been published (McGuckin & Blackshaw, 1987).

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1987 breeding season study

During the 1987 breeding season, samples were obtained from the same population (a large camp at Mt Berryman in south-east Queensland) every 21 days on four occasions from early February until mid-April. Adult males were shot and blood samples were obtained by cardiac puncture, collected in heparinized vials and stored on ice until centrifugation at 4000 g for 15 min to obtain plasma which was kept at −20 °C until thawed for assay. The reproductive tract was quickly dissected and both adrenal glands and testes were frozen for extraction and steroid analysis. One testis and the remainder of the reproductive tract were fixed in Susa’s fixative for histological examination. Plasma, testicular and adrenal testosterone and androstenedione concentrations were measured at intervals of 3 weeks during the breeding season.

Tissue extraction

Pieces of frozen tissue were thawed (~200 mg of testicular parenchyma and bisected adrenal glands) and weighed on a Mettler electronic balance. Tissue was placed in 17 × 98 mm glass tubes containing 500 µl assay buffer and 10 000 c.p.m. 3H-steroid and homogenized using a Polytron homogenizer. Then, 2 ml AR ethanol (May and Baker, Australia) was added and the mixture was vortexed several times in 30 min. Tubes were then centrifuged at 4000 g for 20 min and supernatants decanted into 12 × 75 mm glass tubes. The ethanol was evaporated under a stream of N2 at 40°C until only the aqueous phase remained, when 2 ml diethyl ether was added to the tubes, which were vortexed for 15 min. The aqueous phase was frozen over solid CO2 and the ether extract decanted and dried under a stream of N2 at 40°C. Dried extracts were resuspended in 1 ml assay buffer for assay and recovery determination, or in isoctane for chromatography.

Testosterone and androstenedione radioimmunoassays

Testosterone concentrations in plasma and tissue extracts were determined using the methods validated for use in this species (McGuckin & Blackshaw, 1987, 1991).

The antisera (709) used in the androstenedione assay was prepared by R. I. Cox (CSIRO, Division of Animal Production, Prospect, NSW, Australia), who reported significant cross-reactions with only androsterone (28%) and dehydroepiandrosterone (5.9%). Extraction and assay were performed in 12 × 75 mm glass tubes. Tritiated androstenedione (110 Ci/mmol) was obtained from Amersham Australia and a sufficient quantity dried under a stream of N2 and redissolved in assay buffer to 8000 c.p.m./100 µl. Fifty µl aliquants of unknown plasma samples in triplicate were extracted in 2 ml diethyl ether. Standards 0-2048 pg/tube in triplicate were dried under a stream of N2 for each assay. Dried plasma extracts and standards were resuspended in 300 µl of antisera, at a 1:14 000 dilution in assay buffer, and 100 µl of tracer. Tubes were incubated for 16 h at 4°C, followed by addition of 400 µl of a dextran-coated charcoal suspension for 10 min at 4°C. After centrifugation, supernatants were added to 3 ml of scintillation fluid (5.6% (w/v) acetic acid in toluene containing 0.3% (w/v) 2,5 diphenyloxazole and 0.03% (w/v) 1,5-bis[2-(5-phenyloxazolyl)]-benzene, Syndel Laboratories, Canada) and radioactivity was determined in an LKB liquid scintillation counter. A standard curve was constructed using a logit-log transformation and concentrations in unknowns were determined after correction for recovery. Plasma samples found to contain >2000 pg androstenedione/50 µl were diluted in assay buffer and re-extracted and assayed.

Sensitivity of 8 consecutive androstenedione assays was 34 ± 13 pg/tube, which is equivalent to 0.74 ng/ml of plasma when 50 µl of plasma was assayed and extraction recovery was 92%. Sensitivity of this assay was increased by incubating the unknowns and standards with the antisera for 60 min at 37°C before addition of the tracer. Although sensitivity was increased by this technique to ~4–8 pg/tube, the slope of the standard curve was not steep in this region, resulting in possible errors in calculation of concentration. Therefore, if concentrations were undetectable in a particular sample, it was preferred to re-assay a greater volume of the sample rather than use the precipitation technique. Charcoal-stripped, castrate Pteropus plasma did not cause positive or negative blanking in the androstenedione assay, therefore stripped plasma was not included as blanks in each assay and the zero standard was used for the assay blank, as in the testosterone assay. Recovery of androstenedione was assessed by the addition of 256 pg to a plasma sample containing ~3.5 ng/ml before extraction in 3 consecutive assays. Recovery of added steroid was determined after allowing for extraction recovery and was 111 ± 22 (s.d.) %. Parallelism was demonstrated using serial dilutions of a plasma sample containing ~10 ng androstenedione/ml. Celite chromatography (Thorneycroft et al., 1973) showed that the peaks in immunoreactivity and radioactivity for androstenedione corresponded.

The cross-reactivities of the testosterone and androstenedione antisera with androstenedione and testosterone, respectively, were determined by comparing the amount (in molar concentration) of cross-reacting steroid required to produce 50% inhibition of binding with the amount of standard giving the same inhibition (Abraham, 1969). Cross-reactivity was then expressed as a percentage of the standard; for androstenedione in the testosterone assay it was 2.1% and for testosterone in the androstenedione assay it was <0.1%.

Statistical analysis

The nonparametric Wilcoxon rank sum test (Snedecor & Cochran, 1967) was used to assess the statistical significance of seasonal changes in concentrations of testicular testosterone and androstenedione.
Peak in plasma testosterone in flying foxes

Results

1983–87 study

Peripheral plasma testosterone concentrations showed marked seasonal variations; concentrations attained in March were much higher than in most eutherians (46·3 ± 5·9 ng/ml, mean ± s.e.m., n = 14) and significantly higher (P < 0·01) than during February (17·5 ± 4·4, n = 8) and April (17·6 ± 4·4, n = 9). After the breeding season, from June until December, concentrations were significantly lower (2·18 ± 0·45, n = 12, P < 0·001) than during February to April.

Plasma androstenedione concentrations changed less dramatically with season; they were highest during the breeding season (February, 6·3 ± 1·0 ng/ml, mean ± s.e.m., n = 8; March, 16·1 ± 3·1, n = 12; April, 11·5 ± 2·1, n = 8) and lowest in the nonbreeding season (June to December, 4·0 ± 2·7, n = 8). During the nonbreeding season, plasma androstenedione concentrations were slightly greater than plasma testosterone concentrations, but the peaks during March were lower than for testosterone at this time.

Variations amongst individuals in testicular testosterone and androstenedione concentrations and content were considerable. Mean breeding-season testosterone and androstenedione values, which peaked in March, were significantly greater than the means in regressed testes (July to October, Table 1). Testicular testosterone content was correlated significantly and positively with testicular androstenedione content (r = 0·65, P < 0·001, n = 36).

| Table 1. Testicular testosterone and androstenedione concentrations and total gland contents in adult male *P. poliocephalus* during the breeding months and the nonbreeding season (July to October): means ± s.e.m. |
|-------------------------------------------------------------
<table>
<thead>
<tr>
<th>Month</th>
<th>n</th>
<th>Testosterone (pg/mg)</th>
<th>Testosterone Content (ng)</th>
<th>Androstenedione (pg/mg)</th>
<th>Androstenedione Content (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>February</td>
<td>10</td>
<td>156 ± 51</td>
<td>503 ± 175</td>
<td>58 ± 9b</td>
<td>170 ± 34b</td>
</tr>
<tr>
<td>March</td>
<td>13</td>
<td>366 ± 93</td>
<td>1034 ± 252</td>
<td>70 ± 13</td>
<td>201 ± 33</td>
</tr>
<tr>
<td>April</td>
<td>5</td>
<td>152 ± 58</td>
<td>360 ± 134b</td>
<td>59 ± 13b</td>
<td>139 ± 24b</td>
</tr>
<tr>
<td>July–October</td>
<td>8</td>
<td>78 ± 24a</td>
<td>101 ± 23a</td>
<td>25 ± 5a</td>
<td>35 ± 6a</td>
</tr>
</tbody>
</table>

Statistics: *March (P < 0·01), b> July–October (P < 0·05), Wilcoxon rank sum test.*

Adrenal glands showed a large and significant size change with season. Paired adrenal glands were heavier during the breeding season (February to April; 880 ± 60 mg, mean ± s.e.m., n = 28) than during the nonbreeding season (September to November; 560 ± 36 mg, n = 13, P < 0·01).

1987 breeding season study

Testosterone and androstenedione concentrations showed considerable variation among individuals (Fig. 1). Plasma testosterone concentrations showed a significant peak in mid-March (19 March 1987). Corresponding values of testicular testosterone concentration and content were significantly higher than at the beginning of the breeding season, but not at other times of sampling. Testicular testosterone concentration and content were correlated significantly and positively with plasma testosterone concentrations (r = 0·87 and 0·85, respectively, P < 0·001). Adrenal testosterone concentration and content showed no significant changes during the breeding season. Although adrenal testosterone content was correlated significantly (r = 0·63, P < 0·005) with plasma testosterone concentration, the mean adrenal content was much less than in the testis (being 28, 5, 3 and 6% of the testicular mean at the 4 sampling times, respectively).

No significant changes in plasma, testicular or adrenal androstenedione concentrations were found as the breeding season progressed. Mean adrenal androstenedione content was less than in the testis at all times (39, 13, 9 and 13% of the testicular mean at the 4 sampling times, respectively).
Testicular androstenedione concentrations and contents were weakly correlated with plasma androstenedione concentrations ($r = 0.58$ and $0.52$, respectively, $P < 0.05$). Significant correlations were found between plasma ($r = 0.68$, $P < 0.01$) and testicular ($r = 0.82$, $P < 0.001$) concentrations, and between testicular content ($r = 0.91$, $P < 0.001$) of testosterone and androstenedione.

**Discussion**

Peripheral concentrations of testosterone attained during March in grey-headed flying foxes exceed those found earlier in the year, when testes were already close to their greatest size and spermatogenic activity. High testosterone secretion may be required for accessory gland development,
which peaks in April (McGuckin, 1989) and/or for stimulation of mating behaviour and, perhaps, pheromone production.

The greater testicular testosterone and androstenedione concentrations observed in February–April, compared with those of regressed testes, parallels the changes observed in testicular weight, spermatogenic activity and Leydig cell morphology (McGuckin & Blackshaw, 1987). During testicular recrudescence, size of nuclei in Leydig cells, cytoplasmic volume and total Leydig cell volume/testis increase (McGuckin & Blackshaw, 1987). Presumably, before the breeding season, the appropriate environmental stimulus, which is probably change in photoperiod (McGuckin & Blackshaw, 1988), causes activation of the gonadotrophin-releasing hormone pulse generator and therefore an increase in release of luteinizing hormone (LH) and follicle-stimulating hormone from the pituitary, leading to increased spermatogenesis and testicular size. The concentrations of testosterone measured may be overestimates, because Leydig cells could continue to secrete considerable amounts of testosterone after the blood supply is severed before freezing (Sharpe, 1987). Maddocks et al. (1986) contend, after study of testicular testosterone production using push–pull cannulae in rats, that many of the reported values for testicular testosterone may be overestimates.

The data on steroid concentrations in plasma, testes and adrenals obtained during the 1987 breeding season suggest that the large increase in peripheral testosterone during the mating period is due, at least in part, to increased testicular production. Leydig cells appear fully active, histologically, before the peak (in February) (McGuckin & Blackshaw, 1987), suggesting that further stimulation occurs, resulting in increased production of testosterone. Increased stimulation may involve an alteration in the LH release pattern and/or increased sensitivity of the Leydig cells to LH. Changes in stimulation could occur in response to many factors including environmental zeitgebers, female pheromones or stimulation of the central nervous system associated with mating or territorial behaviour. Although adrenal testosterone content is considerable, its contribution to the rise in peripheral content appears small. Adrenal testosterone content decreased as a proportion of testicular content at the time of the peak in plasma concentration. However, the seasonal change in adrenal gland size indicates that the adrenal may be involved with seasonal reproductive and associated physiological changes. The adrenal increases in size about the time of the breeding season in other species, including the ground squirrel, Citellus tridecemlineatus (Zalesky, 1934), the woodchuck, Marmota monax (Christian, 1962), and the European hamster, Cricetus f rumentarius (Kayser & Aron, 1950). In red deer stags, adrenal glands undergo a seasonal size change, but are largest during the late summer, well before the rut (Lincoln, 1971).

In grey-headed flying foxes, a large increase in plasma testosterone concentration occurs during the mating season associated with increased testicular production. Further experiments are required to determine whether changes in metabolic clearance are also involved in maintaining the high peripheral testosterone concentrations. Measurement of gonadotrophins will clarify the role of the pituitary in increased testicular stimulation at this time.

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References

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