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Spermatogenesis and plasma testosterone levels in Western Australian burrowing desert frogs, *Cyclorana platycephala*, *Cyclorana maini*, and *Neobatrachus sutor*, during aestivation

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Abstract

Changes in testis size, histological status, and plasma levels of testosterone were monitored for males of three species of Western Australian desert frogs, *Cyclorana maini*, *Cyclorana platycephala*, and *Neobatrachus sutor* during aestivation. The frogs were induced to burrow and form cocoons soon after their capture and then disinterred at intervals in order to monitor changes in reproductive activity of the testes. All stages of spermatogenesis were evident in active frogs, which were collected a few days following rain from breeding choruses. Relative testis mass declined gradually in all species during the first 7 months of aestivation and then increased significantly at 16–19 months in the two species for which extended data were available (*C. maini* and *N. sutor*). A decrease in the number of sperm bundles 2–4 months after cocooning was associated with an initial increase in the number of free spermatozoa in all three species, which then returned to the levels seen in active animals after 7 months. Increases in the number of primary and secondary spermatogonia were most evident in *C. platycephala* after 4–7 months of aestivation, but early stages of spermatocytogenesis were evident in all species after 7 months of aestivation, especially in individuals that contained neither sperm bundles nor mature spermatozoa. Changes in plasma testosterone levels correlated significantly with variations in the diameter of the seminiferous tubules and the GSI, suggesting that this hormone plays a major role in controlling testicular recrudescence in aestivating, cocooned, desert frogs. Data from this study show that, in the absence of any external cues, testicular recrudescence is evident after approximately one year of aestivation in desert frogs which prepares them to breed again, once rain falls.

Keywords: Amphibia; Frog; Desert; Burrowing; Testis; Testosterone; Reproduction

1. Introduction

Climatic condition and habitat are known to be important factors controlling the breeding of the Amphibia. Anuran frogs in temperate regions usually breed in spring (Main, 1968) but, in tropical and sub-tropical areas, breeding may be prolonged over several months and in some cases occur throughout the year (Lofts, 1987; Salthe and Mecham, 1974). Amphibians that occupy more arid areas tend to be opportunistic breeders and spawn in response to rainfall (Bragg, 1945; Lofts, 1984). In many amphibian species, reproductive cycles vary according to climatic conditions and time of the year and are associated with changes in plasma testosterone levels, testis structure and spermatogenesis (Lofts, 1984, 1987; Rastogi, 1976; Rastogi and Iela, 1992; Whittier and Crews, 1987). Seasonal variations in plasma testosterone levels and their role in male reproduction has been investigated in many anuran species (Delgado et al., 1989; Guarino et al., 1993; Itoh et al., 1990; Ko et al., 1998; Licht et al., 1983; Pierantoni et al., 1984). Two patterns of testosterone changes have been reported in different frog species: high plasma testosterone levels associated with active spermatogenesis during the middle stage of hibernation and falling

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during breeding (Delgado et al., 1989), and plasma testosterone levels changing in parallel with changes in spermatogenic activity (Rastogi et al., 1986). Thus, changes in the testosterone level are not always associated with spermatogenic activity, or breeding, in frogs.

Western Australian desert frogs, *Cyclorana platycephala*, *Cyclorana maini*, and *Neobatrachus sutor*, breed following significant summer rains induced by tropical cyclones. These animals burrow into soil during dry conditions and have been classified as continuous gametogenic frogs (van Beurden, 1979). Little research has been done on spermatogenesis during aestivation in these species, however, and gonadal sex steroids have yet to be measured in any species. In this study, spermatogenesis changes and gonadal recrudescence have been monitored, along with changes in plasma testosterone levels in these Australian desert frogs during periods of induced aestivation. Our study demonstrates that aestivating frogs held in the laboratory in the absence of any external cues, pass through a period of atrophy lasting approximately a year, which is followed by testicular recrudescence.

2. Materials and methods

2.1. Frogs

Three species of Western Australian desert burrowing frogs (*C. maini*, *C. platycephala*, and *N. sutor*) were collected from an ephemeral creek (Lat 24°26′S, Long 119°41′E) in December 1999 following heavy rain about 70 km south of Newman, Western Australia. Frogs were active on the surface and males were calling when collected. Frogs were returned to Perth within 2 days, along with soil collected at the capture site.

2.2. Experimental design

Frogs were sorted into their respective species and randomly assigned to an 'active' and an 'aestivating' group. The active group was processed within the first week of capture and before any attempt had been made by frogs to enter aestivation. *C. maini*, *C. platycephala*, and *N. sutor* were induced to enter aestivation by placing them individually in plastic containers half filled with soil collected at their site of capture and then placed in a constant room temperature maintained at 20 °C and a 10/14 h light régime with no access to water. Aestivation was considered to commence as soon as the frogs burrowed below the soil surface which was normally within 2 days. Wherever possible, sub-samples of 6 frogs were disinterred at intervals (2, 4, and 7 months for all three species and 16 and 19 months in the case of *C. maini* and *N. sutor*), killed by pithing and then processed. Due to attrition, it was not always possible to include 6 individuals in each sub-sample and the actual numbers processed are shown in each figure legend. All changes over time were compared statistically with the values recorded in active frogs.

2.3. Collection of plasma samples

Following cranial and spinal pithing of the frogs, blood samples were withdrawn by cardiac puncture. The samples were centrifuged using a Beckman Microfuge (TM11). Decanted plasma was transferred to heparinised capillary tubes and kept frozen at −20 °C until analysis.

2.4. Radioimmunoassay of plasma testosterone

Testosterone was measured by radioimmunoassay, using an antibody raised in rabbits against testosterone-3-oxime-bovine serum albumin (Endocrine Sciences, T_3-125). The antiserum exhibited 100% binding with testosterone and cross-reacted 52% with Δ1-testosterone, 20% with dihydrotestosterone (DHT), 3.6% with 4-androsten-3β, 17β-diol, 1.8% with 5α-androstan-3β, 17β-diol, 0.5% with Δ4-andostenedione, 0.3% with 5βandrostan-3α, 17β-diol, and <0.15% with corticosterone, aldosterone, dehydroepiandrosterone, oestradiol, and progesterone. The tracer used was [1, 2, 6, 7-^{3}H] testosterone (Amersham TRK 402). Testosterone was extracted from 10 µl of plasma with 2 volumes (each of 2.5 ml) of diethyl ether in the presence of 0.05 M borate buffer, pH 8. The dried extract was incubated with antibody at 37 °C for 45 min followed by 3 h at 24 °C. The bound radioactive fraction was separated by adsorption to 5 mg dextran-coated charcoal (Norit A, acid washed) at 0 °C and counted in a liquid scintillation counter (Packard Tri-Carb 2300 TR). Testosterone concentration was calculated using a 4-parameter logistic curve after plotting standard amount of testosterone (T) against % radioactivity bound (Fig. 1) the coefficient of variation for the internal standard was 9.3%.

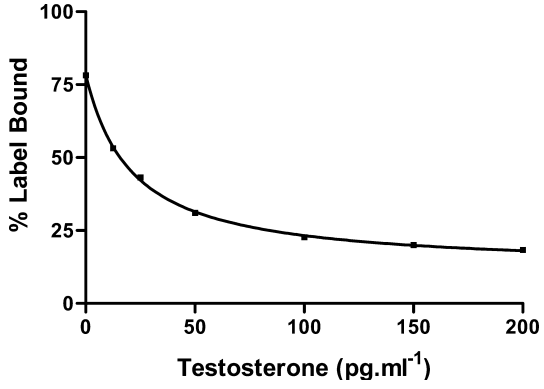

Fig. 1. Representative standard curve for the testosterone radioimmunoassay with percent label bound versus standard ligand. Curve fitting is by 4-parameter logistic equation with $r^2 = 0.96$.

2.5. Histological examination

After pithing, testes were removed, weighed using a Sartorius balance (H 51), fixed in 10% buffered formal saline solution and then embedded in wax. Six-micron thick histological sections were prepared. Sections were stained with haematoxylin and eosin for spermatogenesis determination. Seminiferous tubule diameters were measured using a calibrated software programme (Optimas and Image Pro, Media Cybernetics) for ten randomly selected tubules per section.

2.6. Determination of Gonadosomatic index (GSI)

The Gonadosomatic index (GSI) was calculated for each frog as a percentage of the weight of the two testes, relative to the body mass (g). The body weight was measured to the nearest 0.1 g and testes immediately after dissection to the nearest mg.

2.7. Evaluation of spermatogenic activity

Germ cells in seminiferous tubules of all three species of the frogs were organised into germinal cysts throughout the body of the testis (see Plate 1). The numbers of cysts containing germ cells at different times of aestivation were counted in randomly selected field at low magnification under the microscope, using a standard field of 0.1252 mm^2. The following cell nest types were identified and counted according to the descriptions of Lofts (1974), Rastogi et al. (1976), Harvey et al. (1997), and Ko et al. (1998). (1) *Primary spermatogonia* (*ISPG*), small to large single cells, usually found around the periphery of the tubule. (2) *Secondary spermatogonia* (*IISPG*), clusters of two up to twelve medium-sized cells with similar appearance to I SPG. (3) *Primary spermatocytes* (*I SPC*), groups of medium-sized cells (12–24) irregularly shaped with large, darkly stained nuclei. (4) *Secondary spermatocytes* (*II SPC*), small cells (over 24 cells). (5) *Spermatids* (*SPT*), group of very small cells, in which the groups of 10 have hollow centres and form "rings." (6) *Spermatozoa bundles* (*SB*), spermatozoa with the heads still embedded in Sertoli cells. (7) *Free sperm* (uncounted) in the lumen of the tubules.

2.8. Statistical analysis

Differences between means at different time intervals for *C. maini*, *C. platycephala*, and *N. sutor* were analysed using one-way ANOVA followed by post-hoc Tukey tests. GSI ratios were transformed to logarithms prior to analysis using parametric statistics, and a non-parametric test (Mann–Whitney) was employed when standard deviations for the samples differed significantly.

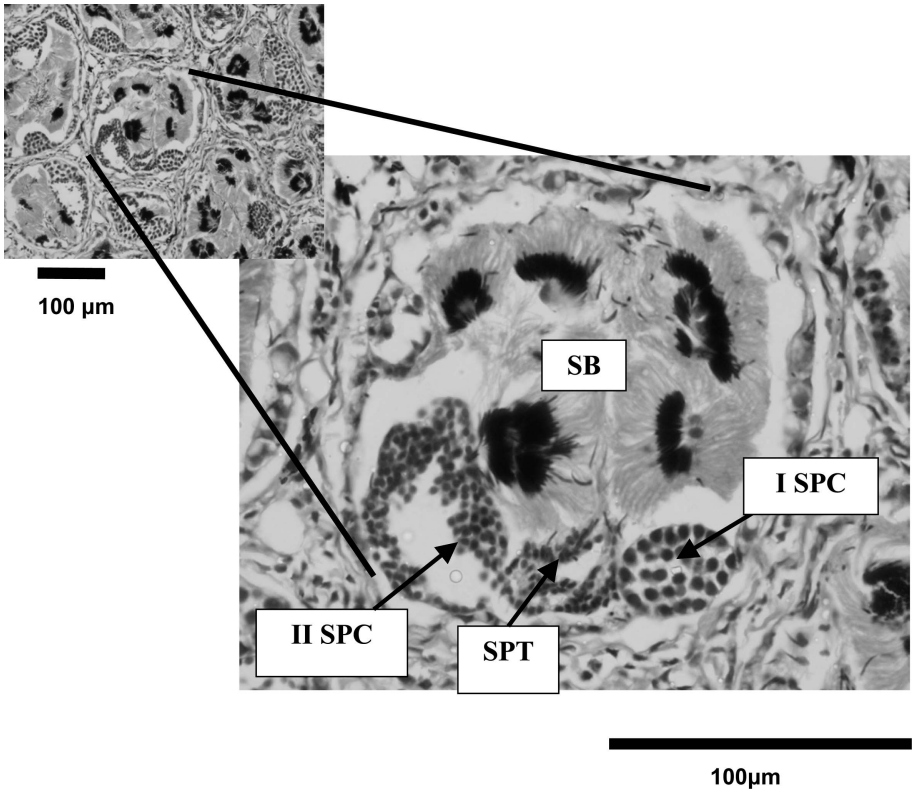

Plate 1. Transverse section of the testis of an active male *Neobatrachus sutor* from a breeding chorus showing primary spermatocytes (I SPC), secondary spermatocytes (II SPC), spermatids (SPT), and sperm bundles (SB). 100 µm scale bar shown.

3. Results

Changes in GSI and seminiferous tubule diameter of the three species of desert frogs are shown in Fig. 2. *C. platycephala* exhibited a significant decrease to $0.07 \pm 0.01\%$ after 4 months of aestivation and to 0.08 ± 0.01 after 10 months ($F_{3,10} = 8.25$, $P = 0.005$). The only significant variation in the GSI for *C. maini* was a fall to $0.05 \pm 0.01\%$ after 10 months, but the 16-month value of $0.23 \pm 0.02\%$ is suggestive of recrudescence. This trend is more apparent for *N. sutor*, where GSI fell significantly after 10 months and then increased at 16 months, and the mean is not significantly different from the GSI value of active animals after 19 months ($0.27 \pm 0.04\%$ versus $0.41 \pm 0.12\%$).

Seminiferous tubule diameter (Fig. 3) was unchanged during 4 months of aestivation for *C. platycephala* and *C. maini*. However, it showed a significant decrease to 48.6 ± 2.5 μm ($P < 0.05$) and 32.9 ± 1.9μm ($P < 0.01$) at the 7th month of aestivation in both species, respectively. For *N. sutor*, the diameter of the seminiferous tubules fell sharply from a mean of 146 ± 4.3 to 94 ± 4 μm after 4 months and 93 ± 6 μm after 7 months of aestivation. The 19-month mean diameter of 157 ± 11 μm for this species does not differ significantly from that seen in active specimens and shows a clear recrudescence.

Changes in plasma testosterone levels in the three frog species over the first 7 months of aestivation are shown in Fig. 4. Testosterone levels for *C. platycephala* fell significantly from a mean of 1047 to 248.8 pg ml^{-1} ($P < 0.01$) in the first 2 months of aestivation and remained at this low level for the next 5 months. Plasma testosterone levels fell in both *C. maini* and *N. sutor* after 2 months, and remained low up until 7 months of aestivation, but the differences were not statistically significant due to high individual variability. There was a significant correlation, however, between individual plasma testosterone levels and both GSI and seminiferous tubule diameter in all frog species, the latter being shown in Fig. 5.

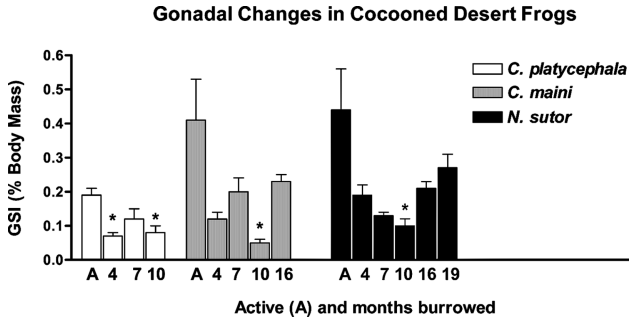

Fig. 2. Changes in the Gonadosomatic Index (GSI) of *C. platycephala*, *C. maini*, and *N. sutor* during 4–19 months underground aestivation. The GSI is expressed as the total mass of both testes (in mg) as a percentage of the body mass (in g). Data presented as the Mean ± SE with (A) active frogs and $^{*}P < 0.05$. Sample sizes for active (A), 4, 7, 10, 16, and 19-month samples are as follows: *C. platycephala* = 6,6,4,4; *C. maini* = 6,6,5,5,4; *N. sutor* = 6,5,5,5,5,9.

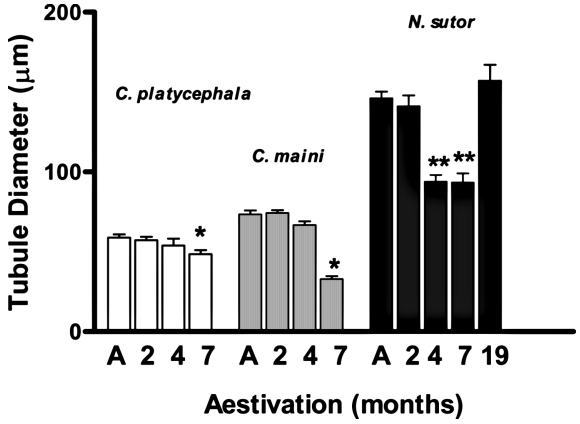

Fig. 3. Changes in testicular seminiferous tubule diameter in three Australian desert frogs during 2–19 months of aestivation. Each column represents the mean diameter in μm ± SE of the tubules. (A) Active frogs with $^{*}P < 0.05$, $^{**}P < 0.01$. Sample sizes for active (A), 2, 4, 7, and 19-month samples are as follows: *C. platycephala* = 6,6,4,4; *C. maini* = 6,6,5,5; *N. sutor* = 6,5,5,5,9.

Changes in numbers of spermatogonia (I SPG and II SPG), spermatocytes (I SPC and II SPC), spermatids (SMT), and sperm bundles (SB) were used to evaluate spermatogenic activity during aestivation (see Figs. 6–8). The presence of free sperm (FS) was observed, but no attempt was made to count numbers; numbers of sperm bundles were counted instead and the percentage of these containing sperm noted. Primary spermatogonia numbers (I SPG) fell in all three species after 2 months and decreased further after 4 months in *C. maini*, but then increased to reach or even exceed (in the case of *C. platycephala*, $P < 0.001$) the numbers found in active frogs (see Fig. 6). Secondary spermatogonia (II SPG) increased in numbers from the start of aestivation in *C. platycephala*, but first fell in the other two species before increasing to reach near active levels after 7 months.

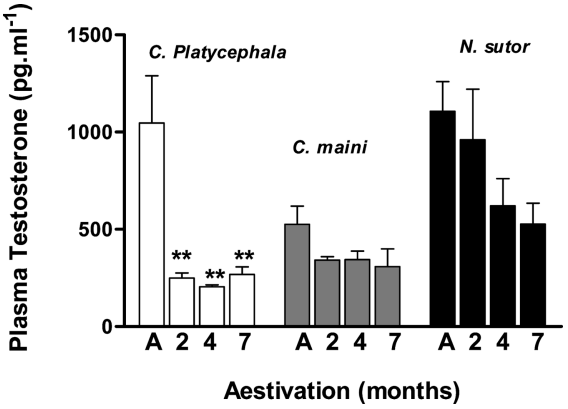

Fig. 4. Change in plasma testosterone concentrations in three species of Australian desert frogs during 7 months of aestivation. Data presented as the Mean ± SE in pg ml^{-1} with $^{**}P < 0.01$. Sample sizes for active (A), 2, 4, and 7-month samples are as follows: *C. platycephala* = 6,6,4,4; *C. maini* = 6,6,5,5,4; *N. sutor* = 6,5,5,5.

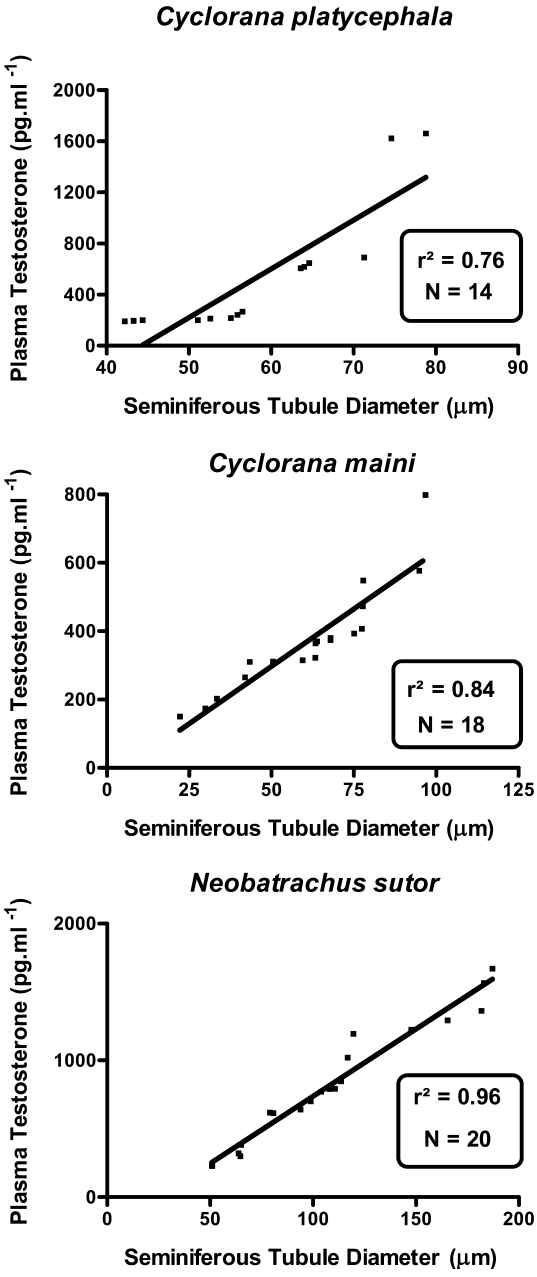

Fig. 5. Correlations between plasma testosterone levels (in $pg\,ml^{-1}$) and the seminiferous tubule diameter (in μm) of the testes of *C. platycephala*, *C. maini*, and *N. sutor*. Sample numbers for each regression are noted on each graph.

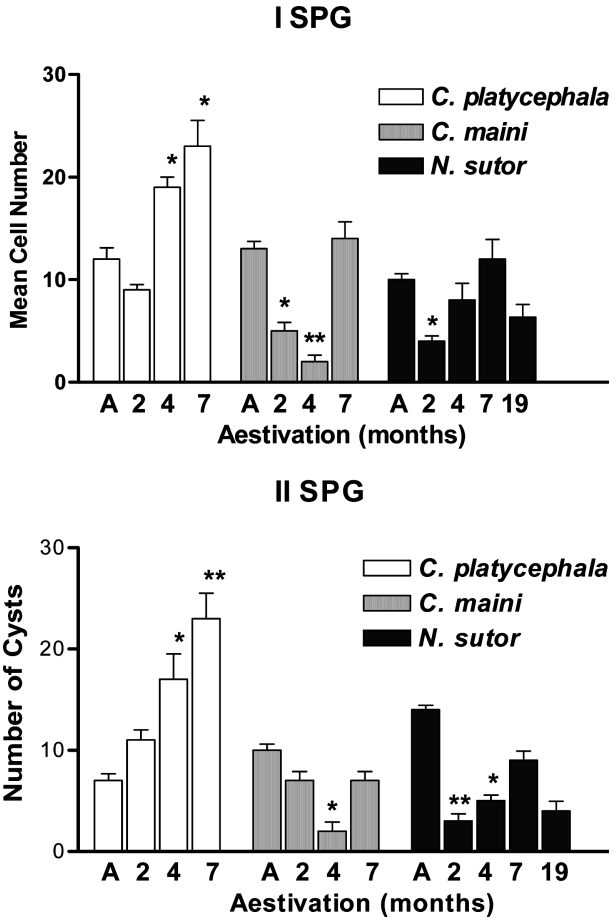

Fig. 6. Changes in mean cell densities of primary (I SPG) and secondary spermatogonia (II SPG) during 2–19 months of aestivation in three species of Australian desert frogs. Data presented as Mean ± SE of the number of cells per $1.252\,mm^2$ of the testis section with $^{*}P < 0.05$ and $^{**}P < 0.01$. Sample sizes for active (A), 2, 4, 7, and 19-month samples are as follows: *C. platycephala* = 5,5,4,4; *C. maini* = 6,4,4,4; *N. sutor* = 6,5,5,5,6.

Numbers of spermatocytes (I SPC and II SPC) showed a significant decrease ($P < 0.05$) in all three species after 2 months of aestivation compared with active frogs and remained low at 4 months, increasing slightly in *C. maini* after 7 months and increasing significantly in *N. sutor* after 7 and 19 months to reach almost 50% of the numbers seen in active individuals. Numbers of spermatids fluctuated during the 7-month aestivation period but did not show any statistically significant changes. The overall number of sperm bundles (SB) fell progressively from the second month of aestivation in all three species ($P < 0.05$) but contrasted with a marked increase in the percentage of those remaining sperm bundles containing free sperm (see Fig. 8). The 19-month data from *N. sutor* are suggestive of effective recrudescence with an enormous increase in the number of sperm bundles containing free sperm to reach 69 ± 11%, compared with only 12.5 ± 2.3% in active individuals (see Fig. 8).

The sampling protocol used in this study (with 5–6 frogs being sacrificed at each time interval) meant that, depending on the initial number of frogs collected, sufficient specimens of each species were not always available for all time periods. Data for *C. platycephala* thus do not exceed 7 months, and 16 months in the case of *C. maini*, but it was possible to collect data after a period of 19 months in the case of *N. sutor*. Plates 1–3 record the histological changes observed in this species over the 19-month aestivation period. These sections, which are all to the same scale, show the significant decline in tubule diameter after 7 months underground, followed by the

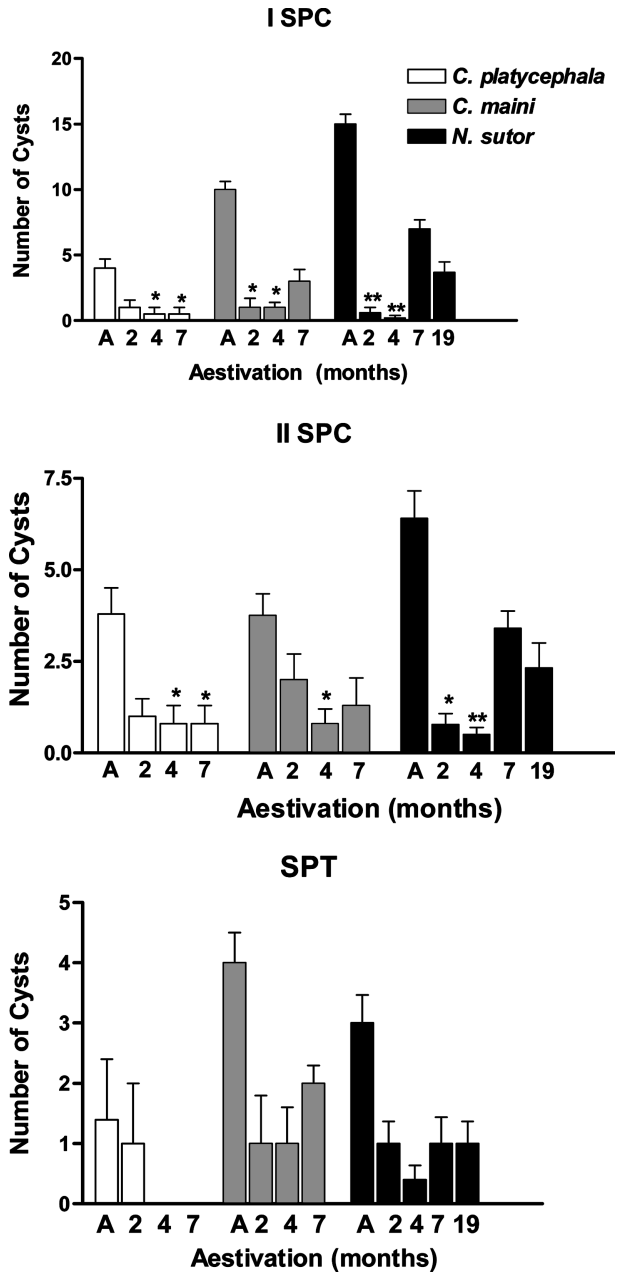

Fig. 7. Changes in mean cell densities of primary (I SPC) and secondary spermatocytes (II SPC) and spermatids (SPT) during 2–19 months of aestivation in three species of Australian desert frogs. Data presented as Means ± SE of the number of cells per 1.252 mm^2 of the testis section with $^*P < 0.05$ and $^{**}P < 0.01$. Sample sizes for active (A), 2, 4, 7, and 19-month samples are as follows: *C. platycephala* = 5,5,4,4; *C. maini* = 6,4,4,4; *N. sutor* = 6,5,5,5,6.

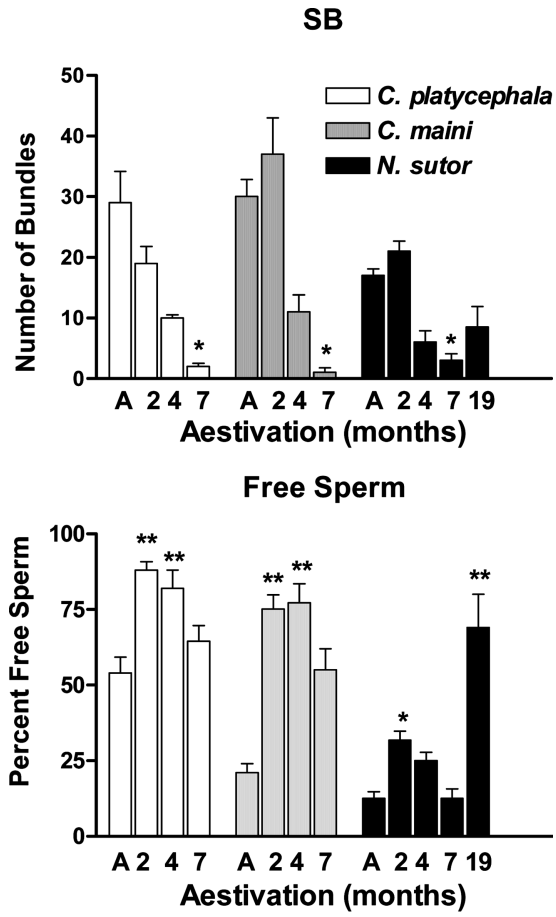

Fig. 8. Changes in numbers of sperm bundles (SB) and the mean percentage of free sperm in bundles (FS) during 2–19 months of aestivation in three species of Australian desert frogs. Data presented as Mean ± SE with $^*P < 0.05$ and $^{**}P < 0.01$. Sample sizes for active (A), 2, 4, 7, and 19-month samples are as follows: *C. platycephala* = 5,5,4,4; *C. maini* = 6,4,4,4; *N. sutor* = 6,5,5,5,6.

massive increase evident after 19 months that is associated with large numbers of free sperm.

4. Discussion

Many Australian desert frogs, in common with desert anurans in a number of other continents, have the ability to burrow into soil during dry periods and survive for extended periods of time until rain falls again. Anecdotal records suggest that they may aestivate for periods of up to 4–5 years, as frogs have been recorded to emerge after rain in central Australia after such extended periods of drought (Heatwole, 1984; van Beurden, 1982) and Predavec and Dickman (1993) even record some individuals of *Neobatrachus centralis* emerging 8 h prior to the rain falling in central Australia. Undoubtedly, the fact that these desert frogs are able to form a cocoon to protect them from desiccation (Withers, 1995, 1998) and dramatically reduce their rate of metabolism (Withers, 1993; Withers and Thompson, 2000), is the reason for their long-term survival under such difficult conditions. The problem of how and when they prepare themselves for their next breeding period has, however, remained unknown, although a recent paper by Storey et al. (1999) has found evidence for the up regulation of riboflavin-binding protein in the liver of aestivating spade foot toads.

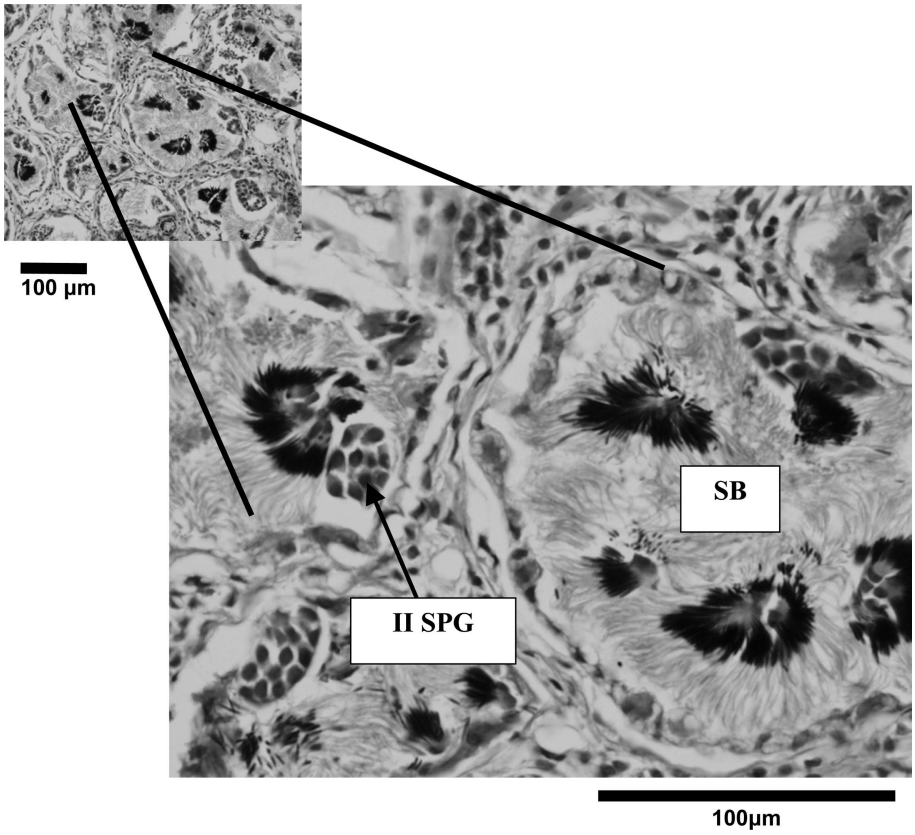

Plate 2. Transverse section of the testis of a cocooned male *Neobatrachus sutor*, after a period of 7 months aestivation in an underground burrow. II SPG, secondary spermatogonia and SB, sperm bundles. 100 µm scale bar shown.

Crews and Moore (1987) and Krohmer et al. (1987) predicted that animals living in harsh climates with predictable, but truncated breeding periods, would display what they termed "dissociated" reproductive tactics—with reproductive steroids being elevated at some time other than during the breeding season. This contrasts with the more common pattern of "associated" reproductive tactics where mating behaviour coincides with maximal circulating levels of sex steroids such as testosterone and oestradiol-17β (E2) (Crews, 1984). Harvey et al. (1997) tested this prediction in the explosively breeding desert spade foot toad, *Scaphiopus couchii*, and found that this was not the case. Levels of testosterone and dihydrotestosterone (DHT) were maximal in amplexing males, as was also the case with testosterone, DHT, progesterone, and E2 in females. All hormones then fell to very low levels in post-breeding frogs and evidence of testicular recrudescence was suggested by an increase in numbers of primary and secondary spermatogonia (I SPG and II SPG) and spermatocytes (SPC) in the testes of these males. This study was carried out over a 2-month period in Arizona during which time the frogs had emerged following monsoonal summer rain and remained active, but had not yet re-burrowed to resume their summer aestivation. In an interesting follow-up paper, Harvey and Propper (1997) found that, although androgens are associated temporally with the onset of reproductive behaviour in this toad, they are not necessary for its expression, with castrated males clasping females normally. A similar conclusion was reached by Saint Girons et al. (1993) in studying the hormonal control of reproductive behaviour in a reptile, the Aspic viper (*Vipera aspis*), where testosterone was found to be a necessary but not a sufficient condition for the display of mating behaviour in males.

Spermatogenic cycles in temperate-breeding frogs have been described in some detail in an extensive series of papers and reviews by Lofts (1964, 1972, 1974, 1984, 1987), van Oordt and van Oordt (1955), van Oordt (1960), Burgos and Vitale-Calpe (1967), Rastogi (1976), Pierantoni et al. (1984), and Pudney (1995). Frogs living in cold climates in the northern hemisphere, such as *Rana temporaria*, typically have a discontinuous spermatogenic cycle with an absence of developmental activity during the winter period. An upsurge in spermatogenic activity takes place some weeks after the spring breeding period so that, by summer, large numbers of germinal cysts are found in different stages of development. Development continues throughout autumn to produce spermatozoa so that, before the onset of the next winter, the testes already contain a

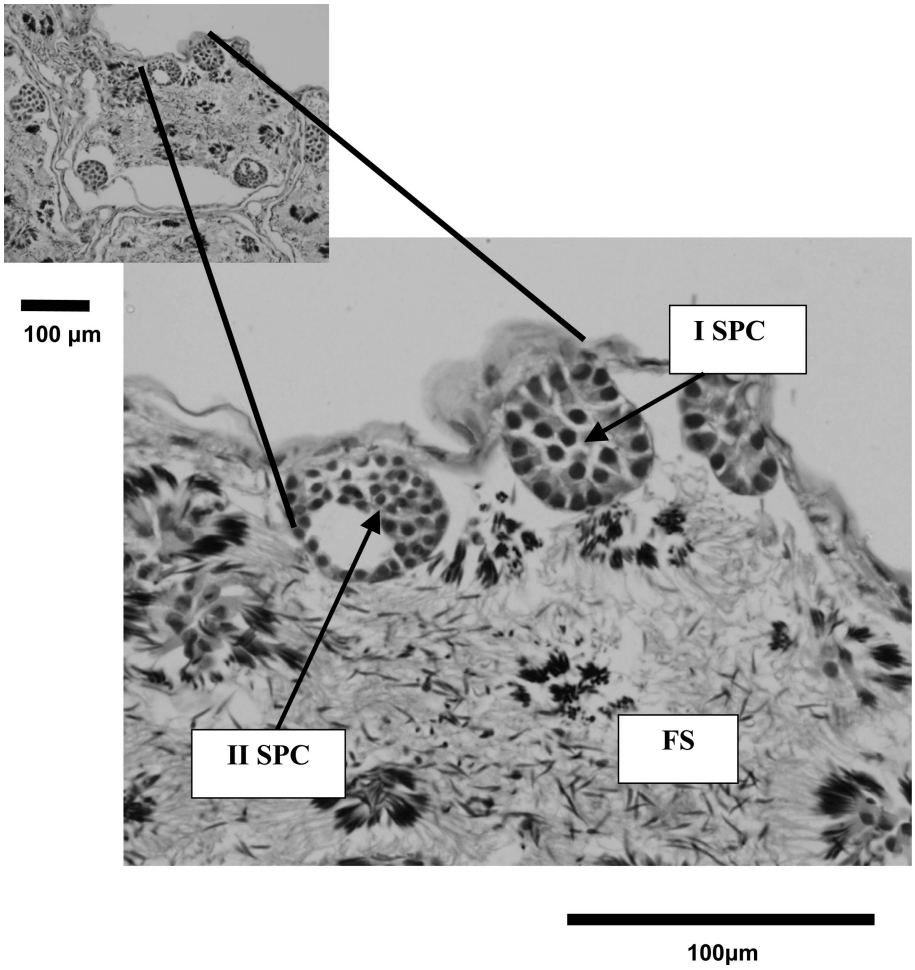

Plate 3. Transverse section of the testis of a cocooned male *Neobatrachus sutor*, after a period of 19 months aestivation in an underground burrow. I SPC, primary spermatocytes, II SPC, secondary spermatocytes, and FS, free sperm. 100 µm scale bar shown.

generation of spermatozoa destined to be used during the succeeding spring breeding season.

The interest of the present study in three species of desert burrowing frogs was to determine whether a similar preparation for future reproduction occurs in the short period of activity before the frogs return underground, or whether testicular recrudescence occurs during the period of aestivation. In this latter case, recrudescence would be expected to be very slow, giving the extreme depression of the metabolic rate that is characteristic of aestivating desert frogs (Flanigan et al., 1991; Guppy and Withers, 1999). The study of Harvey et al. (1997) with *S. couchii* shows that, in this burrowing species, testicular recrudescence commences whilst the animals are post-reproductive but still active above ground—similar to what occurs in temperate-living species. This desert toad differs, however, from the Australian species used in the present study in that *S. couchii* does not form a cocoon when underground and is not known to aestivate for periods of longer than a year, typically emerging to feed and breed with regular midsummer monsoonal rains (Bragg, 1945; Seymour, 1973).

The three species of desert burrowing frogs in this study differ from other species with discontinuous spermatogenic cycles in that the gonadosomatic index (GSI) declined significantly in the first 4 months of aestivation and remained low-up until 10 months. Associated with this reduction in testis mass was a decrease in tubule diameter and a fall in circulating levels of testosterone. Cytological changes were not identical in the three species, *C. platycephala* for example, showing a proliferation of primary and secondary spermatogonia (I SPG and II SPG) after 4 months underground, a change not observed in the other two species until 7 months had passed.

Both primary and secondary spermatocytes fell in *C. platycephala* and *C. maini* from active levels and were still low after 7 months, representing the last samples available for these two species, but both I SPC and II SPC increased noticeably in *N. sutor* after 7 and 19 months underground. There was a monotonic decrease in the number of sperm bundles throughout the period of aestivation in all species, which was mirrored by significant increases in the percentage of SB containing

free sperm that was evident within 2 months of the frogs cocooning. The pattern seen in *N. sutor* appeared to differ, with numbers of free sperm rising after 2 months, then falling after 7 months, to reach a very high peak after 19 months.

These data are open to interpretation. On the one hand, the increase in the number of sperm bundles containing free sperm could be taken as evidence of increased spermiogenesis following the breeding period. On the other hand, it may simply reflect the fact that those sperm bundles not containing free sperm are declining in numbers, and leaving intact those that do contain free sperm which thus increase as a percentage. The presence of free sperm after 2–4 months of aestivation implies their continued nourishment by Sertoli cells (Brökelmann, 1964; Burgos and Vitale-Calpe, 1967; Lofts, 1974) as has been observed in *Rana esculenta* (a potentially continuous spermatogenic frog) by a number of investigators (Lofts, 1964; Rastogi et al., 1976; Sluiter et al., 1950). This raises the interesting possibility that, if rain fell again in the 6-month period after the first breeding episode, the frogs may be able to re-emerge and breed effectively for a second time although, to date, there do not appear to be any instances of this having been recorded (van Beurden, 1979).

These data bring into focus the role of testosterone as a hormonal modulator of spermatocytogenic activity in these desert frogs. Recorded plasma testosterone levels vary markedly between different species of frogs, from as low as $1.2\,ng\,ml^{-1}$ in *Bufo aspis* (Emerson and Hess, 1996) to as high as $130\,ng\,ml^{-1}$ in amplexing *S. couchii* (Harvey et al., 1997). Testosterone levels in the three species in this study fall between 0.5 and $1.0\,ng\,ml^{-1}$ in active breeding males and declined further during the period of aestivation. Unfortunately, plasma samples were not available from those few *N. sutor* that were disinterred after a period of 19 months, but the significant correlation that was found between both tubule diameter and GSI in all three species, suggests strongly that testosterone levels would also have been raised and that this hormone has an overall trophic effect on the male gonad.

It is presumed that levels of plasma testosterone reflect testosterone production by the interstitial (Leydig) cells in the testis in amphibians as observed in *Rana rugulosa* by Kao et al. (1993) and in *R. esculenta* by Pierantoni et al. (1984). Testosterone is considered to play a pivotal role in controlling spermatogenic activity in many amphibians (Guarino et al., 1993; Itoh et al., 1990; Licht et al., 1983; Pierantoni et al., 1984; Rastogi et al., 1976) but its precise mode of action differs between species. In anurans, testosterone has been reported to increase testis size and spermatogenesis in some species (e.g., *Bufo fowleri* and *B. arenarum*) but also to suppress spermatogenesis at the secondary spermatagonia stage in several species of *Rana* and *Bufo melanostictus* (Rastogi and Iela, 1980). It has also been reported that testosterone levels either decline during the late stages of spermatogenesis (during spermatid formation) after increasing during the early proliferative stages (Delgado et al., 1989; Guarino et al., 1993), or they parallel spermatogenic activity (Rastogi et al., 1986). Testosterone is synthesised at a high level by the interstitial tubule cells during periods of high spermatogenic activity when Sertoli cells are largely lipid-free, but levels have been reported to fall during the post-nuptial phase when Sertoli cells are heavily lipoidal and cholesterol-rich (Lofts, 1972). There is evidence that gonadotrophins stimulate the multiplication of spermatogonia in amphibian testes and Minucci et al. (1992) found that the gonadotrophin agonist, GnRHA, significantly increased the mitotic index of hypophysectomised *R. esculenta*. Paracrine and/or autocrine mechanisms are thought to be involved in the regulation of testosterone secretion throughout the reproductive cycle with oestradiol possibly acting to feedback on GnRH secretion after the mating period, leading to falling testosterone levels in summer in frogs such as *R. esculenta* (Minucci et al., 1989).

Although plasma levels of oestradiol-17β (E2) were not monitored in this study of male frogs, E2 is known to have an impact on testicular development, through intra-testicular feedback mechanisms and via interactions with Gonadotrophic releasing hormone (GnRH) from the hypothalamus (Minucci et al., 1992). Fasano et al. (1989) found that levels of testosterone and DHT peaked in autumn and early spring in male *R. esculenta*, whereas E2 reached maximal levels later, in mid-spring, before falling to very low levels. They also found with in vitro incubations of testicular tissue that E2, stimulated by pituitary factors, inhibited androgen synthesis by the testis, in contrast to the effect of testosterone. Polzonetti-Magni et al. (1984) similarly found that E2 levels increased in male *R. esculenta* in early summer, following a decline in levels of testosterone, and they speculated that E2 inhibited GnRH release and ultimately LH secretion in the males at this time, leading to the seasonal refractory period of this species. This is consistent with the results of a study in the same species by Minucci et al. (1992) where GnRH agonists and antagonists were used to show that spermatid formation is dependent on GnRH and, ultimately, gonadotrophic secretion.

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