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# Seasonal Changes in Spermatogenesis (Including Germ Cell Degeneration) and Plasma Testosterone Concentration in the Grey-headed Fruit Bat, *Pteropus poliocephalus*

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*Abstract*

Maximal testicular size of *P. poliocephalus* occurred during the February–March–April period (autumn breeding season) associated with maximal plasma levels of testosterone (121·5 ± 23·4 nmol/l). Testicular size decreased after the breeding season concomitant with a decrease in plasma testosterone (7·6 ± 1·7 nmol/l). Histologically, regressive changes were observed in the testis after the breeding season including a decrease in seminiferous tubule diameter and a decrease in Leydig cell nuclear diameter. Significant germ cell loss occurred during the breeding season (44%) mainly during the final spermatogonial division and meiosis and increased in regressed testes (69%), being accentuated chiefly at meiosis. All regressed testes showed some sperm production although it was much reduced after the breeding season; however, optimal fertility appears to be restricted to autumn by the large increases in testosterone secretion which only occur at this time.

## Introduction

The grey-headed fruit bat, *Pteropus poliocephalus*, is a short-day breeder which inhabits the coastal regions of eastern Australia (Ratcliffe 1931). Adult males show seasonal changes in testis size with maximal testis size occurring during autumn just prior to the time of mating (Nelson 1963). Spermatogenic dynamics and germ-cell degeneration have been examined in laboratory animals, such as the rabbit (Swiestra and Foote 1963) and the rat (Russell and Clermont 1977), domestic species (sheep, Ortavant 1956) and in some wild species (vole, Grocock and Clarke 1975). Although qualitative descriptions of spermatogenesis in chiropterans are available (Krutzsch 1961, 1975; Bradshaw 1962; Racey 1974; Racey and Tam 1974; and Singwi and Lall 1983) no spermatogenic kinetic studies have been performed on this large order of mammals. The present study provides a detailed analysis of the seasonal changes in spermatogenesis in the fruit bat. The microchiropterans in which peripheral plasma testosterone concentrations have been measured have all shown extremely high levels compared with most eutherians, during at least one phase of their sexual cycles (Racey 1974; Gustafson and Shemesh 1976; and Beasley *et al.* 1984), therefore the present study provides a comparison utilising a megachiropteran. Pettigrew (1986) and others have suggested that the Micro- and Megachiroptera evolved separately and that the megachiropterans are possibly prosimian primates. Detailed analysis of reproductive patterns in megachiropterans will provide some useful comparisons with primate and microchiropteran reproduction.

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## Materials and Methods

*Animals and Tissue Collection*

Adult male *P. poliocephalus* were obtained throughout the year from camps in the south-east Queensland region either by hand-capture or by shooting. Blood samples were obtained immediately by cardiac puncture from shot animals, and by either cardiac or venipuncture from animals captured in the wild (within 20 min of capture). Captured animals were killed by barbiturate overdose within a few hours of capture. Reproductive organs were removed immediately after death and either directly immersed in Susa's fixative or alternatively left on ice for less than 2 h prior to immersion.

*Histology*

Tissue was fixed for 24 h and then placed in 70% (v/v) ethanol prior to processing. Epididymis and fat were removed from fixed testes which were then weighed (at the 70% ethanol stage) on a Mettler electronic balance. Testis tissue blocks were then dehydrated in a graded series of alcohols, cleared in toluene, and infiltrated and embedded in Paraplast (Medos). Testis cross-sections 5 $\mu$m thick were stained with Mayer's haematoxylin and alcoholic eosin after treatment with Lugol's iodine and sodium thiosulfate solutions, to remove any remaining mercuric chloride crystals (Humason 1972).

*Stereology and Quantification of Spermatogenesis*

The volumetric proportions of testicular tissue components were determined by point counting utilizing a Weibel 42-point graticule (Weibel 1979) overlying a television monitor carrying an image of the histological sections. One randomly selected testis cross-section from each testis was analysed. Tissue underlying points (average of about 500 per section) was classified as either seminiferous tissue, Leydig cells, interstitial tissue other than Leydig cells (including blood vessels, lymphatic vessels and connective tissue), capsule or space. Counts for space (assumed to be artefact) and capsule were deleted and the percentage of points designated to each tissue component calculated (equivalent to the volumetric proportion).

Other quantitative data were obtained from an analysis of sections from five breeding season testes (March $n = 4$, April $n = 1$) and five non-breeding testes (August $n = 4$, September $n = 1$) randomly selected from those available in the two seasons. One randomly selected tissue section from each testis was analysed. All germ cell nuclei (including fragments) lying within two circular tubule cross-sections of each of the eight stages of spermatogenesis (McGuckin and Blackshaw 1987) were counted. Seminiferous tubule diameter was measured for each analysed tubule as was the diameter of two whole nuclei of each cell type within each tubule. Nuclear counts were transformed by an adaptation of Abercrombie's formula (Abercrombie 1946) developed by Swiestra and Foote (1963) and used similarly for a seasonal analysis of germ cell loss by Grocock and Clarke (1975):

$$P = C[T/(L+T)] \,.\, (D'/D)\,,$$

where $P$ = average number of nuclear points per cross-section (corrected cell count), $C$ = crude count of the nuclear fragments, $T$ = section thickness in micrometres, $L$ = diameter of whole nuclei in micrometres, $D$ = diameter of each particular seminiferous tubule, $D'$ = mean diameter of 80 breeding season (228 $\mu$m) or 80 non-breeding season (164 $\mu$m) tubules. Hence the raw cell counts were corrected for both the bias in counting nuclear fragments from nuclei of different sizes and for individual variation in tubule diameter within classes (breeding and non-breeding). Mean corrected cell numbers per tubular cross-section were therefore calculated for all germ cell types in the eight stages of spermatogenesis during the breeding and non-breeding seasons. Expected germ cell numbers were calculated from the mean number of intermediate spermatogonia in stage 6 assuming mitotic division ($\times 2$) to form B spermatogonia followed by mitotic division to form primary spermatocytes ($\times 2$) and meiotic division ($\times 4$) to form round spermatids.

On the same sections six Leydig cell nuclear diameters were measured, and high-power point counting was performed (as previously) over clumps of Leydig cells to determine the volumetric proportion of Leydig cell nucleus and cytoplasm.

*Testosterone Assay*

The concentration of testosterone in plasma was measured by radioammunoassay using an antiserum to testosterone (prepared by Dr R. I. Cox, Division of Animal Production, CSIRO, Prospect, N.S.W., Australia) with significant cross-reactions to only 5$\alpha$-dihydrotestosterone (31%) and 4-androsten-3$\beta$, 17$\beta$-diol (30%). Celite column chromatography, of ether extracts of plasma samples collected at various times of the year was used to ensure that the immunoreactive substance measured in the routine assay

was testosterone, using the techniques of Thorneycroft *et al.* (1973). Isooctane was used as the mobile phase of the Celite columns and increasing concentrations of benzene (8, 30 and 60% v/v) were used to flush steroids from the columns resulting in a peak of labelled testosterone that corresponded with the peak of immunoreactive substance in all cases. Two pools of plasma containing, respectively, low and high concentrations of testosterone were included in each assay to calculate inter- and intraassay coefficients of variation at two levels on the standard curve. Parallelism was demonstrated using graded dilutions (1/50 to 1/1) of a plasma sample containing approximately 10 ng of testosterone per millilitre.

All solvents used in the routine assay were analytical reagent grade (BDH Chemicals). [$^3$H]Testosterone (3·81 TBq/mmol) was obtained from Amersham Australia. Triplicates of 50 $\mu$l of plasma were extracted in 2·0 ml diethyl ether and dried over nitrogen (average extraction efficiency 92%). Standards were prepared and stored in ethanol and various concentrations (0–1024 pg/tube) were blown dry over nitrogen in triplicate, for each assay. Extracts and standards were resuspended in 300 $\mu$l of antiserum, at a 1 : 30 000 dilution in 0·1 M phosphate–gelatin buffer (pH 7·4), and 100 $\mu$l of [$^3$H]testosterone (10 000 cpm) in the same buffer. Tubes were incubated overnight at 4°C followed by separation of the bound and free steroid with 400 $\mu$l of 0·625% charcoal–0·0625% dextran for 10 min at 4°C. Following centrifugation the supernatant was removed, added to 3 ml of scintillation fluid (toluene, containing 0·3% PPO, 0·03% POPOP and 5% acetic acid) and radioactivity was measured in a LKB scintillation counter. A standard curve was calculated according to a logit transformation and testosterone concentrations in unknown plasma samples were determined from this curve and expressed as nanograms of testosterone per millilitre of plasma. If the testosterone concentration was above the upper limits of the standard curve, plasma was appropriately diluted in the assay buffer and 50 $\mu$l of the diluted plasma re-extracted and assayed. The sensitivity of the assay (calculated as two standard deviations from the blanks) was typically less than 4 pg/tube (0·08 ng/ml plasma). The intra-assay coefficients of variation were 8·3 and 10·4% at the low and high levels of the standard curve, respectively. The interassay coefficients of variation were 20·3 and 18·7% at the low and high levels of the standard curve, respectively.

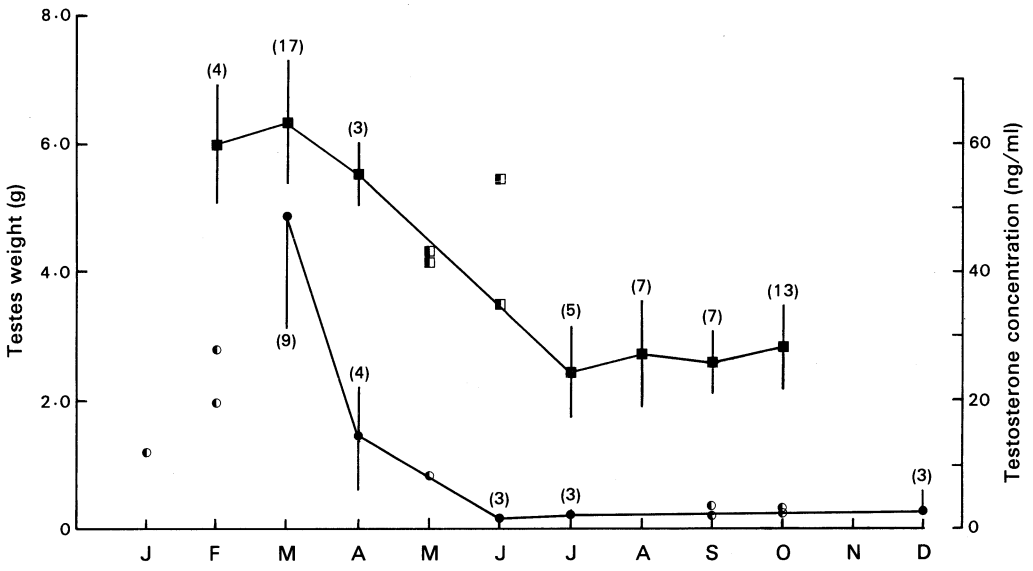
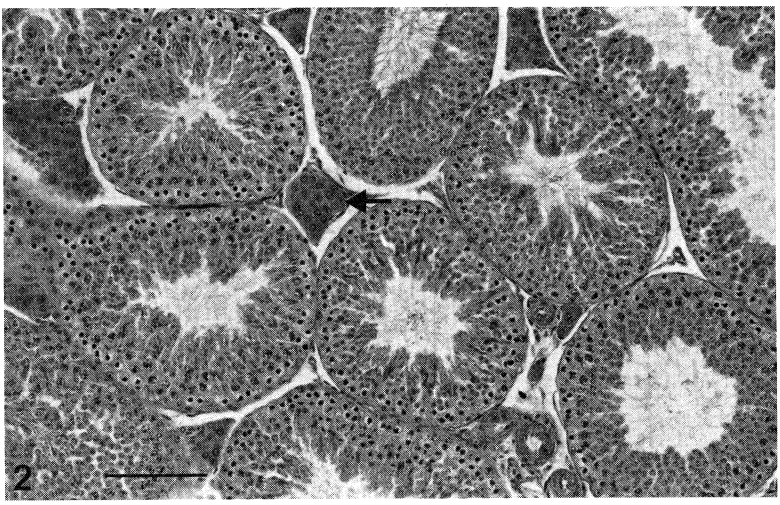



**Fig. 1.** Seasonal changes in testes weight (■) and plasma testosterone concentration (●) in wild adult male *P. poliocephalus*. Solid symbols are monthly means ± s.d. (*n*), half-symbols are individual values.

## Results

### *Testicular Size*

Testicular weight was maximal during the autumn mating season and decreased significantly after the breeding season reaching minimal levels during the months July to October (Fig. 1). Body weight appeared to alter with season being maximal in February and least at the time of testicular regression.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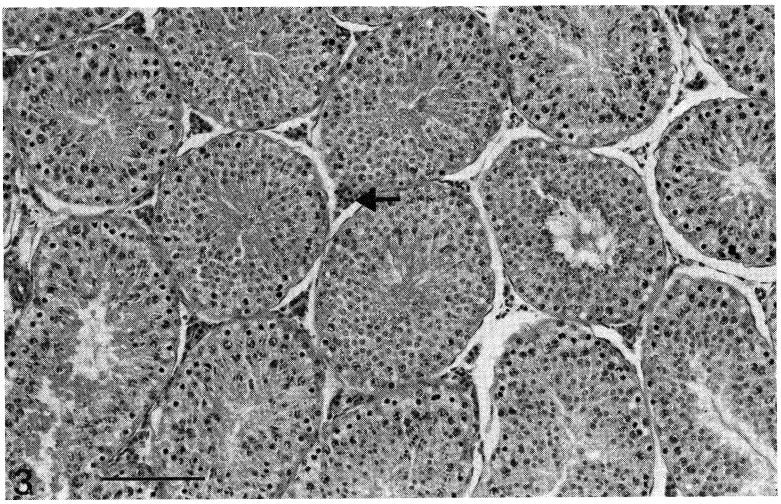
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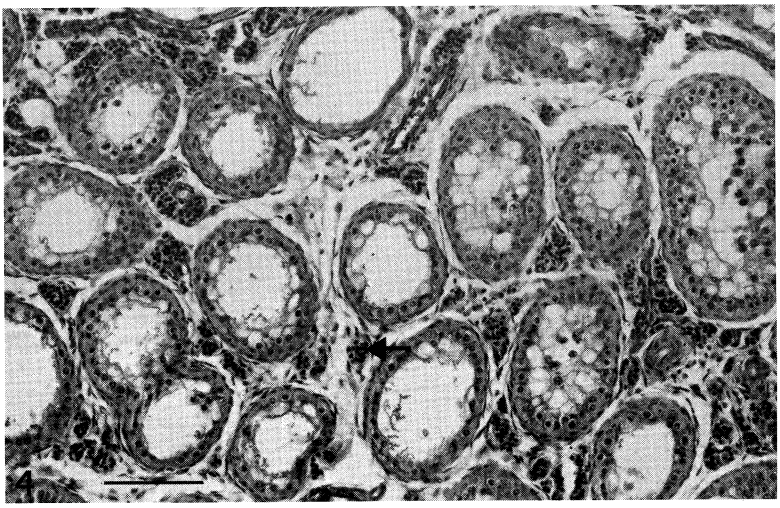
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*Spermatogenesis*

At the time of minimal testis size (July–October) fruit bat testes showed regressive histological changes (Figs 2–4). The volumetric proportions of seminiferous tissue, Leydig cells and other interstitial tissue did not vary greatly with season (Table 1). There was a small but significant decrease in the volumetric proportion

**Table 1. Volumetric proportions of fruit bat testicular tissue components**
Tissue was classified as either seminiferous tissue, Leydig cells or interstitial tissue other than Leydig cells (including blood vessels, lymphatic tissue and connective tissue). Results are mean ± s.d. of the volumetric proportions expressed as a percentage. $*P < 0{\cdot}05$, ANOVA

| Month | *n* | Seminiferous tissue (%) | Leydig cells (%) | Other interstitial (%) |
|---|---|---|---|---|
| Jan. | 2 | 88·6, 90·2 | 3·4, 2·5 | 8·0, 7·3, |
| Feb. | 3 | 90·4±3·8 | 4·3±2·3 | 5·4±1·9 |
| Mar. | 12 | 91·3±1·6 | 3·9±1·0 | 4·8±1·2 |
| Apr. | 3 | 89·6±0·8 | 4·8±0·3 | 5·6±0·3 |
| May | 2 | 88·7, 90·7 | 2·3, 4·5 | 9·0, 4·8 |
| July | 5 | 86·3±7·0*[A] | 4·0±2·9 | 9·7±4·3*[A] |
| Aug. | 6 | 88·7±3·2*[A] | 3·7±0·8 | 7·6±3·2*[A] |
| Sept. | 3 | 91·2±1·6 | 2·9±1·3 | 5·9±0·3 |
| Oct. | 3 | 90·1±5·3 | 2·9±0·9*[B] | 7·1±4·7 |

[A] Compared with value for March.
[B] Compared with value for April.

**Table 2. Seasonal changes in seminiferous tubule diameter, Leydig cell nuclear diameter and volumetric proportion of Leydig cell nucleus to cytoplasm in the fruit bat testis**
Values are mean ± s.e.m. $**P < 0{\cdot}01$, ANOVA

| Season | Seminiferous tubule diam. (μm) | Leydig cell nuclear diam. (μm) | Vol. propn of Leydig cell nucleus to cytoplasm (as % nucleus) |
|---|---|---|---|
| Breeding | 228·3±2·3** | 5·7±0·1** | 25·6±1·5** |
| Non-breeding | 163·7±2·5 | 4·5±0·1 | 34·8±1·7 |

of seminiferous tissue after the breeding season (highest in March and significantly lower during August and July). The fall was accompanied by an increase in the proportion of interstitial tissue other than Leydig cells (lowest in March and significantly higher in August and July) and a small significant decrease in the proportion of Leydig cells (highest in April and significantly lower during October). Seminiferous tubule diameter decreased significantly (Table 2), and tubules appeared less active spermatogenically. After the breeding season Leydig cell nuclear diameter

**Figs 2–4.** Cross-sections of *P. poliocephalus* testes showing the typical appearance during the breeding season (*2*, March) and examples of the range of regression found after the breeding season from a fairly active testis (*3*, September) to the most severe regression observed (*4*, July). Note the change in appearance of the Leydig cells (arrows) after the breeding season. Haematoxylin and eosin. Scale bars = 100 μm.

decreased significantly along with a significant increase in the volumetric proportion of nucleus when compared with cytoplasm (Table 2).

Spermatogenic regression varied in degree between individual bats although all testes examined possessed some long spermatids in stage 8 tubules (spermiation). Germ cells (chiefly pachytene primary spermatocytes and round spermatids) were often seen being shed from tubules and also were observed in the initial segment of the epididymis. Estimation of germ cell loss allowed quantification of seasonal changes in spermatogenesis. Germ cell loss was observed in both the breeding (44%) and non-breeding (69%) seasons (Table 3). Most germ cell loss occurred during the final spermatogonial division to form the primary spermatocytes and during meiotic division to form the round spermatids. Regressed testes showed greater cell loss at meiosis than testes during the breeding season.

**Table 3. Mean germ cell numbers per seminiferous tubule cross-section at the eight stages of the seminiferous epithelial cycle in the fruit bat during the breeding and non-breeding season**

Cell counts are expressed as mean ± s.e.m. ($n$ = 5). Predicted cell numbers were calculated from the mean number of intermediate spermatogonia in stage 6

| Cell type[A] | Stage[A] | Breeding | | Non-breeding | |
|---|---|---|---|---|---|
| | | Cell count | Predicted | Cell count | Predicted |
| A spermatogonia | 1 | 2·6±0·5 | – | 2·4±0·7 | – |
| | 2 | 3·9±0·5 | – | 3·2±0·4 | – |
| | 3 | 5·8±0·6 | – | 4·9±1·4 | – |
| | 4 | 5·1±0·4 | – | 4·4±0·8 | – |
| | 5 | 6·1±0·3 | – | 4·5±0·6 | – |
| I spermatogonia | 6 | 8·3±0·6 | – | 5·4±0·4 | – |
| B spermatogonia | 8 | 15·7±0·7 | 16·5 | 10·7±1·0 | 10·7 |
| Preleptotene spermatocyte | 1 | 20·5±1·0 | | 13·7±0·7 | |
| Leptotene spermatocyte | 2 | 21·6±1·1 | | 12·6±1·1 | |
| Zygotene spermatocyte | 3 | 23·9±1·0 | | 13·1±1·4 | |
| | 4 | 22·5±1·5 | | 14·4±1·2 | |
| | 5 | 23·2±2·8 | | 14·0±0·6 | |
| Pachytene spermatocyte | 6 | 22·9±1·3 | 33·0 | 15·5±1·1 | 21·4 |
| | 7 | 24·0±2·0 | | | |
| | 8 | 23·5±1·8 | | 14·9±0·8 | |
| | 1 | 25·6±1·6 | | 13·8±0·7 | |
| | 2 | 25·2±1·1 | | 10·9±1·6 | |
| | 3 | 27·8±1·5 | | 14·2±0·7 | |
| Round spermatid | 5 | 91·4±7·7 | | 36·3±3·7 | |
| | 6 | 88·8±5·7 | | 33·3±5·1 | |
| | 7 | 81·0±4·0 | | | |
| | 8 | 87·5±4·7 | | 31·3±5·5 | |
| | 1 | 80·9±3·8 | 132·0 | 35·6±4·9 | 85·8 |
| Elongating spermatid | 2 | 78·1±3·3 | | 35·4±4·2 | |
| Elongated spermatid | 3 | 77·4±4·0 | | 29·3±4·9 | |
| | 4 | 80·3±6·3 | | 30·8±4·6 | |
| | 5 | 80·1±5·5 | | 31·6±3·0 | |
| | 6 | 73·9±4·2 | | 26·8±3·8 | |

[A] See McGuckin and Blackshaw (1987, fig. 1).

### *Plasma Testosterone Concentration*

Peripheral plasma testosterone concentrations showed marked seasonal variations (Fig. 1). Levels reached during March were very high compared to most eutherians

(169·3 ± 29·1 nmol/l. mean ± s.e.m., $n = 9$). During the February to April period concentrations (121·5 ± 23·4 nmol/l, $n = 15$) were significantly greater ($P < 0·01$, $t$-test), than after the breeding season, June to December (7·6 ± 1·7 nmol/l, $n = 11$). Immunoreactivity in the DHT Celite chromatography fractions of March plasma samples was below the detectability of the assay.

## Discussion

The timing of maximal testicular size found in this study during February–April is slightly inconsistent with the observations of Nelson (1963, 1965) who reported peak testis weight to occur during February. Testicular regression in this species is not as marked as in some seasonal breeders which show complete cessation of spermatogenesis such as the grey squirrel, *Sciurus carolinensis* (Tait and Johnson 1982); masked civet cat, *Paguma larvata larvata* (Tsui *et al.* 1974); stoat, *Mustela erminea* (Gulamhusein and Tam 1974); fallow deer, *Dama dama* (Chaplin and White 1971); badger, *Taxidea taxidus* (Wright 1969); Californian leaf-nosed bat, *Macrotus californicus* (Bradshaw 1962); and the pipistrelle bat, *Pipistrellus pipistrellus* (Racey and Tam 1974); but similar to regression shown by the vole (Grocock and Clarke 1975); red deer *Cervus elaphus* (Hochereau-de Reviers and Lincoln 1978); and ram (Ortavant 1956) which exhibit reduced sperm production outside the breeding season. Seasonal testicular regression in the fruit bat encompasses both a decrease in the amount of seminiferous tissue and a decrease in the productivity of the tissue as evidenced by the decrease in testis weight and increase in germ cell loss, respectively. The small seasonal variation in the volumetric proportions of seminiferous and interstitial tissue show that the significant fall in testis size occurred as a result of roughly equivalent decreases of these two components. Germ cell loss (44%) occurs in active testes of this bat as compared to 24% in the rabbit (Swiestra and Foote 1963) and 40% in mature vole testes (Grocock and Clarke 1975). This value should represent an underestimate as expected germ cell numbers were predicted from the number of intermediate spermatogonia in stage 6, and observations in other species, including the rabbit (Swiestra and Foote 1963) and rat (Russell and Clermont 1977), indicate that degeneration occurs during spermatogonial divisions prior to this stage. Degeneration appeared to occur chiefly during the final spermatogonial division to form the primary spermatocytes (37·5%) and meiotic division to form the round spermatids (17·8%). Regressed testes showed an increased germ cell loss, 69% compared to 81% in regressed vole testes (Grocock and Clarke 1975); degeneration being accentuated only during meiosis (36·1%) whilst remaining similar to that found in the breeding season during the final spermatogonial division (36·0%). Increased germ cell loss indicates a decreased sperm production per volume of seminiferous tissue in addition to the significant decrease in the amount of seminiferous tissue.

The marked seasonal changes in peripheral plasma testosterone concentration are consistent with observed morphological changes in the Leydig cells. The decrease in Leydig cell nuclear diameter and increase in proportion of nucleus compared to cytoplasm after the breeding season indicates regression of both the nucleus and cytoplasm. Changes in Leydig cell characteristics have been reported in some seasonal breeders including the red deer, *Cervus elaphus* (Hochereau-de Reviers and Lincoln 1978); masked civet cat, *Paguma larvata larvata* (Tsui *et al.* 1974); southern elephant seal, *Mirounga leonina* (Griffiths 1980); and the bats, *Pipistrellus*

*pipistrellus* (Krutzsch 1975); *Myotis velifer* (Krutzsch 1961); *M. lucifugus lucifugus* (Gustafson and Shemesh 1976); and *M. adversus* (Loh and Gemmell 1980) but do not occur in other species such as the stoat (Gulamhusein and Tam 1974) and the European mole, *Talpa europa* (Suzuki and Racey 1978). The regressive changes observed in the Leydig cells of the fruit bat suggest a decreased capacity to synthesize steroids although these changes need to be confirmed by ultrastructural examination. High plasma concentrations of testosterone, comparable to those observed during autumn in the fruit bat, although uncommon, have been reported in other mammalian species including the noctule bat, *Nyctalus noctula* (Racey 1974); pipistrelle bat, *Pipistrellus pipistrellus* (Racey and Tam 1974); a hibernating bat, *Myotis lucifugus lucifugus* (Gustafson and Shemesh 1976); pallid bat, *Antrozous pallidus* (Beasley *et al.* 1984); armadillo, *Dasypus novemcinctus* (Czekala *et al.* 1980); lesser mouse lemur, *Microcelous murinus* (Perret 1985); and several species of New World monkeys including *Saimiri sciureus* (Wilson *et al.* 1978); *Ateles geoffroyi* and *Cebus apella* (Snipes *et al.* 1969). High circulating testosterone levels may be a characteristic of the Chiroptera as five out of six species (a very small sample of this large order) in which testosterone concentration has been measured have exhibited high levels during at least one phase of their seasonal cycles. Loh and Gemmell (1980) report comparatively low levels (3–10 nmol/l) of testosterone during the breeding season in the Australian microchiropteran bat *Myotis adversus*, although this study did not include samples taken throughout the entire reproductive cycle. It appears the levels reached during March in the fruit bat exceed those required for normal spermatogenesis, as those observed earlier are lower at a time when testes are close to maximal size and spermatogenic activity. High testosterone secretion may be required for accessory gland development, which peaks in April (Blackshaw and McGuckin 1984), and/or for stimulation of mating behaviour and perhaps pheromone production. These large increases in peripheral levels may be due to increased testicular secretion, a change in metabolic clearance, adrenal contribution, or a combination of these factors. Adrenal gland size appears to increase during the breeding season in this species (McGuckin and Blackshaw, unpublished observations) indicating a possible role of the adrenal in steroid production at this time. The low levels of testosterone production after the breeding season appear sufficient to support spermatogenesis but to a reduced degree resulting in germ cell loss particularly at meiosis. The testosterone concentrations obtained during this study result from single (usually mid-morning) samples and provide no information on the probable episodic nature of, and possible diurnal variations in, testosterone concentrations.

In conclusion, the grey-headed fruit bat shows a peak in spermatogenic activity and testosterone production during autumn at the time of mating; although some sperm production occurs throughout the entire year. Initial evidence suggests that photoperiod influences reproduction in this species (McGuckin and Blackshaw 1985), therefore a comparison of the reproductive endocrinology of this species and another member of the same genus *P. scapulatus*, which is a long-day breeder (Nelson 1965), could provide an interesting contrast of long- and short-day breeders.

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