The effect of temperature and photoperiod on selected male reproductive characteristics in two seasonally breeding (*Cryptomys hottentotus hottentotus* and *Cryptomys hottentotus pretoriae*) and one aseasonally breeding mole-rat species (*Cryptomys damarensis*).

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Abstract

Reproduction was investigated in three species of social subterranean mole-rats, one breeding aseasonally and two seasonally. Body mass, reproductive tract morphometrics, testicular histology, sperm motility and plasma testosterone concentrations were studied under four simulated seasons in male mole-rats paired with females. The four seasons were simulated under controlled lighting (as occurring above ground) and temperature (as occurring in the burrow) to mimic natural seasonal variation (LD, HT summer; LD, LT autumn; SD, LT winter and SD, HT spring). Urinary melatonin concentrations were determined at four time points in the day, T9 (09h00), T15 (15h00), T21 (21h00) and T3 (03h00) respectively.

The findings from this study suggest that the Damaraland mole-rat does not utilize changes in photoperiod but may use temperature to determine the time of year to breed. In contrast, the common mole-rat uses photoperiod to prime reproduction with winter and spring variables altered by changes in temperature. While the highveld mole-rat appears to rely on both photoperiod and temperature to cue reproduction. Interestingly, all three species exhibit year round manifestation of spermatogenesis and lack testicular regression. They all show active sperm motility and produce viable spermatozoa throughout the different seasons. In all three species the types of spermatozoa abnormalities found are not tertiary and therefore do not affect their fertilizing capability. The three species also exhibit no difference in plasma testosterone secretions in all simulated seasons, which indicates that they are physiologically primed to reproduce throughout the year.

All three species appear capable of responding to changes in photoperiod, manufacturing pineal melatonin in dark conditions with a reduction in manufacture during exposure to light. However, intra-specifically (for all three species investigated) there was little difference in the daily melatonin profiles between each particular season, suggesting that all three species are capable of responding to photoperiod, but not necessarily utilizing it to cue reproduction. The subterranean niche possess similar pressures on the three species investigated and it is proposed that the invasion of each species into a different habitat has resulted in each species modulating its reproduction to maximize the lifetime reproductive success by cueing into different environmental variables. These results suggest that although these species have different biological coping systems, they have the same basic adaptations regardless of their mode of reproduction.

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Chapter 1

Introduction

Since subterranean mammals rarely, if ever, are exposed to light it is extremely unlikely that photoperiod plays a role in reproduction (Bennett and Faulkes, 2000). Rainfall, however, softens the soil enabling mole-rats to extend their burrow systems to increase access to their underground food resources. The burrow systems of subterranean rodents exhibit marked seasonal differences in temperatures in both mesic and arid habitats, but not in tropical habitats (Kennerly, 1964, Bennett, Jarvis and Davies, 1988). The presence or absence of seasonal temperature differences may be important in determining the onset of reproduction in seasonally reproducing subterranean mammals (Bennett, Faulkes and Molteno, 2000). However temperature and photoperiod may act synergistically to fine tune the cues that time reproduction and thereby anticipate the time most appropriate for procreation for now and future environmental conditions (Tamarkin *et al.*, 1985; Yu Hing-Sing and Reiter, 1993; Kriegsfield *et al.*, 2000).

The ideal method of evaluating the fertility of a male other than his ability to produce pregnancy is to examine the semen. Spermatogenesis, the process by which spermatogonial stem cells undergo several processes of multiplication, division and proliferation resulting in the formation of spermatozoa, together with the annual progression of the change in gonads provide a potentially valuable source of information that can be used to determine and thereby predict which cues animals use to ready themselves for optimal reproduction (Clermont, 1962; Johnson *et al.*, 1997). Seasonal fluctuations in reproductive activity and associated changes in the levels of sex steroid hormones are amongst the most commonly observed photoperiodic responses in mammals (Reiter, 1991; Wehr, 1991; Yu Hing-Sing, *et al.*, 1993).

Rhythmicity is a basic characteristic of all organic life (Lofts, 1970). The earth's rotation around the sun as well as its own rotation causes oscillations in the environment on a seasonal as well as a daily basis. These oscillations include abiotic factors such as ambient illumination or temperature and biotic factors such as activity of predators and sexual partners. In order to cope with these rhythms in the outside world any organism needs a matching (in case of mate activity) or an avoiding (in case of predators) rhythmicity in the internal organization of its physiology and/or behavoiur (Steinlechner, 1996).

General Introduction: Seasonality in African mole-rats.

1. Theory of seasonality in breeding:

Reproduction is the means by which individuals perpetuate copies of their genes and it is through differences in reproductive success that natural selection acts to shape phenotypes (Bennett and Faulkes, 2000). Most temperate and polar animals employ seasonal reproduction to avoid environmental conditions that might be detrimental to the survival of young and/or adults themselves (Nelson *et al.*, 1989). Animals can rely on a variety of seasonal and environmental variables to determine their reproductive status. These factors or cues include: photoperiod, rainfall, humidity, quantity and quality of food and temperature (See Reiter and Follett, 1980 for a review). Most animals restrict reproduction to times of the year when food is abundant and therefore ensuring that survival of their offspring is optimal (Nelson *et al.*, 1989). In most mammalian orders where seasonality in male reproductive function is expressed, the non-breeding periods are characterized by testicular regression and cessation of spermatogenesis (Woodwall *et al.*, 1989; Gottreich, 2000). Seasonal phenomena offer an ideal opportunity to investigate the central mechanisms that regulate the fertility of an individual and the species. In seasonal breeders the spermatogenic output should vary in parallel with the changing endocrine patterns. In several species there are variations in both the quality and quantity of semen (Hafez, 1987).

While temperature may have a very direct and obvious relationship to certain seasonal adaptations, such as changes in the insulating properties of fur or the timing of reproducing, photoperiod on the other hand would appear to have little or no direct importance for most organisms occurring underground (Richter, 1998; Richter *et al.*, submitted). Although the light:dark cycle is an important factor affecting reproduction in most mammals, photoperiodic effects are often mitigated by the effects of changes in temperature which brings about seasonal breeding (Kriegsfield, 2000, Steinlechner and Niklowitz, 1992).

In mammals the circadian system is employed in the regulation of reproductive, physiological and behavioural functions in two very important ways. Firstly, in some species there is a very strong circadian component in the timing of ovulation and reproductive behaviour ensuring that these events occur at a time when the animal is most likely to encounter a mate. Secondly, many mammals exhibit seasonal reproductive rhythms that are largely under photoperiodic regulation, in these species the circadian system and pineal gland are crucial components of the mechanisms that are employed by the species to measure day length (Goldman, 1999). Circadian rhythms probably evolved as adaptations that allowed organisms to prepare for relatively predictable environmental changes associated with the day-night cycle. Subterranean organisms are subjected to far less circadian variation in habitat character as compared to organisms above ground (Riccio *et al.*, 2000). In subterranean animals with degenerate eyesight one might expect photoperiod not to influence reproduction. However, it has been shown in the Mediterranean mole-rat, *Spalax ehrenbergi* which has subcutaneous eyes, that it is not only able to perceive light, but can also entrain daily activity patterns to a circadian light: dark cycle (Cooper *et al.*, 1993). Light should therefore not be dismissed as a potential entrainer of reproductive processes in other small subterranean mammals!

Environmental stimuli that are integrated to initiate reproduction can be divided into three general categories: social, physiological and physical (i.e. all the biotic and abiotic factors) that an individual experiences during its lifetime (Schoech *et al.*, 1996). All mammals regardless of their activity patterns (i.e. diurnal, nocturnal or crepuscular) exhibit a rhythm of melatonin synthesis (Goldman, *et al.*, 1993). Photoperiod determines the secretion of a number of endogenous substances that exhibit diurnal variation. For instance, the secretion of the hormone melatonin is far greater at night when animals are in the dark, than during the day, when animals are exposed to light (Arendt, J, 1988,1995, Reiter, 1991, Malpaux *et al.*, 1999, Cooper *et al.*, 1993, Goldman *et al.*, 1993).

The nocturnal pattern of melatonin secretion varies among species with respect to, the amount of total melatonin synthesized and released; the day/night amplitude; the phase of the peak to scotophase of the daily light-dark cycle and the duration of elevated melatonin levels. All of these parameters convey information about the length of the day and thus may be involved in conveying the photoperiodic message to neuroendocrine regulatory centers (Goldman, 1983; Steinlechner and Niklowitz, 1992).

Despite being widely distributed in seasonal habitats, most strictly subterranean mammals do not restrict reproduction to a particular time but are active throughout the year (Gottreich *et al.*, 1995; Shanas, *et al.*, 1995; Spinks, *et al.*, 1997; Gottreich *et al.*, 2000). Although seasonal fluctuations in some reproductive parameters have been reported in some species (Pevet *et al.*, 1984; Spinks, *et al.*, 1997).

One explanation for the absence of seasonal breeding in subterranean mammals is that they cannot interpret and respond to seasonal changes in daylength because there is no appropriate photoperiodic signal underground. The effect of photoperiod on reproductive parameters in subterranean rodents is unknown, although one study failed to find any effect of photoperiod on reproduction in the blind mole-rat (*Spalax ehrenbergi*) (Shanas, *et al.*, 1997).

2.Overview of the Bathyergidae:

The family Bathyergidae is endemic to Africa south of the Sahara and comprises five genera exhibiting a wider range of mean body mass (30g –1000g) and social structure (solitary to eusocial) than of any of the seven families or sub-families of subterranean rodents. African mole-rats are subterranean rodents occurring in a variety of habitats ranging from mesic to arid areas (Jarvis and Bennett 1990, Spinks *et al.*, 1997). In general, the solitary genera (*Bathyergus*, *Georychus* and *Heliophobius*) are larger and occur in regions with a higher rainfall (over 350mm/yr).

The social genera, *Cryptomys* and *Heterocephalus*, tend to be smaller, but there are exceptions to this pattern. The solitary species inhabit mesic regions where rainfall is fairly seasonal and predictable (Bennett *et al.*, 1999). The polyspecific genus *Cryptomys* has species that are sympatric with solitary species. However, some species are found inhabiting arid areas with sparse and unpredictable rainfall (< 200 mm/yr) (Jarvis and Bennett, 1990, 1991).

All bathyergids are herbivorous, feeding upon the underground storage organs of geophytes. This food resource is biologically inactive for much of the year and is packed with energy rich nutrients and constitutes an invaluable water resource (Bennett, 1988). Indeed, mole-rats drink no free water and rely on the consumption of geophytes for a positive water balance. The Bathyergidae live completely underground and rarely, if ever, emerge on to the surface, except during times of dispersal and heavy rainfall when they are flooded out from their burrow systems. They remain underground when foraging for their food resource and when disposing of the excavated soil onto the surface. The burrow is an important refuge in that it provides the mole-rat with protection against environmental extremes and predators.

Mole-rats encounter their food resources as they burrow. Their diet predominantly comprises tubers, corms and bulbs. However, two genera, *Bathyergus* and *Georychus* supplement their diet with the aerial portions of plants, which they pull underground into the burrow. In general smaller-sized geophytes are translocated to a designated food store in the burrow system. While, the larger tubers encountered by the mole-rats tend to be eaten *in situ*.

Anatomical and morphological adaptations to a subterranean lifestyle

Morphologically and anatomically, mole-rats are adapted for their existence underground. The mole-rat is a streamlined organism that can move forwards and backwards with equal dexterity (Genelly, 1965; Jarvis and Sale, 1971). Stiff hairs fringing the outer borders of both the hind feet and the short compressed tail facilitate the collecting and transportation of soil.

The mole-rat has undergone dramatic morphological modifications to cope with an underground existence. The animals are equipped with extrabuccal incisors that are ever growing and used exclusively for excavation in all genera. In *Bathyergus*, the fore claws may also be used for displacing the loose sandy soils (Jarvis and Bennett, 1990). The mole-rat has a low body carriage which is streamlined to provide minimum friction as the animal passes along the burrow. The external ear pinnae are reduced to small shells that encase the auditory opening, whereas, the nose is broad and horseshoe-shaped and the eyes microphthalmic with a thickened cornea (Bennett and Faulkes, 2000).

Physiological adaptations to a subterranean existence

Due to their small size and thermal sensitivity, many rodents have a totally subterranean existence. The burrows provide a niche that is relatively thermo-stable with respect to ambient temperatures, but at the same time is characterized by a high relative humidity as well as high carbon dioxide and low oxygen concentrations (Darden, 1972, Kennerly, 1964, Roper *et al.*, 2001). Indeed, physical conditions such as light, temperature and partial pressures of gases deviate considerably from those values normally experienced above ground. The most extensive portion of their burrow is superficial occurring 20cm below the ground surface. Although the burrows show some diurnal and seasonal fluctuations in temperature, they are considerably muted as compared to those above ground (Bennett *et al.*, 1988). Although in the nest, the mole-rats are probably exposed to hypercapnic and hypoxic conditions, their metabolic rate will however be at its lowest. It is possible that the haemoglobin of the mole-rat has a high affinity for oxygen, as is certainly the case for the naked mole-rat. At thermoneutrality (the temperature range within which metabolic heat production is unaffected by the ambient temperature change), most subterranean rodents have resting metabolic rates that are substantially lower than those of surface dwelling rodents whose body temperatures are higher and their thermal conductance lower.

Activity patterns in African mole-rats

Many subterranean rodents exhibit an evenly distributed activity pattern. Laboratory studies conducted on the pocket gopher, *Geomys busarius* (Vaughan and Hansen, 1961) and radiotelemetry studies on free ranging pocket gophers, *Thomomys talpoides* (Andersen and MacMahon, 1981) and *T. bottae* (Gettinger, 1984) suggest activity is evenly distributed over the 24h cycle. In the bathyergids, field studies on the Silvery mole-rat, *Heliophobius argenteocinereus* (Jarvis, 1973) and laboratory studies on the Natal mole-rat *Cryptomys hottentotus natalensis* (Hickman, 1979) and the common mole-rat *Cryptomys hottentotus hottentotus* (Bennett, 1992) have shown that activity is dispersed over the 24 h period. Lovegrove, *et al.*, (1988) have shown that in the Damaraland mole-rat, *Cryptomys damarensis,* activity is more readily distributed during the day. In contrast, in the Naked mole-rat, *Heterocephalus glaber* locomotor activity is more nocturnal (Riccio and Goldman, 2000).

It can therefore be that due to a lack of photoperiodic cues mole-rats should select other environmental factors to cue their activity patterns. Temperature is a very good candidate for such an environmental *zeitgeber*. Although showing some diurnal and seasonal fluctuations in temperature (Bennett *et al.*, 1988), superficial burrow temperature fluctuations are far more muted than those occurring on the surface. Despite the fact that the burrows show similar overall daily patterns of temperature change with respect to soil surface and air, the magnitude of the changes and the times of day at which maximal and minimal temperatures occur are different.

3.The Study Animals:

The Damaraland mole-rat, *Cryptomys damarensis,* is a eusocial species that lives in colonies of up to 41 individuals (Bennett and Jarvis, 1988; Bennett, 1990; Jarvis and Bennett, 1993). The mean body mass is 131g. A single female and one or two males are responsible for procreation whilst the rest of the members are subordinate and reproductively quiescent (Bennett, 1994). In addition to a reproductive division of labour, there is a secondary division of labour based on burrow maintenance activity. The Damaraland mole-rat occurs in semi-arid to arid regions of Southern Africa, which experience sporadic and unpredictable rainfall patterns. It is an aseasonal breeder producing a maximum of 3 litters per annum. Litter size ranges between 1-6 (mean ± 3) and the gestation period ranges from 78-92 days (Bennett and Jarvis, 1989).

The non-reproductive females of the species have been shown to be physiologically and behaviorally suppressed from breeding. In contrast, there is no physiological component to suppression in subordinate males, and it is proposed that males refrain from breeding due to incest avoidance (Molteno *et al.*, 1996; Maswanganye *et al.*, 1999).

The common mole-rat, *Cryptomys hottentotus hottentotus* is a social species living in colonies of between 2-14 individuals with one female and up to two males responsible for breeding (Bennett, 1989; Spinks, *et al.*, 1999). It inhabits mesic to semi-arid regions of the Western and Northern Cape Province (South Africa). They occur in a wide diversity of substrates ranging from sandy loams to heavier, more compact soils such as exfoliated schists and stony soils, and are not fond of heavy clay or brecciated soils. The common mole-rat is small with a mean body mass of 77g for males and 57g for females. It is an obligate outbreeder occurring in a winter rainfall region, where the rainfall patterns are fairly predictable with up to 600mm of rainfall/annum (SA Weather Bureau).
The common mole-rat is a seasonal breeder with mating mainly restricted to the summer months. It has a gestation period of 55-65 days, the young are mostly born in early spring to summer with a maximum of two litters/year (Bennett, 1989; Spinks *et al.*, 1997). The common mole-rat also exhibits a reproductive division of labour (Spinks *et al.*, 1997, 1999). Mating in *C. h. hottentotus* is not confined to a particular season but mostly occurs in the summer months (Bennett, 1989). The common mole-rat occurs in sympatry over its range with *Georychus capensis* and often with overlapping burrow systems (Davies and Jarvis, 1986).

The highveld mole-rat, *Cryptomys hotentotus pretoriae* is a loosely social species that occurs in colonies of 2-12 individuals with reproduction restricted to one female and one/two males (Moolman *et al.*, 1998). It occurs in a mesic area with predictable rain, which mostly falls in summer. This area is characterized by cold dry winters and hot moist summers (Janse van Rensburg *et al.*, 2002). They are obligate outbreeders with breeding confined to the months of May to November. Colonies have neither a distinct social hierarchy, nor a presence of any distinct working castes (Moolman, *et al.*, 1998). The highveld mole-rat is a seasonal breeder. It has a gestation period of around 66 days; the young are born in the winter months (Janse van Rensburg *et al.*, 2002). Litter size ranges between 1-3 (mean 2). The small colony size implies frequent dispersal events, facilitated by a habitat of moist workable soils for much of the year.

4. Aims of study:

The underground lifestyle of African mole-rats, while solving many problems from a thermoregulatory and predatory point of view, begs the question "What external cues do these animals use to set their endogenous biological clock?" They provide an excellent model to try to elucidate which environmental variables have been selected to provide signals of the time of day and season in their underground niche. The physiological mechanisms that are utilized by mole-rats to enhance reproductive success have thus far only been speculated upon.

All the solitary bathyergid species studied to date exhibit strict reproductive cyclicity whereas the majority of social species do not. Reproductive cyclicity in *C. h. hottentotus* and *C. h. pretoriae* is presumably a result of having invaded a seasonal habitat, but it is not manifested in male gonadal changes (Bennett *et al.*, 1999, Spinks *et al.*, 1997).

In the bathyergids, a great deal of attention has been given to studies on social suppression in non-reproductive females (see Bennett, *et al.*, 1999 for review), but reproduction in males has received little or no attention. Seasonality in temperature and rainfall are important determinants of seasonal breeding in the solitary Batherygidae. Reproductive cyclicity outside the breeding period is uncommon amongst seasonally breeding mammals. Most seasonal breeders use environmental cues, e.g., photoperiod, temperature, rainfall, food quantity and/or quality, to produce and give birth to young at the most opportune times of the year.

Therefore the main aims of this study were primarily to determine whether male mole-rats utilize light and/or temperature to cue their reproduction. The evaluation of sperm cell motility and morphology is an essential parameter in the examination of sperm quality and in the establishment of correlations between sperm quality and fertility (Verstegen *et al.*, 2002). *Cryptomys damarensis* was used as a control species since it is aseasonal.

This thesis sets out to investigate the effect of temperature and light on the reproductive biology of three species of male mole-rat from the genus *Cryptomys*. In particular, the study set out to assess the effect of temperature and photoperiod on the quality and quantity of sperm produced during the different seasons (vitality and sperm motility), which has thus far not been investigated. In addition the affect of season on sperm morphology and plasma testosterone titres was investigated. Finally, four seasons were simulated under controlled lighting and temperature regimes to mimic natural seasonal variation. Urinary melatonin concentrations were investigated at four different time points during a 24hour cycle and the amount of melatonin manufactured noted.

Chapter 2

Materials and Methods

Animal collection:

A total of 96 male mole-rats were used in this study comprising 32 animals per species. The mole-rats were collected using Hickman live-traps baited with sweet potatoes (Hickman, 1979). The Damaraland mole-rats (*Cryptomys damarensis*) were captured at Dordabis (22°58′S, 17°41′E) and Hotazel (Namibia) (27°17′S, 22°58′E). The common mole-rats (*C. h. hottentotus)* were captured at Sir Lowry's Pass, Western Cape (18°55′E, 34°17′S) and at Springbok, Northern Cape (29°40′W, 17°33′S) (South Africa). The highveld mole-rats (*C. h. pretoriae*) were captured in Tygerpoort (26°S, 28′E) and Garsfontein (25°45′S, 28°10′E) East Pretoria, Gauteng province, South Africa. All animals used were mature, according to criterion of Bennett, (1989). Animals were considered adult if they approximated the following masses C. *damarensis* ±80g, (Rickard and Bennett, 1997), *C. h. pretoriae* ± 60g (Moolman *et al.*, 1998), *C. h. hottentotus* ±40g (Spinks *et al.*, 1999).

The animals were caught during the appropriate seasons to reduce time needed for acclimatization. All the animals were then transported to the laboratory for experimentation. The animals were housed in plastic crates (25cm by 45cm) provided with wood shavings and tissue paper as nest material. Small plastic containers were provided as nests. Food was provided *ad libitum* and comprised chopped sweet potatoes, squash, carrots and apples. No free water was provided.

Males were paired with unrelated females from distinctly different colonies, to ensure that mating would occur and that incest is avoided. On initial pairing, the animals were observed for 1-2 hours to record copulation or aggressive interaction between individuals. If the mole-rats showed any evidence of aggression, they were immediately separated. Subsequent pairings were performed two days later, using a different partner. The mole-rats were acclimatised for two months under their respective light and temperature regimes before sampling occurred. Males and females were separated 3 days prior to sperm motility analyses to avoid ejaculation through copulation.

The mole-rats were divided into 4 groups comprising ±8 pairs/ per species. The groups were placed into pre-set temperature and photoperiod controlled rooms of long day (LD) with 14L:10D, low (18°C) & high (26°C) temperatures lights on at 5am off at 7pm and short day (SD) rooms with 10L:14D, low /high (18°C/26°C) temperatures lights on at 7am off at 17pm. A few mole-rats died during the experiments and consequently these could not be incorporated into the final analysis.

The cages were cleaned once weekly during daylight hours (Rickard and Bennett, 1997, Bennett 1989, 1990).

Urine samples for analysis of melatonin were collected over a 24 hour period, divided into four main collection periods: From (09h00-15h00 = R1) collection at T15, (15h00-21h00 = R2) collection at T21, (21h00-03h00 = R3) collection at T3 and (03h00-9h00 = R4) collection at T9. Collection at night was aided using a torch fitted with red cellophane paper to reduce irradiance (0.1lux) or in complete darkness. The animals were placed in urine collection chambers with food *ad libitum*. The urine was vortexed for 1 minute and then stored at -40°C until required for radioimmunoassay.

Removal of reproductive tracts and tissue preparation:

Prior to euthanasia, the animals were weighed using a Sartorius balance. Animals were euthanised by inhalation and subsequent overdose of halothane anaesthetic. Blood samples were collected in heparinized vacu-tainers from the left ventricular region of the heart. Testes were dissected out, weighed and placed in Bouins fixative for a maximum of 7 days before being transferred into 70% ethanol for storage until histological processing.

Histology:

Tissue sections were processed using standard histological procedures (Drury and Wallington, 1967). Whole testes were dehydrated through a series of ascending percentages of ethanol (70%-100%) and embedded in wax. Sections of approximately 6μm were cut through the whole testis and epididyme. Anterior, equatorial and posterior sections were selected for analysis. They were mounted on slides and stained with haematoxylin and eosin stains (Drury and Wellington, 1967). For each group of animals, prepared slides were viewed and the diameters of the seminiferous and epididymal tubules measured. All measurements were done with the aid of a dissecting microscope fitted with a graduated ocular micrometer. Thirty-five measurements were recorded per slide. The slides were then viewed using a light microscope fitted with a camera under oil immersion (100 x). Photographs were taken using a computerised program linked to the microscope and manually by camera using an AS100 film. The photographs were scanned and saved as jpeg images (Kushida, 1974, Trump *et al.*, 1961). Areas of the seminiferous tubules and the epididyme were calculated using the following formula for the area of an ellipsoid (A) = πab, where π = 3.14, a = maximum length and b = maximum width of the tubules.

Anatomy:

It was assumed that any change in the size of the gonads resulting from fixation would be constant for all samples analysed (Spinks, *et al.*, 1997). Tissues were blotted dry to remove excess fixative and weighed to the nearest milligram (mg) using a Sartorius scale. The maximum testis length and width were measured using a dissecting microscope fitted with a graduated eyepiece. Testicular volume was then calculated using the formula for volume of an ellipsoid $V = 4/3\ \pi ab^2$, where π = 3.14, a = maximum length and b = maximum width (Woodall and Skinner, 1989).

Sperm motility quantification:

The testis and epididyme were dissected out and cleaned of all surrounding fatty and connective tissues. They were then transferred into a warmed Hams F10 culture medium supplemented with 1.2g/l sodium bicarbonate (Sigma Cat no. N6635) to avoid shock to the sperm. The epididyme were cut from the testis and the spermatozoa were aspirated into the medium. The epididyme were cut into thin sections to allow maximum sperm aspiration from all regions including the cauda, caput and corpora epididymus.

A 10µl drop of the sperm suspension was placed onto a pre-warmed microscopic slide and covered with a cover slip. The slide was then placed on a pre-heated microscopic stage (34°C) for motility analysis, using negative phase contrast and a magnification of 100x. The images of the moving sperm were recorded on videotape using a VHS recorder. Sperm motility was analyzed using a computerized image analysis system (sperm motility quantifier (CASA), (Wirson Scientific and precision equipment, Auckland Park, Johannesburg). A series of frames were recorded for later analysis.

The sperm recordings were captured at a frame skip of zero and an analysis rate of 50Hz. The minimum and maximum numbers of frames analyzed were 10 and 31, respectively, to measure sperm trajectory.
The following sperm motility parameters were measured as defined by Katz (1991):

1) Curvilinear velocity (VCL): time-averaged velocity of sperm head along its actual path or curvilinear trajectory.
2) Straight-line velocity (VSL): time-averaged velocity of sperm head as projected along the straight line between its first and final detection positions.
3) Average path velocity (VAP): time-averaged velocity of sperm head projected along its spatial average trajectory.
4) Beat cross frequency (BCF): the beat cross frequency of the sperm head.

5) Linearity (LIN): a ratio of projected length of the curvilinear trajectory (LIN = VSL/VCL).
6) Amplitude of lateral head displacement (ALH): maximum amplitude of lateral distances of the sperm head trajectory about its spatial average path.
7) Wobble (WOB): ratio of VAP to VCL and is an expression of the degree of oscillation of the curvilinear path about its spatial average path (WOB = VAP/VCL).
8) Straightness (STR): ratio of VAP to VSL and is an expression of the straightness of the average path (STR = VAP/VSL).
9) Dance (DNC): defined by the product of VCL and ALH, and describes sperm motion as the space occupied by the sperm head path during one second (DNC = VCL x ALH).
10) Radian (RAD): the radius of the circle of which the total curvilinear track is an arc. By using the radian, circling sperm can be detected (RAD = (radius/□) x 180°).
11) Curvature (CURV): it reflects the progressiveness of movement (CURV = 1-(VSLpath/VCLpath).

A further 10µl of sperm suspension was pipetted into an eppendorf to assess vitality. This suspension was mixed with 10µl of negrosine-eosin stain. A 10µl drop was then placed on a slide to make a smear, covered with a cover slip and left to air-dry. The percentage of live and dead cells were analyzed under oil immersion using a light microscope (1000x). Dead sperm stained pink and the live were clear of any stain. A total of 100-200 sperm were analyzed.

The classification of live and dead sperm types were as follows:

1) Live with an intact acrosome
2) Live with reactive acrosome
3) Dead with an intact acrosome
4) Dead with a reactive acrosome

The first two denotes live spermatozoa, whereas the latter two denote dead spermatozoa.

Sperm morphology:

Sperm morphology was analyzed using SEM (Scanning electron microscopy). A 50µl sample of sperm suspension was fixed in 2% glutaldehyde and stored at 20°C prior to analysis. Individual semen samples from the different animals were analyzed and the samples grouped in order to give larger averages within groups and between the different regimes. The spermatozoa were washed using microfilters (0.2mm) (Millipore membrane filters). The microfilters were connected to a 2.5ml syringe. The samples were first shaken by hand to ensure that all spermatozoa were dislodged and the samples were then pulled into the syringe. Dilute phosphate butter (10:10 phosphate butter and double distilled water) was used to wash the spermatozoa, so they could settle on the microfilter. The samples were washed 3 times and left for 15 minutes/wash. The samples were then washed in an ascending series of ethanol from 70% to a 100%, with a 15-minute waiting period between each wash. They were then washed three times in 100% ethanol with a period of 15 minutes between each wash. The samples were then dried to critical point in liquid CO_2.

To prevent tissue collapse due to surface tension, liquid CO_2 was flushed every 5 minutes at 15 minutes intervals, and left in the chamber for 2 to 3 hours. The samples were finally mounted on a stub and spluttered with gold. The samples were analyzed with a Joel 840 scanning electron microscope at 5Am (Beesley, 1989, Hayat, 1981, Meek, 1976). Photographs were taken and analyzed using a computer program (Uthesca Image Tool). The frequency of certain head shapes was recorded and the whole sperm morphology noted. A comparison was then made between the different regimes for the types of abnormalities found. The different polymorphic head forms were noted and described. The spermatozoa were quantified by percentage for every abnormality viewed.

A minimum and maximum of 5 to 160 spermatozoa were analyzed within each species. Spermatozoa were categorized as normal with certain types of head shapes being oval or pear shaped. They were also categorized as having the following structural defects: head defects, tail/headless, cytoplasmic droplet (distal, proximal), bi-flagellated sperm, immature sperm and sperm precursors.

Testosterone Assay:

The testosterone assay was performed with the Coat-A-Count (Total testosterone kit) (Diagnostic Products Corporation, USA), which is a solid phase (I^{125}) RIA designed for the quantitative measurement of testosterone in unextracted serum or heparinized plasma. Due to its simplicity neither extraction nor chromatography is required.
The solid phase is based on a test-specific antibody immobilized to the wall of the polypropylene tubes. Radio labeled iodine (I^{125}) competes for a fixed time with testosterone in the sample for antibody sites. A 100µl plasma sample is pipetted into the tubes and 1ml of the label added. The samples are then covered with parafilm to avoid evaporation and vortexed at low speed for one minute. They are finally incubated at room temperature for three hours. The tubes are decanted, to separate bound from free testosterone and counted in a gamma counter. The amount of testosterone present in the sample is then calculated from the standard curve.

The kit is equipped with human serum based calibrators having testosterone concentration from 20 to 1600ng/dl (0.7-55nmol/l). The tracer has a high specific activity, with total count of approximately 60.000 cpm at iodination. Maximum binding is 30-40%, with detection limits of about 4ng/dl (0.14nmol/l). It is highly specific with cross-reactivity less than 5% with other naturally occurring steroids, which may be present in the plasma.

The assay was validated using unknown samples chosen at random. A serial dilution of reproductive male plasma testosterone paralleled with the standard curve, thus validating the assay (ANCOVA, F=0.18, P>0.05). The curve of parallelism for *C. damarensis* (Molteno *et al.*, 1999), for *C. h. hottentotus* (Spinks *et al.*, 1997), and for *C. h. pretoriae* (Van Rensburg *et al.*, 2002) (Fig 1-3). A log-logit transformation of the data followed (Chard, 1987). The inter-assay coefficient of variation was 4.9 (n=6). The sensitivity of the assay was 2.011nmol/l.

Creatinine Determination:

All urine samples were analyzed for melatonin concentration. These concentrations had to be corrected, since then urine concentration was variable due to varied fluid intake. The correction was undertaken by analyzing each urine sample for createnine concentration. Creatinine is used to correct for urine concentration as it is a breakdown product of tissue proteins and is excreted at a relatively constant rate. Final results are therefore not expressed as ng melatonin/ml urine but rather as ng melatonin/mg creatinine. This method therefore gives an index by which melatonin concentration can be measured, standardizing all melatonin concentration to that of 1mg creatinine.

The creatinine concentration of each sample was determined using a modified Jaffe reaction (Folin, 1914). Creatinine standards were made up at concentrations of 0, 0.05, 0.1, 0.5, 1.0, 1.5, 2.0, and 2.5 mg creatinine/ml distilled-deionized water (DDIW) from a stock solution of 3mg creatinine/ml DDIW. Ten microlitres of standard or sample was added to the wells of a microtitre plate in duplicate leaving two wells empty as a duplicate blank. To each well including blanks 300µl of picrate reagent was added. Picrate reagent was made up fresh and consisted of saturated picric acid solution, alkaline triton and DDIW (1:1:10). The alkaline triton, was in turn made up of 4.2 ml triton X-100, 12.5 ml 1NaOH and 66.0 ml DDIW. Alkaline triton was only used when homogenous by continuous stirring. The microplates was then left in the dark, at room temperature, to allow colour development for a period of 1.5 hours. The absorbance of

the standards, controls, blanks and samples were measured at optical density of 492 nm using a microplate reader.

A straight line was plotted (absorbance vs. creatinine concentration) for the means of the standards. Using linear regression, the equation of the straight line was determined. Rearranging the equation for the standard curve and substituting absorbance values for y-values, independent values were interpolated (creatinine concentration) for each sample. Descriptive statistics (mean ± SD) were calculated.

Urinary Melatonin Assay:

Melatonin is secreted only during periods of dark hence melatonin provides an accurate neuroendochemical index of season. Urine was collected under four experimental regimes LD 14L :10D with high and low temperatures and under SD 10L :14D with high and low temperatures. The urine was collected over four 6 hour periods (T9, T15, T21 and at T3). The animals were placed in urine collection chambers and provided with food *ad libitum*. Collection of urine during the night was undertaken in darkness. Samples were collected from 32 males per species. The urine was lightly vortexed and stored at –40°C until required for melatonin assay.

Urine was assayed in duplicate using a previously described method with a double antibody radioimmunoassay technique (Frazer *et al.*, 1983) with the antibody raised by Tillet *et al.*, (1986).

The urine samples were defrosted in luke warm water and vortexed for 1min at 3000 rpm. A volume of 50µl of urine was pipetted into tubes in duplicate. Tricine buffer (50ul) was added to the urine sample.

A further 100µl of buffer solution containing the antibody in tricine buffer was pipetted into all the tubes, excluding the tubes containing the 1st and 2nd standards. A further 300µl of radiolabeled melatonin was pipetted into all samples and vortexed for a minute further.

Lids were placed on the tubes containing Tr melatonin and all samples transferred into a 4°C fridge to incubate for 18hours.

For the standard curve: Pipette 100µl of the standards into 5 separate tubes each(5/Stand).

1. Tr (radiolabelled mel)
2. BNS (non-specific binding)
3. BO (Total binding of ligand100%)
4. Four different conc of melatonin until 0% binding (concentration :8; 16; 32; 64; 125; 250; 500; 1000).

A volume of 100µl of the tricine buffer was then pipetted into the standard tubes. After the designated incubation period 1ml of the 2nd antibody was pipetted into all tubes. This reacts with the bound melatonin leaving free antibody in the supernatant. The pipetting was done at 4°C. The samples were then re-incubated for 1hr in a fridge at 4°C.

The samples were then centrifuged for 30min at 3000rpm and 4°C, excluding the Tr samples. The supernatant was then discarded using a rack to balance samples. The tubes were left inverted for 2-3 hours to dislodge any further supernatant from the pellet. This also reduces the noise or variability when the count is done. The amount of melatonin in the pellet is then counted using the counter. The results were then checked against the standard curve. The counter providing values of melatonin and the appropriate %Coefficient of variation within samples. This provides an indication of sensitivity. Samples with a %CV more than 15% were re-assayed.

The samples were corrected for the melatonin concentration using the createnine results. Melatonin concentration (ng/ml) were divided by creatinine concentration (mg/ml) for each sample. This results in a final, corrected melatonin concentration expressed as ng melatonin/mg createnine. Parallelism was demonstrated between a standard curve in

ovine plasma and urine samples with 0, 25, 50 and 100 pg/ml of melatonin added. Samples from the different species were assayed separately. The inter- and intra-assay coefficient of variation for the combined assays was 10 and 7% for *C. damarensis*, 8.8% for *C. h. hottentotus* and 9.8% for *C. h. pretoriae*, respectively. Sensitivity averaged 4pg/ml.

Statistical Analysis:

All the samples were tested for homoscedecity. The seminiferous tubule and epididymal diameters were tested using the Generalized Linear Model (GLM) (Zar, 1984). Anatomical data was also tested for normality. To reduce variability within the data sets and type 1 errors, the values for body weights and testis weights were ranked using Scheffes Test. All statistical analyses were done with respect to body mass. The sperm motility data were tested using GLM. The pooled endocrine data was also tested using GLM and analysis of variance to test for differences between the various regimes. A 95% confidence limit applies to all the statistical analysis of the data sets.

Chapter 3

Results

When pairing males for the experiments care was taken to ensure that the mean body mass of the males placed on one protocol was not disparate from that of males placed on alternative lighting and temperature regimes. However, this was not always possible because of the difficulty in obtaining sufficient numbers of males from each species. The mean ± SEM and range of body masses of males of each species placed on the respective regimes are presented in Table 1.

Table 1: The mean body masses of males under the different regimes.

Species	Simulated seasons	Body mass (g)	SEM (g)	Range (g)
C.damarensis	LD 26	145.17	10.69	126-191
	SD 26	129.12	11.22	104.3-180
	LD 18	162.38	8.95	138-209
	SD 18	131.00	19.36	73-181
C.h.hottentotus	LD 26	84.50	5.19	63-104
	SD 26	108.00	11.24	78-180
	LD 18	77.25	7.68	49-114
	SD 18	71.71	5.76	49-91
C.h.pretoriae	LD 26	141.57	15.69	91-215
	SD 26	137.00	9.49	104-180
	LD 18	142.12	22.99	61-266
	SD 18	132.50	18.50	81-181

In the melatonin study, samples were collected over the 24h period at four main collection periods. From 09h00-15h00 (urine collected at 15h00 or T15); 15h00-21h00 (urine collected at 21h00 or T21); 21h00-03h00 (urine collected at 03h00 or T3) and finally 03h00-09h00 (urine collected at 09h00 or T9).

Appropriate temperatures for the four simulated seasons were derived from the mean temperatures normally experienced in superficial foraging tunnels of the three species (Table 2).

Table 2: Mean burrow air temperatures of mole-rat tunnel systems taken at a depth of 20cm during the height of the summer and winter periods.

Species	Summer	Winter	Differences
C. damarensis	31.9°C	18.6°C	13.9°C
C. h .hottentotus	25.1°C	17.5°C	7.6°C
C .h. pretoriae	24°C	15°C	9°C

The Damaraland mole-rat, *Cryptomys damarensis*:

Comparative histology and anatomy:

Male Damaraland mole-rats selected for the study differed significantly between one another with respect to body mass in all four experimental protocols. The body mass of males caught during winter was small, compared to males caught during summer (GLM: $F = 4.06$, $P = 0.05$). Temperature had no significant effect on body mass (GLM: $F = 1.64$, $P = 0.213$). There was no significant interaction between the two variables temperature and photoperiod (GLM: $F = 0.08$, $P = 0.78$, Table 3, Fig. 1). The mean relative testis mass as expressed against body mass was not significantly affected by changes in daylength (GLM: $F = 0.11$, $P = 0.741$). However, it was significantly affected by temperature, high temperatures resulting in a reduced testis mass and low temperatures bringing about a concomitant increase in mass (GLM: $F = 8.52$, $P = 0.008$, see Fig. 2). There was no significant interaction between the two variables (GLM: $F = 0.65$, $P = 0.430$, Table 3, Fig. 2).

The seminiferous tubule area expressed as a function of body mass was not significantly affected by temperature nor by photoperiod (F = 0.535, P = 0.472 and F = 2.296, P = 0.143, respectively). There were no significant interactions between the two variables (F = 0.475, P = 0.497). Likewise, the epididymal area was also not affected by changes in temperature or photoperiod (F = 0.924, P = 0.346, F = 0.401, P = 0.057, respectively). There was no significant interaction between the two variables (F = 0.261, P = 0.614) (Plate 1). Testis volume did not differ significantly during the various lighting regimes (GLM: F = 0.01,P = 0.983, Table 4). Whereas, temperature had a significant effect on testicular volume of the mole-rats (GLM: F = 7.88,P = 0.014). There was no significant interaction when the two parameters (light and temperature) were considered together (GLM: F = 2.78, P = 0.1174, see Table 4).

Table 3: Body and testis mass for *C.damarensis* under different temperatures and photoperiodic regimes (* = significant and Ns = not significant).

	Body mass			Testis mass		
	F-value	P > F	Comment	F-value	P > F	Comment
Temperature	4.06	0.0562	*	0.11	0.7411	Ns
Photoperiod	1.64	0.2133	Ns	8.52	0.008	*
Combination T & P	0.08	0.78	Ns	0.65	0.4302	Ns

Table 4: Comparative testicular volume of *C. damarensis* (n = 18) during the different light and temperature regimes (* = significant and Ns = not significant).

Variable	F-value	Pr > f	Comment
Photoperiod	0.01	0.9383	Ns
Temperature	7.88	0.0140	*
Interaction // T & P	2.78	0.1174	Ns

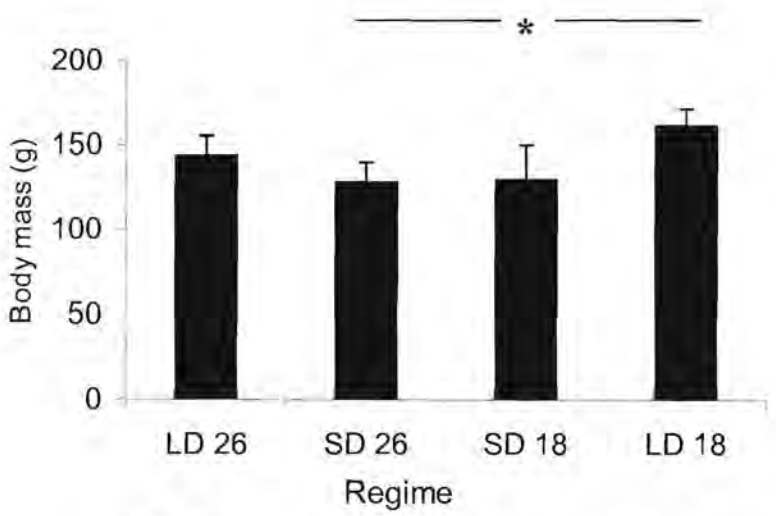


Fig 1: Comparative body masses for *C.damarensis* during the different regimes (X ± SD, * = significant p<0.05).

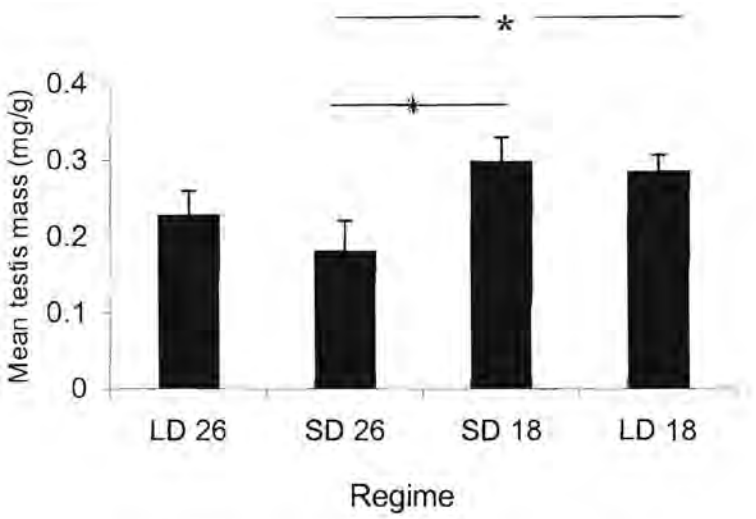


Fig 2: Comparative testis masses for *C.damarensis* during the different regimes (X ± SD, * = significant $p<0.05$).

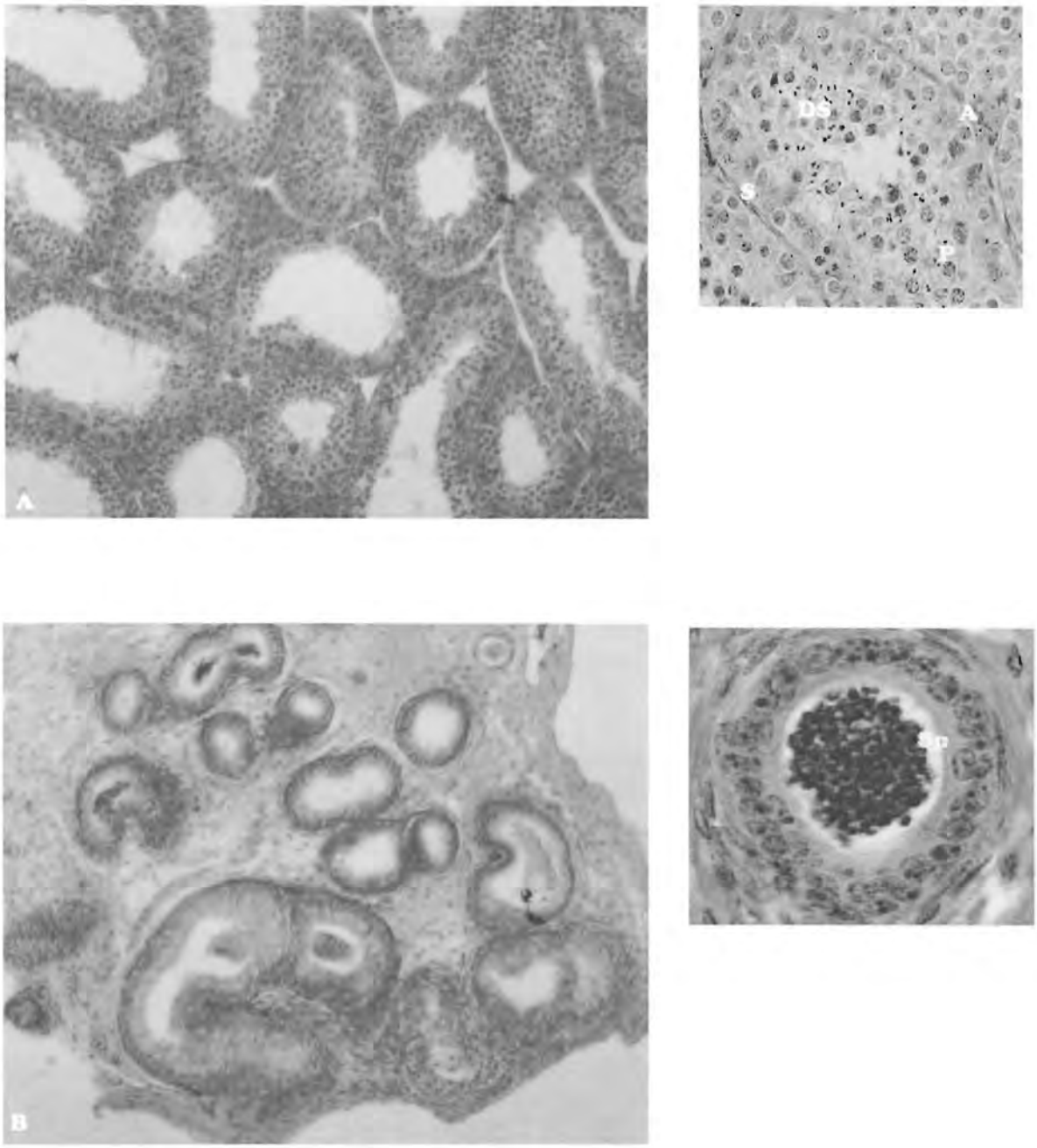


Plate 1(a) simulated summer LD 26°C

Plate 1(a-d): Transverse sections through the (A) seminiferous tubules and (B) epididyme of *C.damarensis* using phase contrast light microscopy x10 under the four regimes (LD:HT, SD:HT, SD:LT, LD:LT) showing active spermatogenesis. At higher mag x20 showing most germ cell types present, (S) sertoli cells, Type A/B spermatogonia, (L) leptotene, (Z) zygotene, (P) pachytene, (DS) differentiating spermatids and (Sp) spermatozoa.

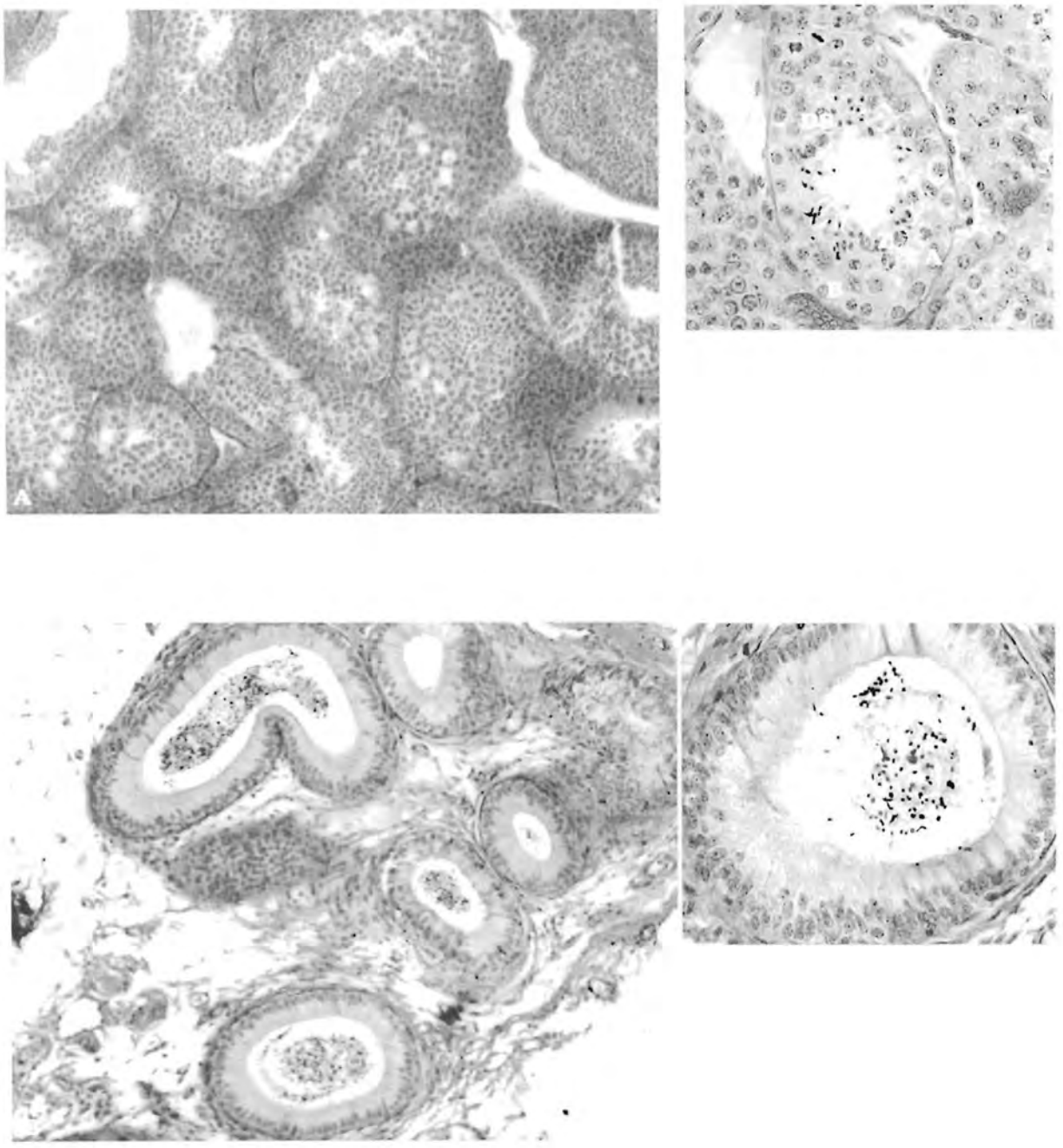
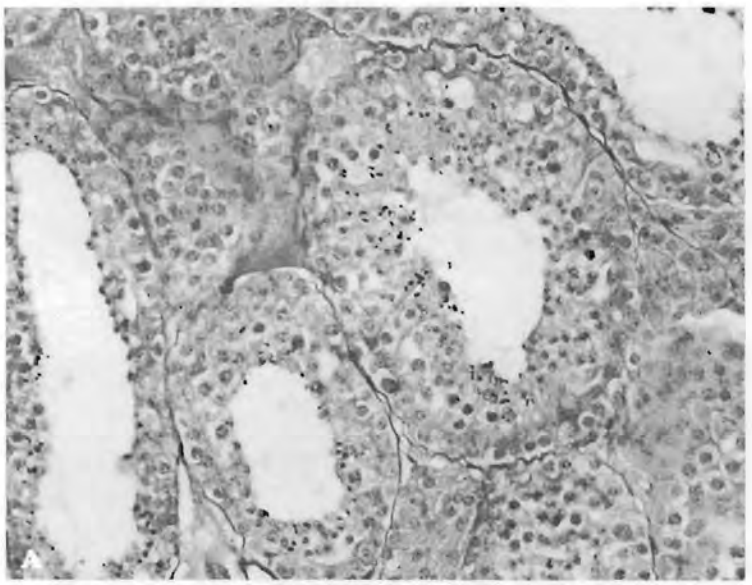
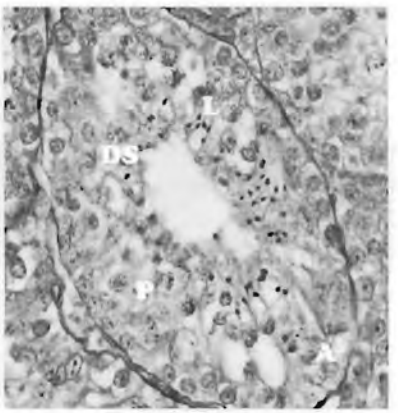
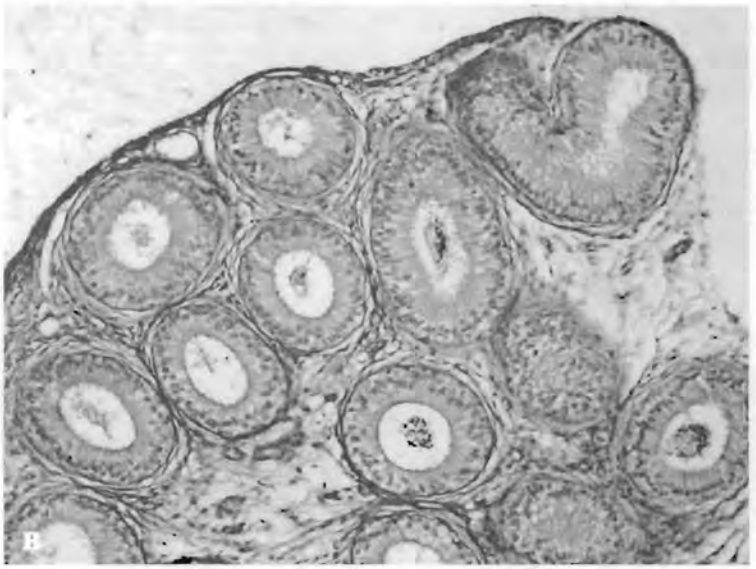


Plate 1(b) simulated autumn SD 26°C







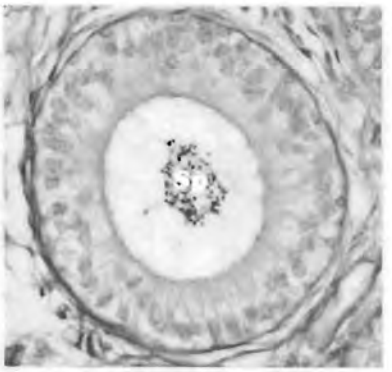


Plate 1(c) simulated winter SD 18°C

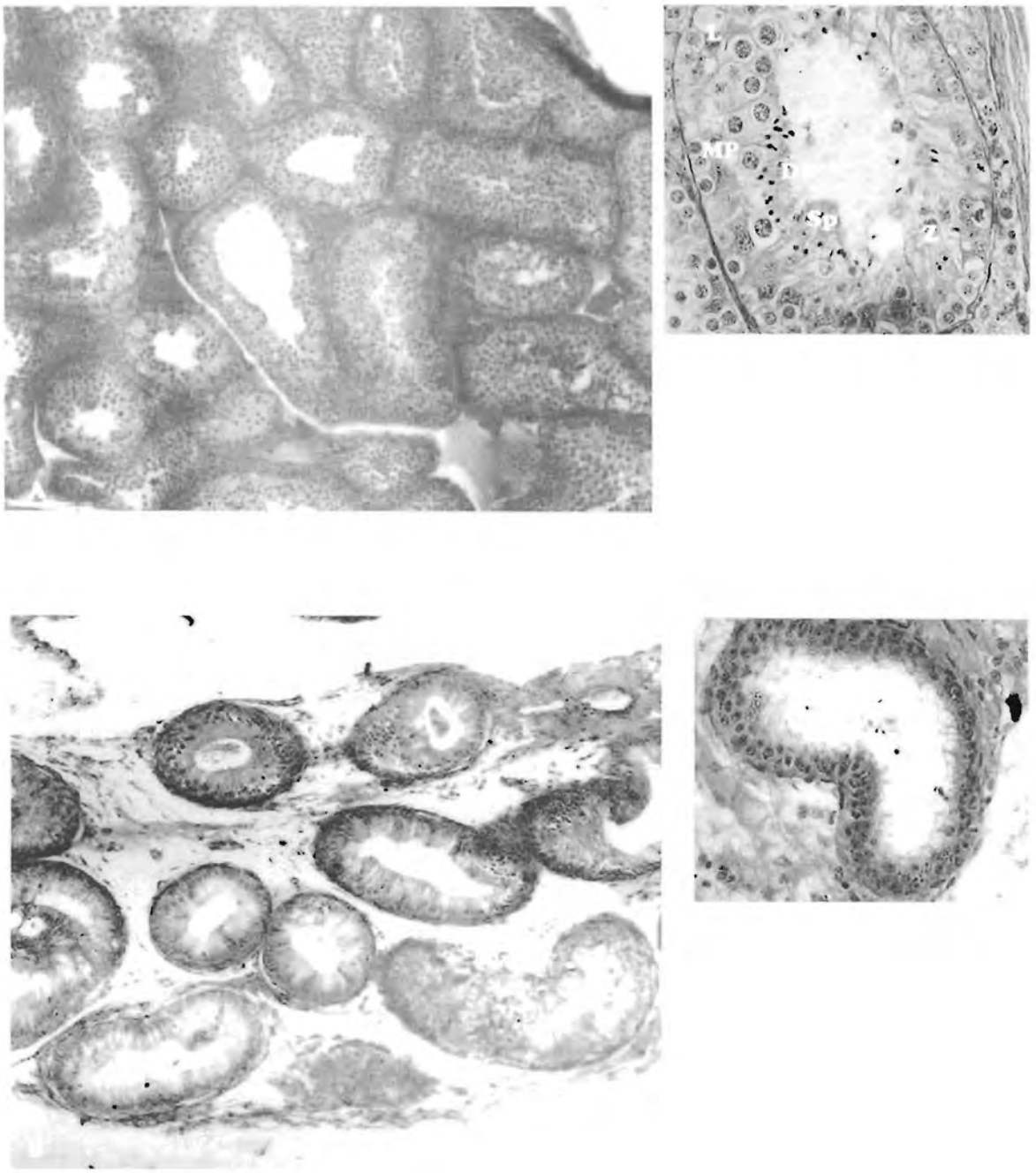


Plate 1(d) simulated spring LD 18°C

Comparative sperm motility and vitality parameters:

Overall there were no significant changes in the sperm motility parameters of mole-rats housed on the four light and temperature regimes (GLM: $F = 0.68, P = 0.359$). However, when looked at in context of an individual parameter, it was found that many of the motility parameters (VCL, LIN, etc) were affected by temperature (Table 5); In contrast, no sperm motility parameter was affected by a change in photoperiod (Table 5). Straight-line velocity (VSL) is probably the most important of the variables and quantifies the ability of the sperm to move swiftly. However, it was not affected by a change in either photoperiod or temperature (GLM: $F = 0.79, P = 0.376$ and $F = 1.03$, $P = 0.31$, respectively).

A comparison of the numbers of live to dead sperm (sperm vitality) revealed that there was no significant change in the numbers of live spermatozoa produced during the four experimental regimes used to mimic the different seasons (Fig. 3). When the effect of potential season was investigated with respect to the numbers and types of abnormal sperm produced, it was found that in simulated summer (LD, HT) a higher percentage of sperm precursors and giant cells were present, but fewer sperm were produced (Fig. 4a).

In autumn (SD, HT) there was a reduction in the percentage of abnormal sperm with the different classes of structural abnormalities being similarly distributed (Fig. 4b). In winter (SD, LT) there were a number of sperm precursors and a generally reduced percentage of the other types of sperm abnormalities (Fig. 4c). Finally, in spring (LD, LT) there was an enhanced number of spermatozoa with head defects and a slightly increased number of spermatozoa produced (Fig. 4d). The types of abnormalities found were uniform across all simulated seasons with a few sperm precursors and giant cells evident in the epididyme (Plate 2 and 3).

Table 5: Comparative sperm motility characteristics for *C.damarensis* during different temperature and photoperiod regimes (* = significant).

Variable	Temperature		Photoperiod	
	F-value	P > fr	F-value	P > fr
VCL	20.98	0.0001*	2.63	0.109
VSL	0.79	0.376	1.03	0.314
LIN	4.5	0.037*	0.06	0.81
MnALH	31.71	0.0001*	2.93	0.091
MxALH	18.69	0.0001*	2.14	0.148
BCF	4.34	0.041*	0.06	0.802
DNC	18.93	0.0001*	0.5	0.48
DNCmn	18.14	0.0001*	0.8	0.373
VAP	2.12	0.15	0.63	0.431
WOB	9.54	0.03*	0.04	0.843
STR	0.04	0.847	2.14	0.148
RAD	0.01	0.933	0.91	0.344
CURV	1.46	0.231	0.41	0.526

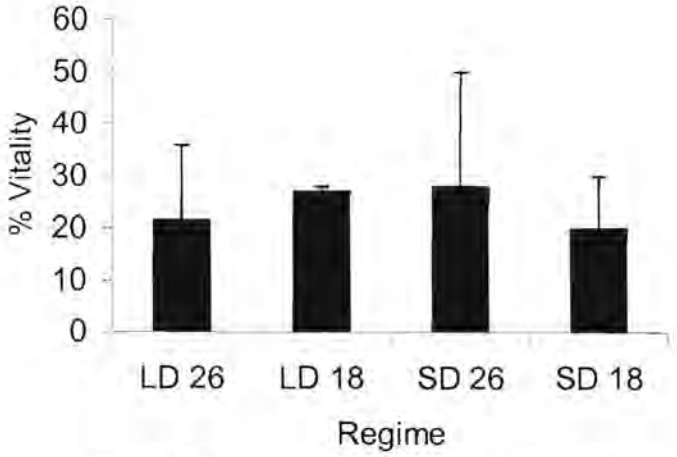


Fig 3: Comparative vitality for *C.damarensis* during the different lighting and temperature regimes (X ± SD).

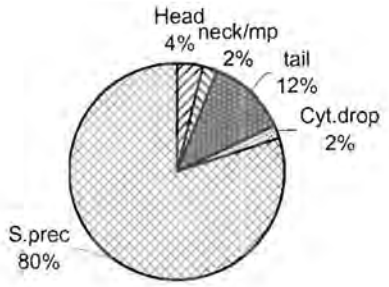


Fig 4(a) simulated summer

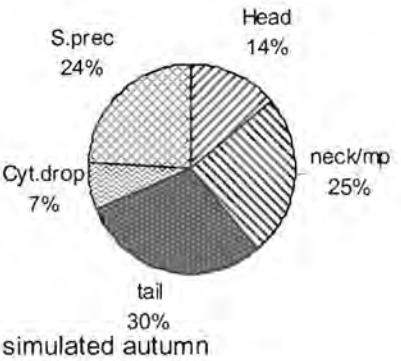


Fig 4(b) simulated autumn

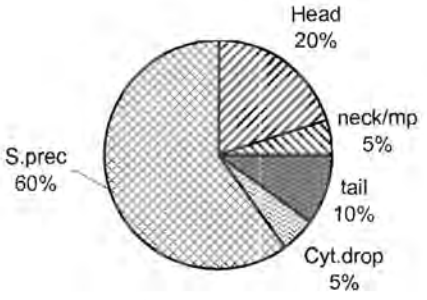


Fig 4(c) simulated winter

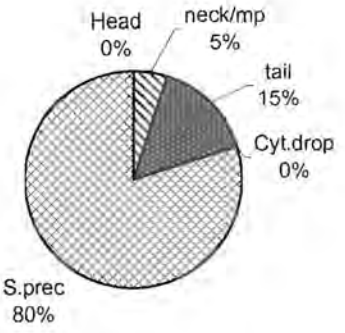


Fig 4(d) simulated spring

Figure 5(a-d): Percentage of abnormal spermatozoa types found in the epididyme of *C.damarensis* during simulated summer, autumn, winter and spring.

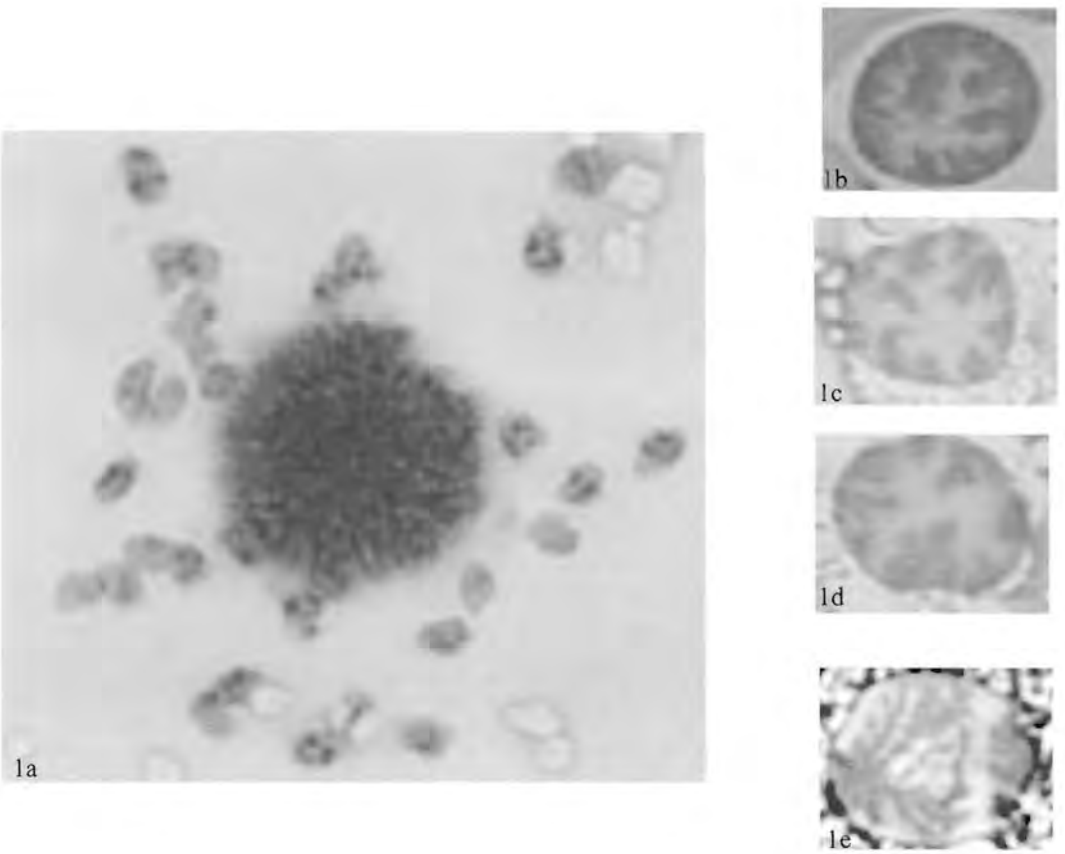


Plate 2: Photomicrographs of the different types of sperm precursor found in the epididymal aspirate in *C.damarensis* under light microscopy x10, x20, 1(b) pachytene, 1(c) zygotene, 1(d) early pachytene and 1(e) leptotene stages x20.

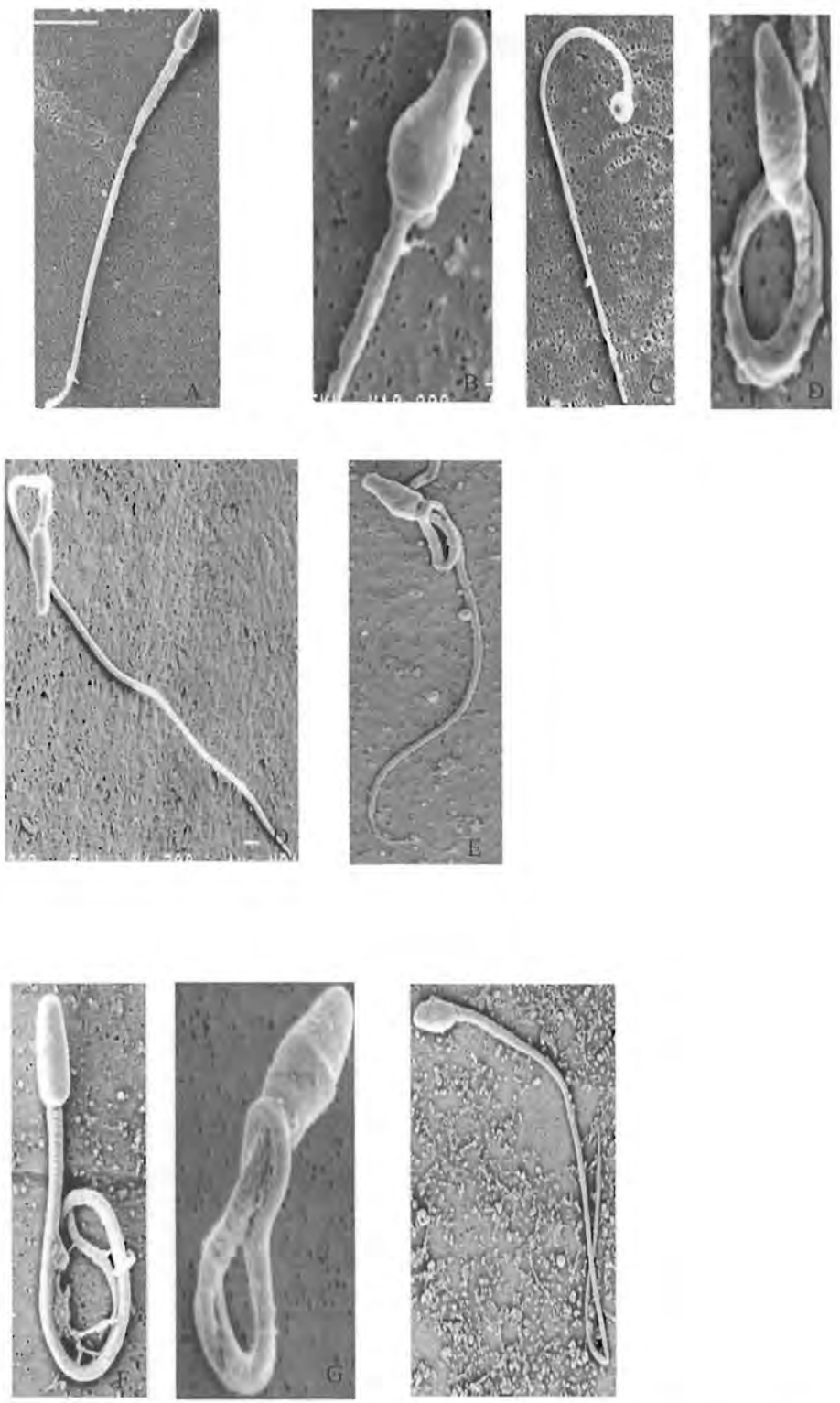


Plate 3: Scanning electron microscopy (Jeol 840) of aspirated epididymal spermatozoa in *C.damarensis* showing the normal morphology and the types of abnormal spermatozoa prevalent in the tubules, (a) normal, (b) head region, (c) pin head, (d) tapered head, (e) bent midpiece, (f-g) anomalies of the tail section.

Comparative endocrinology:

Plasma testosterone concentrations:

There were no significant differences in the mean concentrations of plasma testosterone secreted during the four different temperature and photoperiodic regimes (Fig. 5). Photoperiod was found to have no significant effect on the amount of testosterone manufactured (GLM: F = 0.15, P = 0.699, Table 6). Similarly, temperature had no effect on testosterone secretion (GLM: F = 3.2, P = 0.09, Table 6). Not surprisingly, there was no interaction between the two environmental variables on circulating testosterone concentrations (GLM: F = 0.38, P = 0.543, Table 6).

Table 6: Testosterone profiles during different combinations of photoperiod and temperature.

Variable	F-value	P>f
Photoperiod	0.15	0.699
Temperature	3.2	0.09
Interaction P & T	0.38	0.543

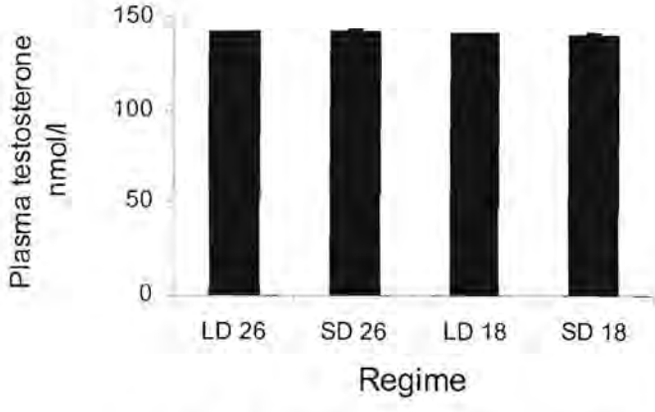


Fig 5: Plasma testosterone titres for *C.damarensis* under the different light and temperature regimes (n = 22).

Urinary melatonin concentrations:

There were no significant differences in the amounts of urinary melatonin concentrations during the different temperature and photoperiodic regimes. Melatonin concentration was not significantly affected by differences in temperature (GLM: $F = 0.14$, $P = 0.705$). Likewise there was no significant effect of daylength on urinary melatonin concentration (GLM: $F = 0.44$, $P = 0.509$). However, there was a significant difference in the concentration of melatonin between the light and dark periods with an increase in melatonin concentration with the lengthening of the dark period (GLM: $F = 0.17$, $P = 0.0007$,). There was no significant interaction between the two parameters (GLM: $F = 0.37$, $P = 0.547$).

There was a clear rhythm of melatonin concentration during summer (LD, HT), with a steady rise of urinary melatonin concentration from early afternoon T15 (41.9 ±13.0 ng/mg creatinine) through late afternoon and evening, with a peak at T9 (71.7 ±12.8 ng/mg creatinine). There was a significant difference in the concentration of urinary melatonin during the light when compared to the dark phase (GLM: $F = 15.49$, $P = 0.0013$) but there was no significant difference in concentration between T3 and T9 (GLM: $F = 1.32$, $P = 0.27$, Fig. 6a).

During simulated autumn (SD, HT) melatonin secretion rose steadily with a single peak at T3 (65.5 ± 6.85 pg/ml) and then it lowered again towards subjective dawn and early morning T9 (44.7 ±13.7 ng/mg creatinine). There was a significant difference in the concentration of urinary melatonin produced during the light and dark period with a clear diurnal rhythm (T15 vs. T3, GLM: $F = 9.51$, $P = 0.0022$), (T21 vs. T3, GLM: $F = 3.33$, $P = 0.0087$) and there was no significant differences between T3 and T9 (GLM: $F = 1.07$, $P = 0.091$, Fig. 6b).

During simulated winter (SD.LT) melatonin concentration rose from the daytime levels at T15, peaks at T3 and remained elevated through to T9. There was a significant difference in the concentration of melatonin during the day and early evening T15 vs. T21 (GLM: F = 7.28, P = 0.0245), the concentration at late evening T21 vs. T3 (GLM: F = 26.25, P = 0.0006) and between T21 vs. T9 (GLM: F = 8.7, P = 0.016). There was no significant difference in the concentration of urinary melatonin produced during the dark period and subjective dawn T3vs T9 (GLM: F = 0.31, P = 0.591, Fig. 6c).

During spring (LD, LT) melatonin secretion rose steadily and the levels stabilized throughout the night. No significant differences in urinary melatonin concentrations were recorded during T15 and T21 (GLM: F = 0.65, P = 0.433). There was, however, a significant difference in melatonin concentration at T15 vs. T3 (GLM: F = 5.29, P = 0.0368) and T15 vs. T9 (GLM: F = 6.12, P = 0.0258). There was also a difference in concentration at T21 vs. T3 (GLM: F = 5.09, P = 0.0395) and at T21 vs. T9 (GLM: F = 4.4, P = 0.0532). But there was no significant difference in urinary melatonin concentration at night and at subjective dawn T3 vs. T9 (GLM: F = 1.07, P = 0.318, Fig. 6d)

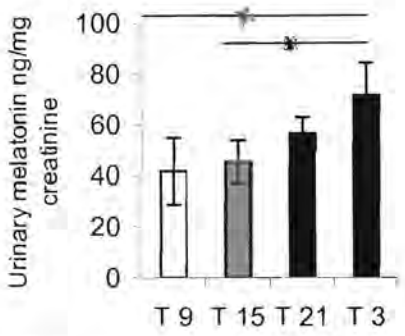


Fig 6(a) simulated summer

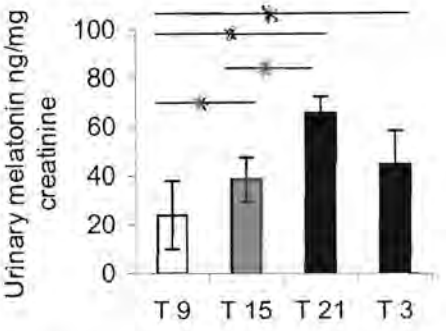


Fig 6(b) simulated autumn

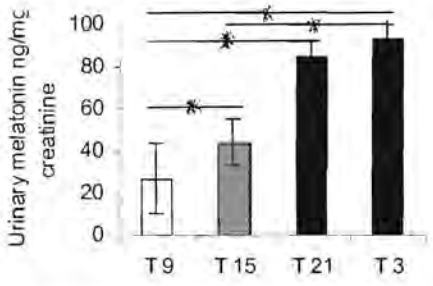


Fig 6(c) simulated winter

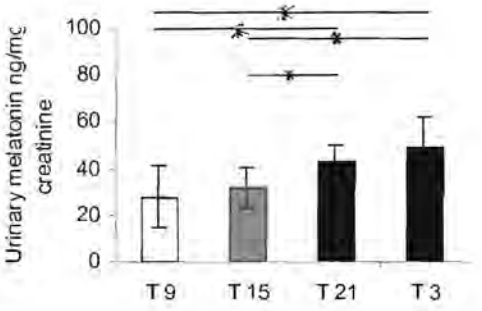


Fig 6(d) simulated spring

Figure 6(a-d): Melatonin titres of male Damaraland mole-rats kept under the different simulated seasons (ng/mg creatinine, * = significant $p < 0.05$).

The common mole-rat, *Cryptomys hottentotus hottentotus*:

Comparative histology and anatomy:

The body masses of male common mole-rats caught (GLM: F = 0.90, P = 0.351, Fig 7) during summer were higher as compared to males caught in winter (GLM: F = 8.14, P = 0.0082, Fig 7), but there was no significant interaction between the two parameters (GLM: F = 2.23, P = 0.147, Table 7). The mean relative testis mass as expressed against body mass was significantly affected by a change in photoperiod (GLM: F = 4.82, P = 0.036, Fig. 8). A change in temperature had no significant affect on the mean relative testis mass (GLM: F = 2.33, P = 0.138, Fig 8). There was no significant interaction between the two parameters (GLM: F = 0.01, P = 0.918, Table 7).

The seminiferous tubule areas were not significantly affected by temperature (F = 0.032, P = 0.855) nor by photoperiod (F = 2.869, P = 0.101), but there was, however, a significant contribution made by the two parameters (F = 18.232, P = 0.00029). The epididymal area was, however, significantly affected by a change in photoperiod (F = 10.65, P = 0.0029), as the days become long the area decreases with an increase in epididymal area when the days become short. The epididymal area was not affected by temperature changes, (F = 3.324, P = 0.08) with no significant interaction between the two variables (F = 2.829, P = 0.104) (Plate 4).

Testicular volume was significantly affected by a change in photoperiod (GLM: F = 9.31,P = 0.0076, see Table 8). Whilst temperature change did not affect testicular volume at all (GLM: F = 2.76, P = 0.1101, Table 8). Not surprisingly, there was no significant interaction between the two parameters (GLM: F = 0.001, P = 0.9763) (Table 8).

Table 7: Body and testis mass for *C.h.hottentotus* under different temperature and photoperiodic regimes (* = significant and Ns = not significant).

	Body mass			Testis mass		
	F-value	P > F	Comment	F-value	P > F	Comment
Temperature	0.9	0.3512	Ns	2.33	0.1388	Ns
Photoperiod	8.14	0.0082	*	4.82	0.0369	*
Combination T & P	2.23	0.1473	Ns	0.01	0.9187	Ns

Table 8: Comparative testicular volume of *C.h.hottentotus* (n = 20) during the different light and temperature regimes (* = significant and Ns = not significant).

Variable	F – value	Pr > F	Comment
Photoperiod	9.31	0.0076	*
Temperature	2.76	0.1101	Ns
Interaction // T & P	0.00	0.9763	Ns

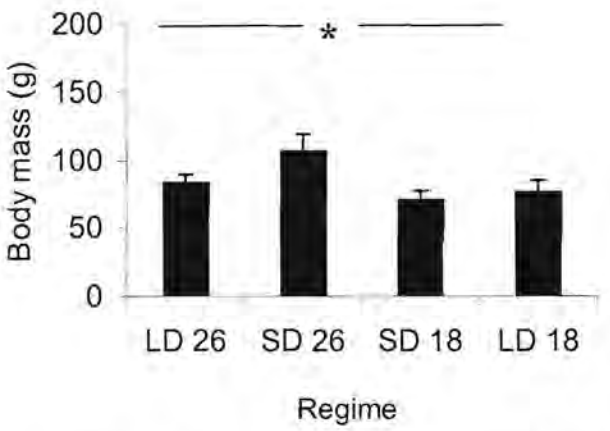


Fig 7: Comparative body masses for *C.h.hottentotus* during the different regimes (X ± SD, * = significant $p < 0.05$).

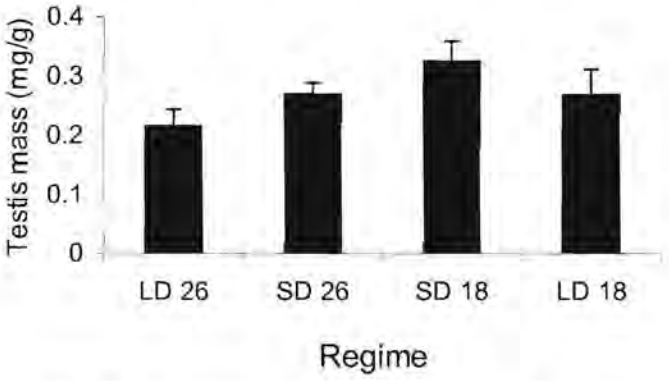


Fig 8: Comparative testis masses for *C.h.hottentotus* during the different regimes (X ± SD).

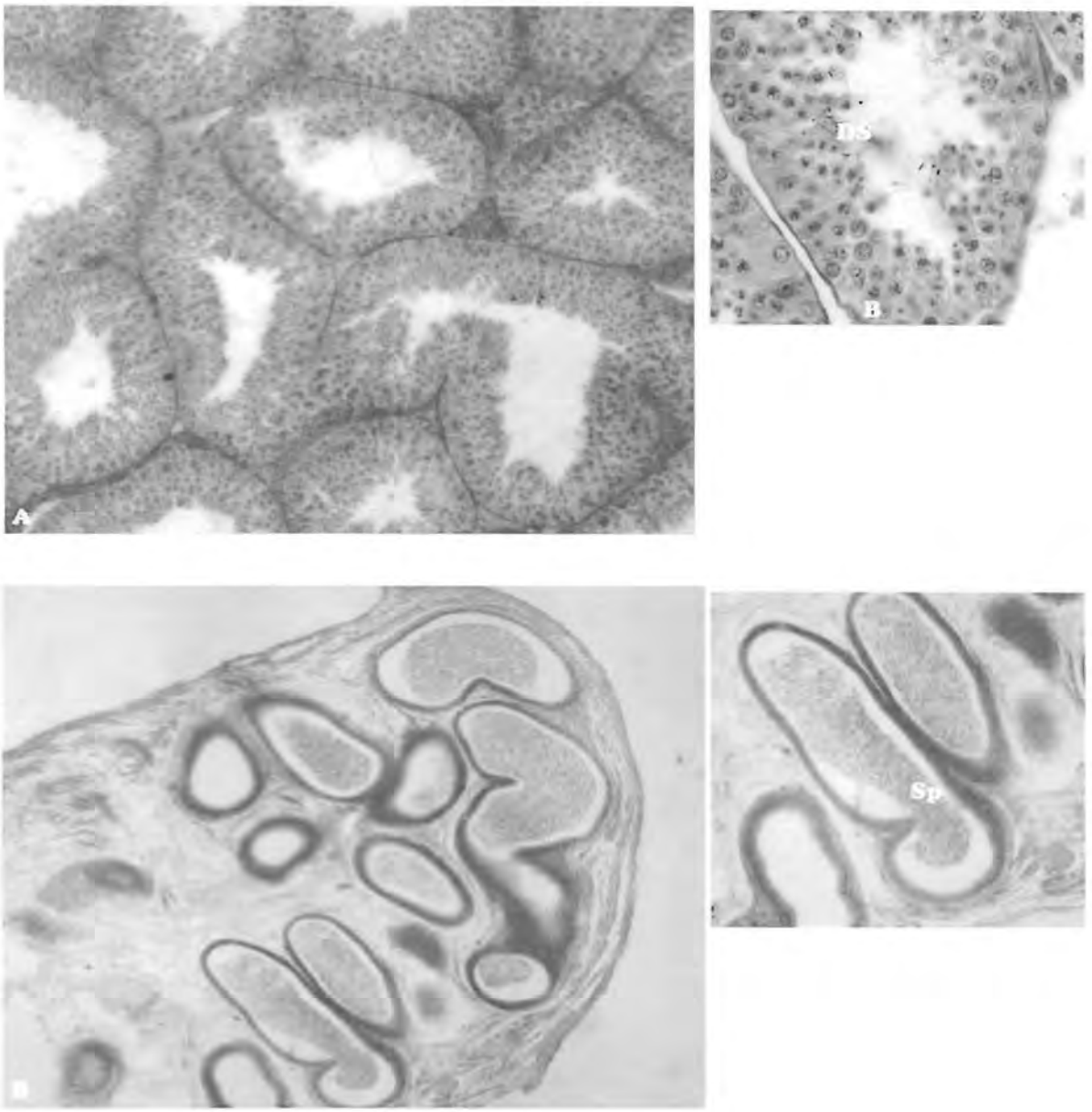


Plate 4(a) simulated summer LD 26°C

Plate 4a-d: Transverse sections through the (A) seminiferous tubules and (B) epididyme of *C.h.hottentotus* using phase contrast light microscopy x10 under the four regimes, (LD:HT, SD:HT, SD:LT, LD:LT) showing active spermatogenesis. At higher mag x20 showing most germ cell types present, (S) sertoli cells, Type A/B spermatogonia, (L) leptotene, (Z) zygotene, (P) pachytene, (DS) differentiating spermatids and (Sp) spermatozoa.

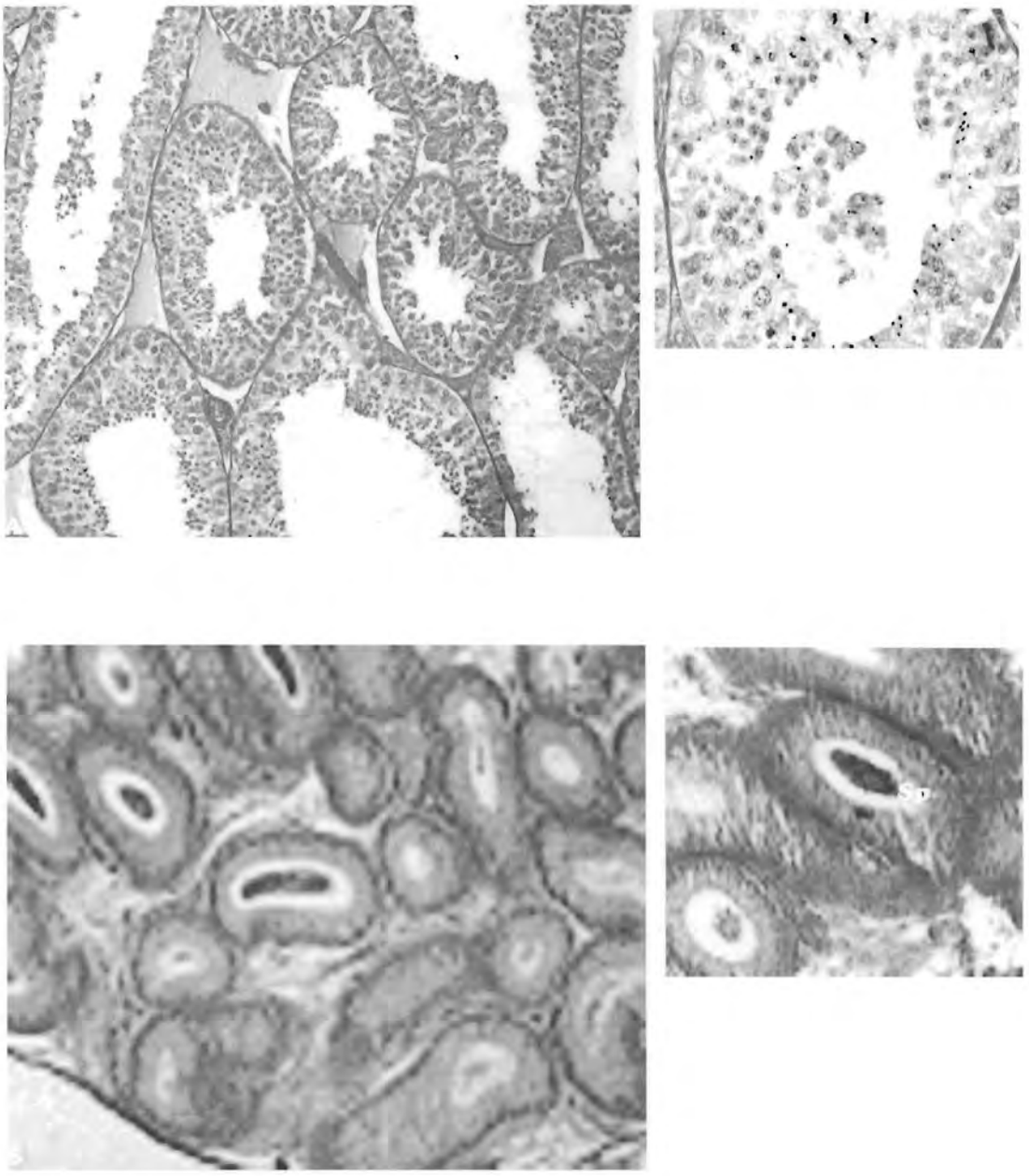


Plate 4(b) simulated autumn SD 26°C

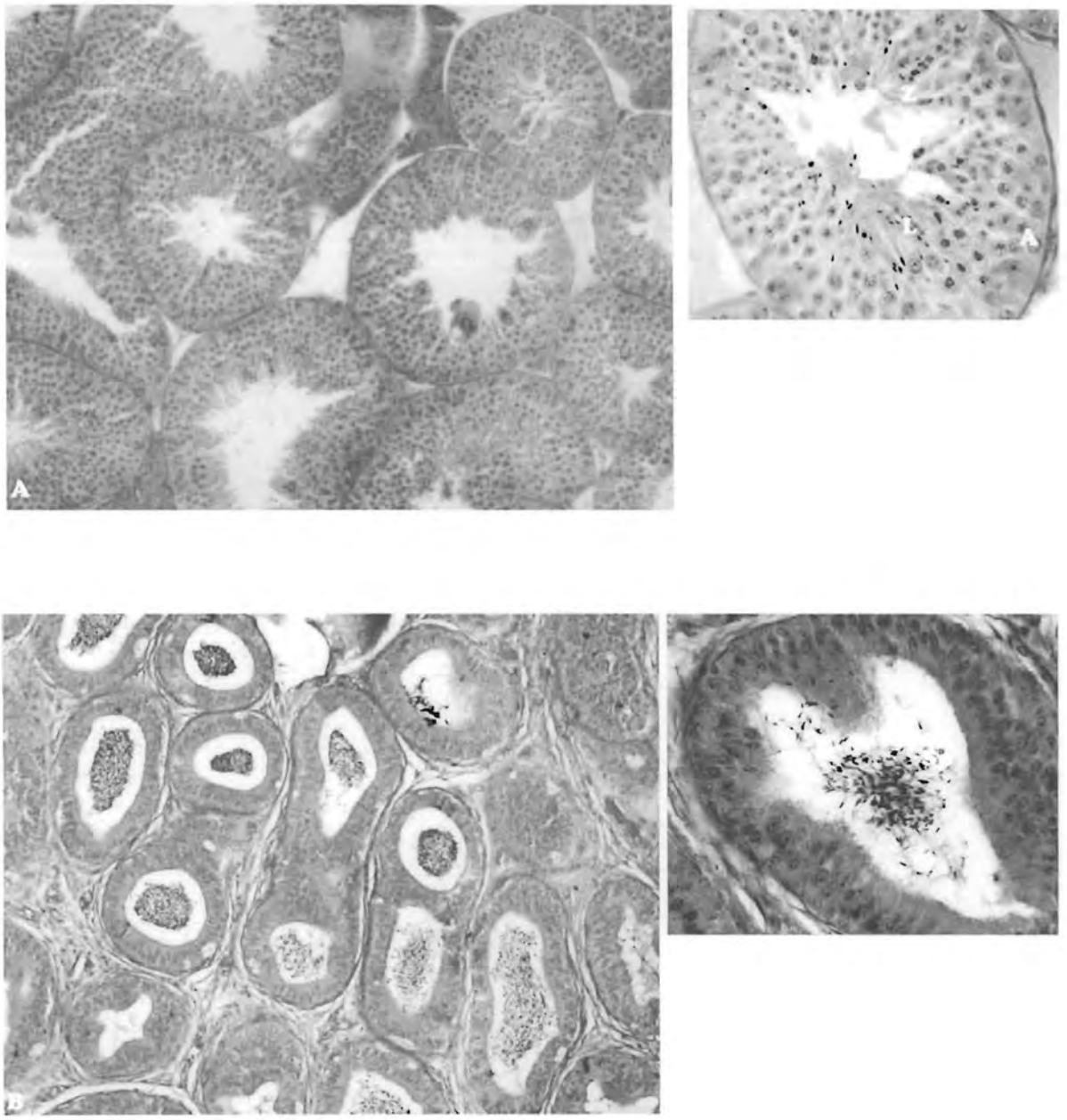


Plate 4(c) simulated winter SD 18°C

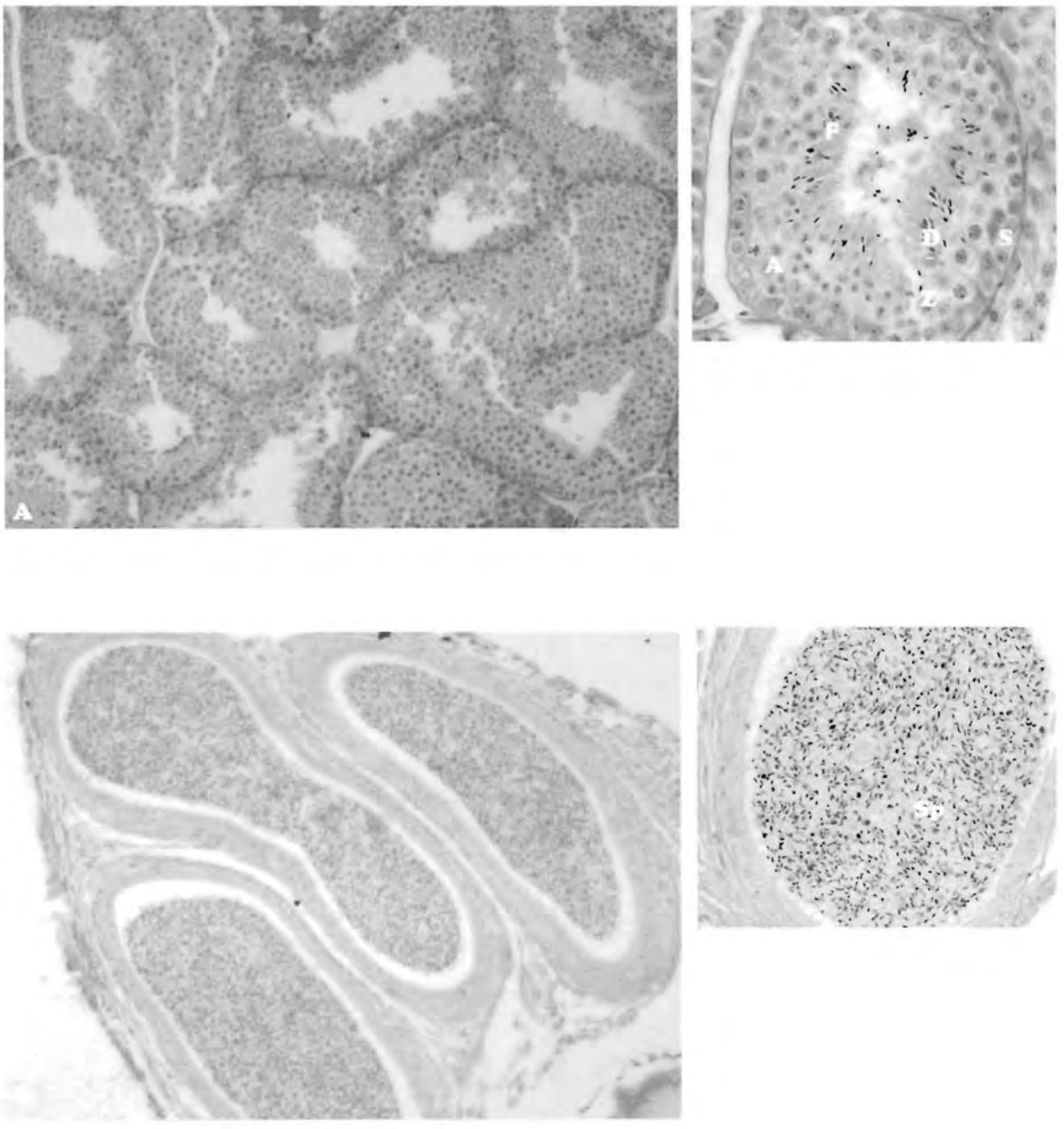


Plate 4 (d) simulated spring LD18°C

Comparative sperm motility and vitality parameters:

Overall, the majority of the motility parameters investigated in the individual context exhibited a distinct change when exposed to a combination of long days and high temperature. An increase in straightness of motion as daylength became shorter and temperature lower was noticeable (Table 9). One of the most important variables, that of straight-line velocity was not significantly affected by temperature (GLM: $F = 2.83, P = 0.092$). Interestingly it was affected by photoperiod (GLM: $F = 22.11$, $P = 0.0001$, Table 9). VAP and LIN were also affected by a change in photoperiod, but not temperature (Table 9).

There were no significant differences in the vitality of spermatozoa during the different seasons (Fig. 9). When the effect of potential season was investigated with respect to the numbers and types of abnormal spermatozoa produced, it was found that in simulated summer (LD, HT) there was an increase in the various categories of abnormalities with a higher number of sperm with cytoplasm droplets and generally equal distribution of the remaining types of abnormalities (Fig. 10a). In simulated autumn (SD, HT) a reduction in the number and types of abnormalities occurred with a surge in spermatozoa with tail defects (Fig. 10b). In simulated winter (SD:LT) an overall increase in the percentages of abnormalities and sperm production occurs (Fig. 10c). The types of abnormalities found were uniform across all seasons (Plate 5). In simulated spring (LD, LT) there was an increase in sperm with neck, midpiece and tail deformities, but there was a decrease in the number of sperm with cytoplasmic droplets found (Fig. 10d). The majority of the sperm possessing cytoplasmic droplets had the cytoplasmic droplet found at the base of the acrosome, indicating that the spermatozoa were still in the process of final maturation on short days. Cytoplasmic droplets during long days and high temperatures were found either proximal, distal to the midpiece or at the tip of the tail, which shows defects in these sperm, the mitochondrial sheath and ring being absent.

Table 9: Comparative sperm motility characteristics for *C.h.hottentotus* during different combinations of temperature and photoperiod (* = significant).

Variable	Temperature		Photoperiod	
	F-value	P > fr	F-value	P > fr
VCL	1.44	0.229	88.12	0.0001*
VSL	2.83	0.092	22.11	0.0001*
LIN	10.25	0.0014*	14.84	0.0001*
MnALH	73.64	0.0001*	88.14	0.0001*
MxALH	45.87	0.0001*	56.18	0.0001*
BCF	7.4	0.006*	0.36	0.548
DNC	33.12	0.0001*	45.69	0.0001*
DNCmn	23.11	0.0001*	27.57	0.0001*
VAP	2.32	0.127	43.37	0.0001*
WOB	13.38	0.0003*	8.64	0.003*
STR	6.03	0.014*	5.98	0.014*
RAD	16.63	0.0001*	0.53	0.467
CURV	1.26	0.262	12.3	0.0005*

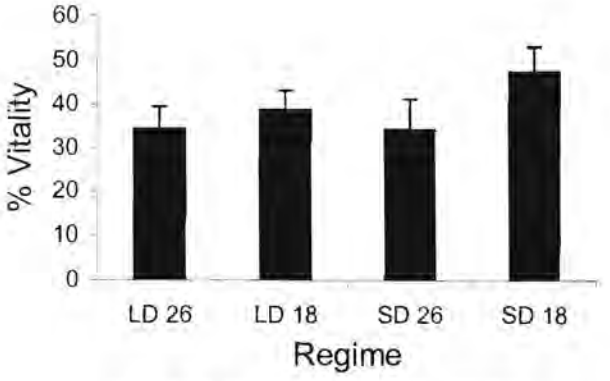


Fig 9: Comparative vitality for *C.h.hottentotus* during the different regimes (X ± SD).

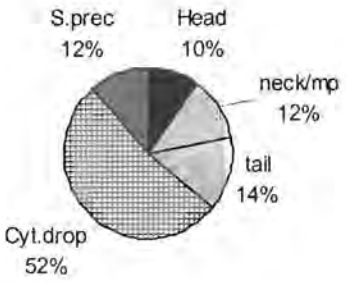


Fig 10(a) simulated summer

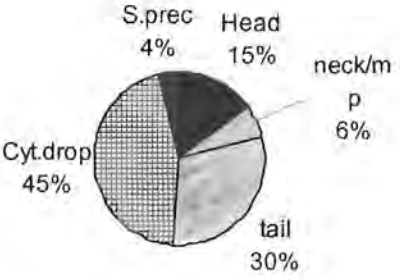


Fig 10(b) simulated autumn

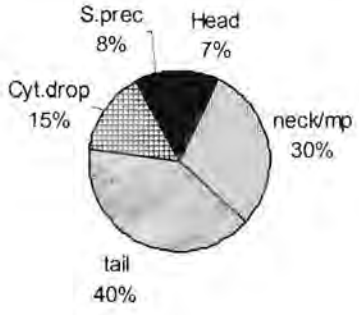


Fig 10(c) simulated winter

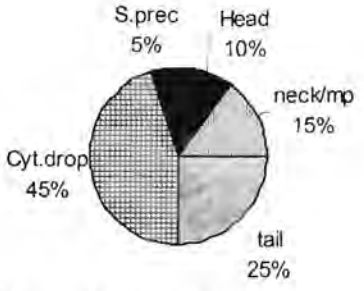


Fig 10(d) simulated spring

Figure 10(a-d): Percentage of abnormal spermatozoa types found in the epididyme of *C.h.hottentotus* during simulated summer, autumn, winter and spring.

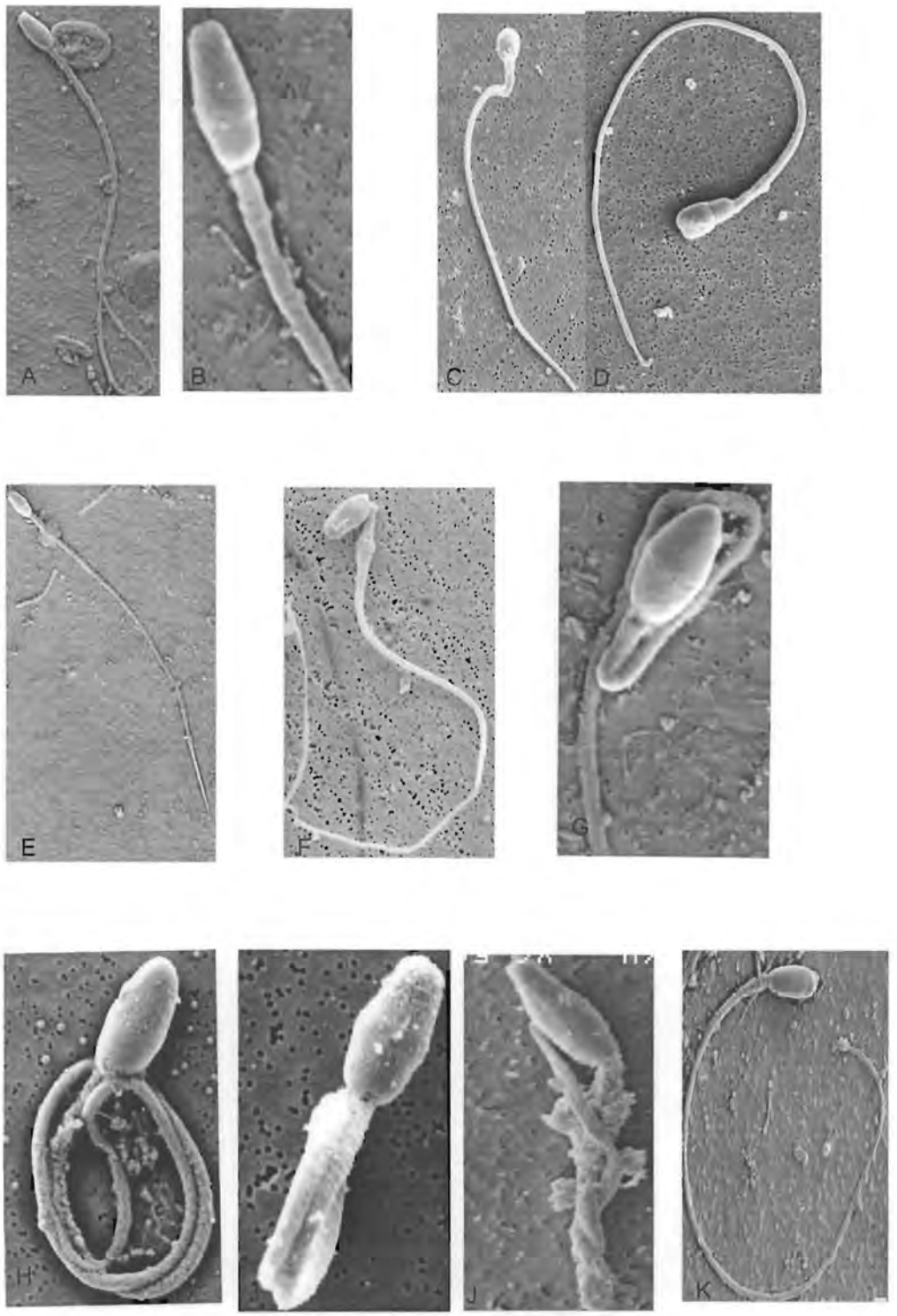


Plate 5: Scanning electron microscopy (Jeol 840) of aspirated epididymal spermatozoa in *C.h.hottentotus* showing the normal morphology and the types of abnormal spermatozoa prevalent in the tubules, (a) normal, (b) head region, (c) pin head, (d) macro cephalic head, (e) distal cytoplasmic droplet, (f) broken midpiece, (g) bent midpiece, (h-i) coiled tails, (j) folded tail and (k) pine cone shaped tail tip.

Comparative endocrinology:

Plasma testosterone concentrations:

There were no significant differences in the concentrations of circulating plasma testosterone secreted during the four different regimes (Fig. 11). Photoperiod was not found to have a significant effect on the amount of plasma testosterone secreted (GLM: $F = 0.33$, $P = 0.569$, Table 10). Similarly, temperature had no effect on testosterone secretion (GLM: $F = 3.18$, $P = 0.084$, Table 10). Predictably, there was no interaction between the two variables (GLM: $F = 0$, $P = 0.991$, Table 10).

Table 10: Testosterone profiles during different combinations of photoperiod and temperature.

Variable	F-value	P>f
Photoperiod	0.33	0.569
Temperature	3.18	0.084
Interaction P & T	0	0.991

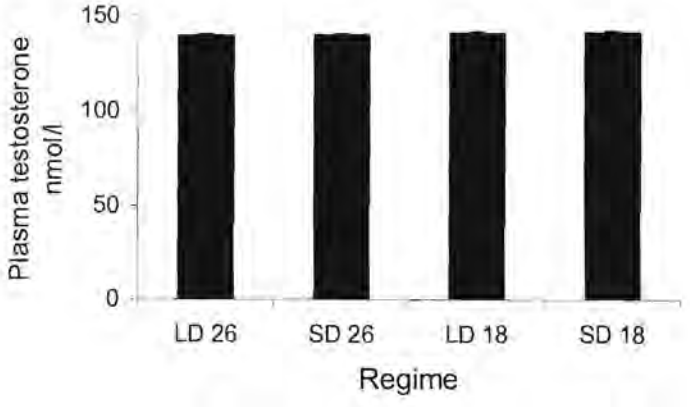


Fig 11: Plasma testosterone titres for *C.h.hottentotus* under the different light and temperature regimes (n = 34).

Urinary melatonin concentrations:

Temperature had a significant effect on the concentrations of urinary melatonin during the light period (GLM: $F = 5.08$, $P = 0.03$), but had no effect on the concentration during the dark (GLM: $F = 0.83$, $P = 0.367$). Photoperiod did not affect the urinary melatonin concentration (GLM: $F = 2.28$, $P = 0.139$) and there was no significant interaction between the two parameters (GLM: $F = 3.39$, $P = 0.073$).

During LD 26°C (simulated summer) melatonin concentration rose steadily from early afternoon T15 with a single peak during the subjective dawn at T9. There was no significant difference in the amount of urinary melatonin concentration at T15 vs. T21 (GLM: $F = 0.63$, $P = 0.446$), T15 vs. T3 (GLM: $F = 3.03$, $P = 0.112$). There was a significant difference in urinary melatonin concentration at T15 and T9 (GLM: $F = 5.4$, $P = 0.042$). There was no significant difference in concentration between T21 vs. T9 (GLM: $F = 4.04$, $P = 0.072$) or at T3 vs. T9 (GLM: $F = 3.47$, $P = 0.092$, Fig 12a).

During SD 26°C (simulated autumn) there was a clear diurnal rhythm in urinary melatonin concentration with secretion rising from mid morning early afternoon and peaking at T3 and stabilizing through to T9. There was no significant difference in the amount of urinary melatonin during the day and early evening T15 vs. T21 (GLM: $F = 0.1$, $P = 0.996$). There was no significant difference in secretion at night T3 vs. T9 (GLM: $F = 0.02$, $P = 0.893$). There was, however, a significant difference in melatonin concentration at T15 vs. T3 (GLM: $F = 5.86$, $P = 0.033$) and at T15 vs. T9 (GLM: $F = 5.68$, $P = 0.036$). There was also a difference in urinary melatonin concentration at T21 vs. T3 (GLM: $F = 5.86$, $P = 0.0333$) and at T21 vs. T9 (GLM: $F = 10.5$, $P = 0.0079$, Fig 12b).

During SD 18°C (winter) there is no clear rhythm in melatonin secretion, with urinary melatonin concentrations being similar over the four sampling time points. There was a slight increase at T21 with stability occurring through to T3 and T9; T15 vs. T21 (GLM: $F = 0.08$, $P = 0.793$); T15 vs. T3 (GLM: $F = 0.06$, $P = 0.823$) and T15 vs. T9 (GLM: $F = 0.24$, $P = 0.987$). There was no significant difference in concentration at night T21 vs. T3 (GLM: $F = 11$, $P = 0.823$) and T3 vs. T9 (GLM: F 0.12, $P = 0.756$, Fig 12c).

During LD18°C (simulated spring) melatonin concentration started to rise at T21 and remained elevated through to T3 and T9.There was no significant difference in the amount of urinary melatonin during T15 vs. T 21 (GLM: $F = 0.94$, $P = 0.349$) and T21 vs. T3 (GLM: $F = 3.66$, $P = 0.078$), T21 vs. T9 (GLM: $F = 0.8$, $P = 0.385$). There were significant differences in secretion at T15 vs. T3 (GLM: $F = 9.04$, $P = 0.01$) and T15 vs. T9 (GLM: $F = 6.49$, $P = 0.024$, Fig 12d).

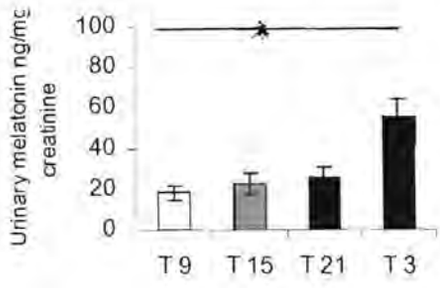


Fig 12(a) simulated summer

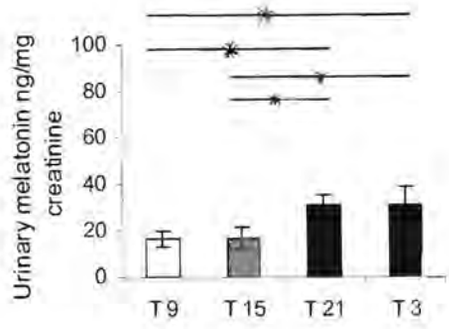


Fig 12(b) simulated autumn

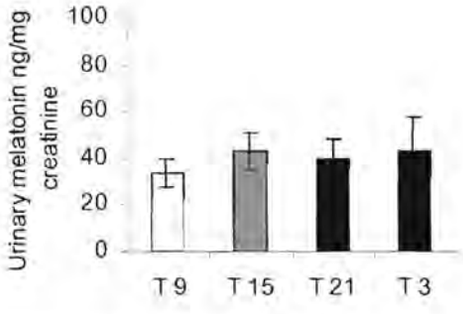


Fig 12(c) simulated winter

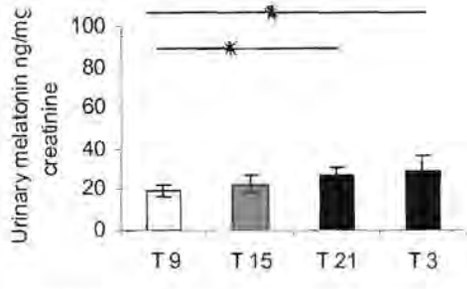


Fig 12(d) simulated spring

Figure 12(a-d): Melatonin titrers of male common mole-rats kept under the different simulated seasons (ng/mg creatinine, * = significant $p<0.05$).

The highveld mole-rat, *Cryptomys hottentotus pretoriae*:

Comparative histology and anatomy:

The body masses of the male highveld mole-rats did not differ significantly between the different capture periods. All animals exhibited similar mass when they were caught, signifying that they were neither affected by daylength (GLM: F = 0.0, P = 0.968), or by temperature changes (GLM: F = 0.07, P = 0.806, Fig 13). There was no significant interaction between the two parameters (GLM: F = 0.01, P = 0.929, Table 11).

The mean relative testis mass expressed as a function of body mass was not significantly affected by daylength (GLM: F = 0.04, P = 0.834, Fig 14). However, it was significantly affected by temperature, with the relative testicular mass of the animals under low temperatures increasing and those under high temperatures decreasing (GLM: F = 17.42, P = 0.0003, Fig 14, Table 11). There was no significant interaction between the two parameters (GLM: F = 0.15, P = 0.702, Table 11). The seminiferous tubule areas were not significantly affected by temperature (F = 3.634, P = 0.069), but by photoperiod when the days are long the tubule areas decrease with a concominant increase when the days become short (F = 11.994, P = 0.002). There was however a significant interaction between the two variables this indicates a transition from long days high temperatures to low temperatures (F = 5.848, P = 0.02). The epididymal area was not significantly affected by temperature (F = 0.1962, P = 0.661), but by photoperiod with an increase in epididymal area when the days are short and a decrease when days are long (F = 37.71, P = 0.00000062). There was no significant interaction between the two variables (F = 0.781, P = 0.385) (Plate 6).

Neither temperature nor light had any appreciable effects on testicular volume (GLM: F = 1.27, P = 0.2787, F = 2.20, P = 0.162). The combination of both variables was also not significantly different (GLM: F = 0.97, P = 0.3414, Table 12).

Table 11: Body and testis mass for *C.h.pretoriae* during the different temperature and light regimes (* = significant and Ns = not significant).

	Body mass			Testis mass		
	F-value	P > F	Comment	F-value	P > F	Comment
Temperature	0	0.968	Ns	17.42	0.0003	*
Photoperiod	0.07	0.8	Ns	0.04	0.834	Ns
Combination T & P	0.01	0.929	Ns	0.15	0.702	Ns

Table 12: Comparative testicular anatomy of *C.h.pretoriae* (n = 18) during the different light and temperature regimes (* = significant and Ns = not significant).

Variable	F-value	Pr > F	Comment
Photoperiod	1.27	0.2787	Ns
Temperature	2.20	0.1602	Ns
Interaction // T & P	0.97	0.3414	Ns

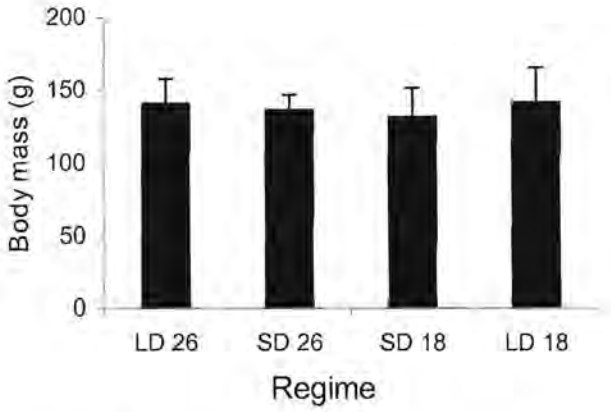


Fig 13: Comparative body masses for *C.h.pretoriae* during the different regimes (X ± SD).

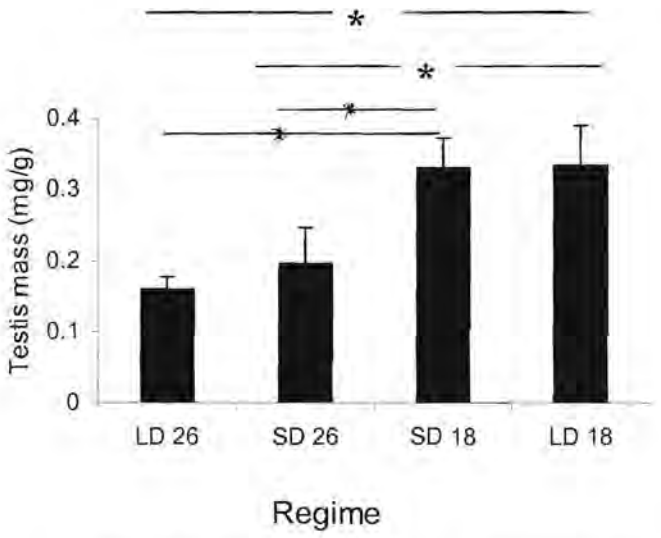


Fig 14: Comparative testis masses for *C.h.pretoriae* during the different regimes (X ± SD, * =significant o<0.05)

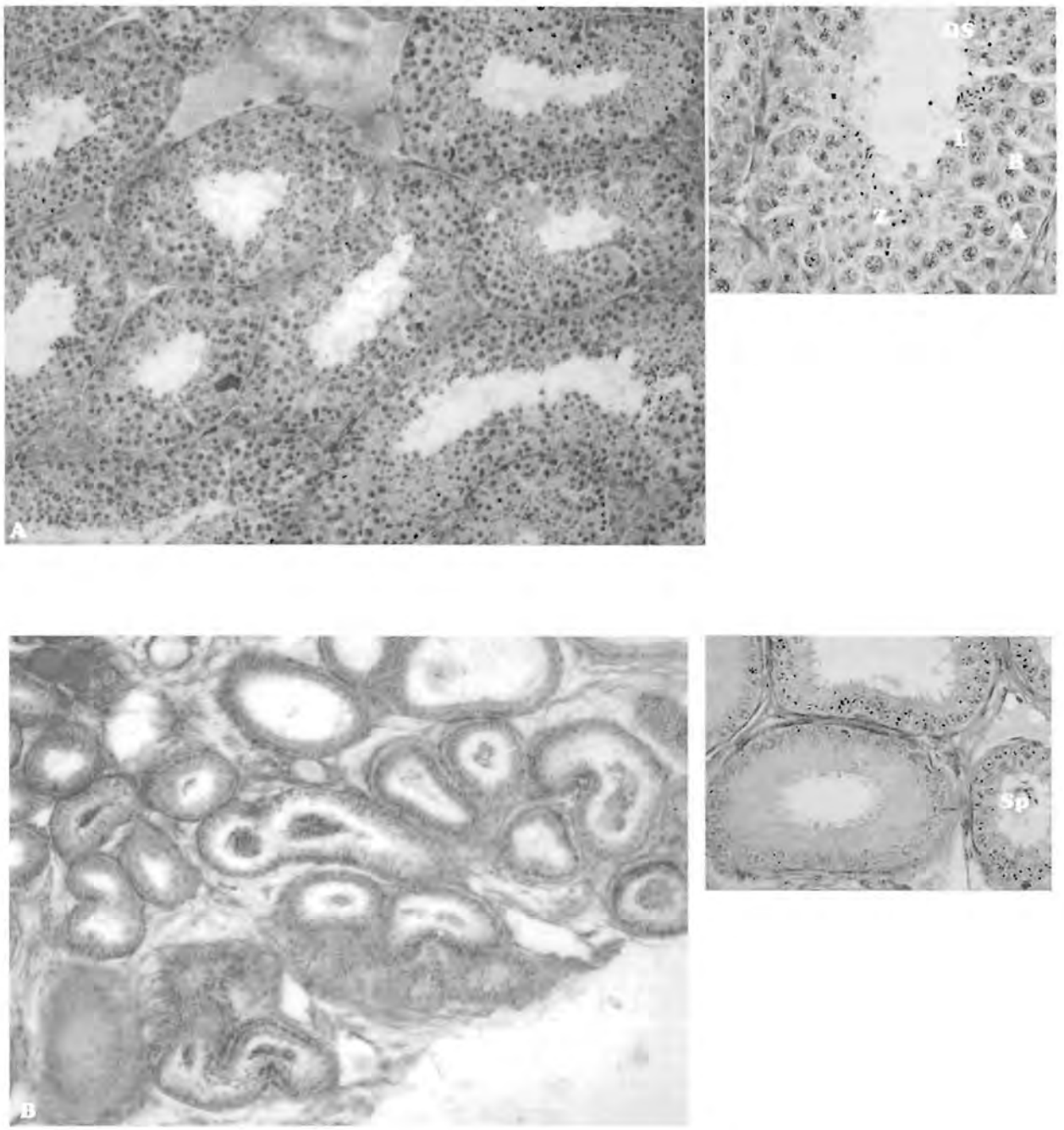
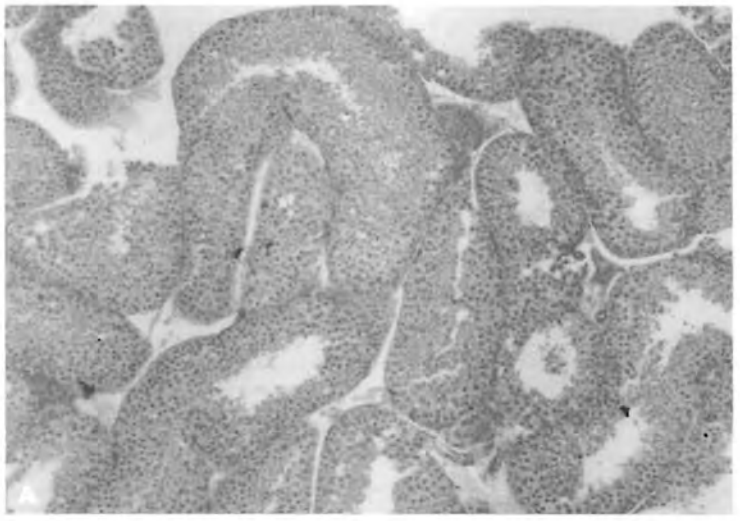
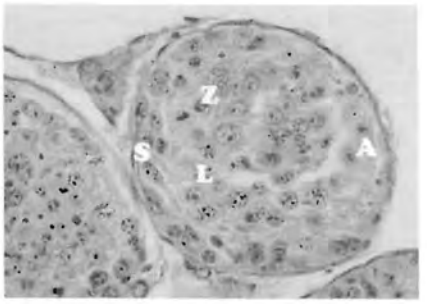
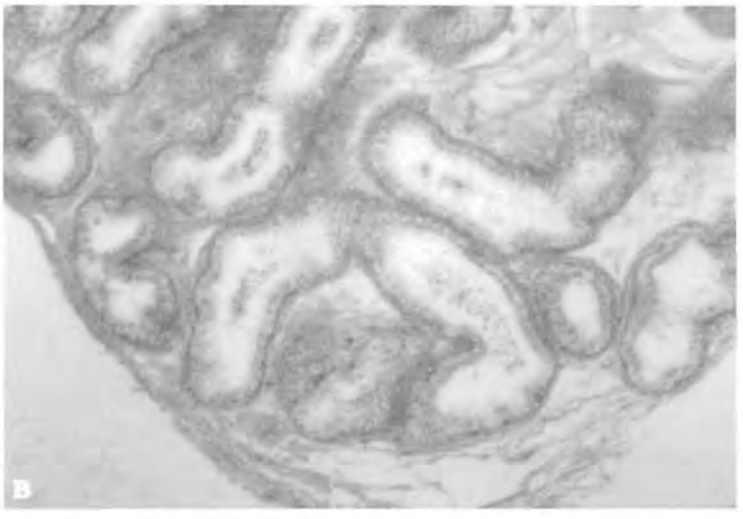


Plate 6(a) simulated summer LD 26°C

Plate 6(a-d): Transverse sections through the (A) seminiferous tubules and (B) epididyme of *C.h.pretoriae* using phase contrast light microscopy x10 under the four regimes, (LD:HT, SD:HT, SD:LT, LD:LT) showing active spermatogenesis. At higher mag x20 showing most germ cell types present, (S) Sertoli cells, Type A/B spermatogonia, (L) leptotene, (Z) zygotene, (P) Pachytene, (DS) differentiating spermatids and (Sp) spermatozoa.







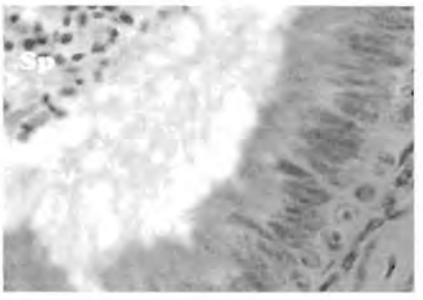


Plate 6(b) simulated autumn SD 26°C

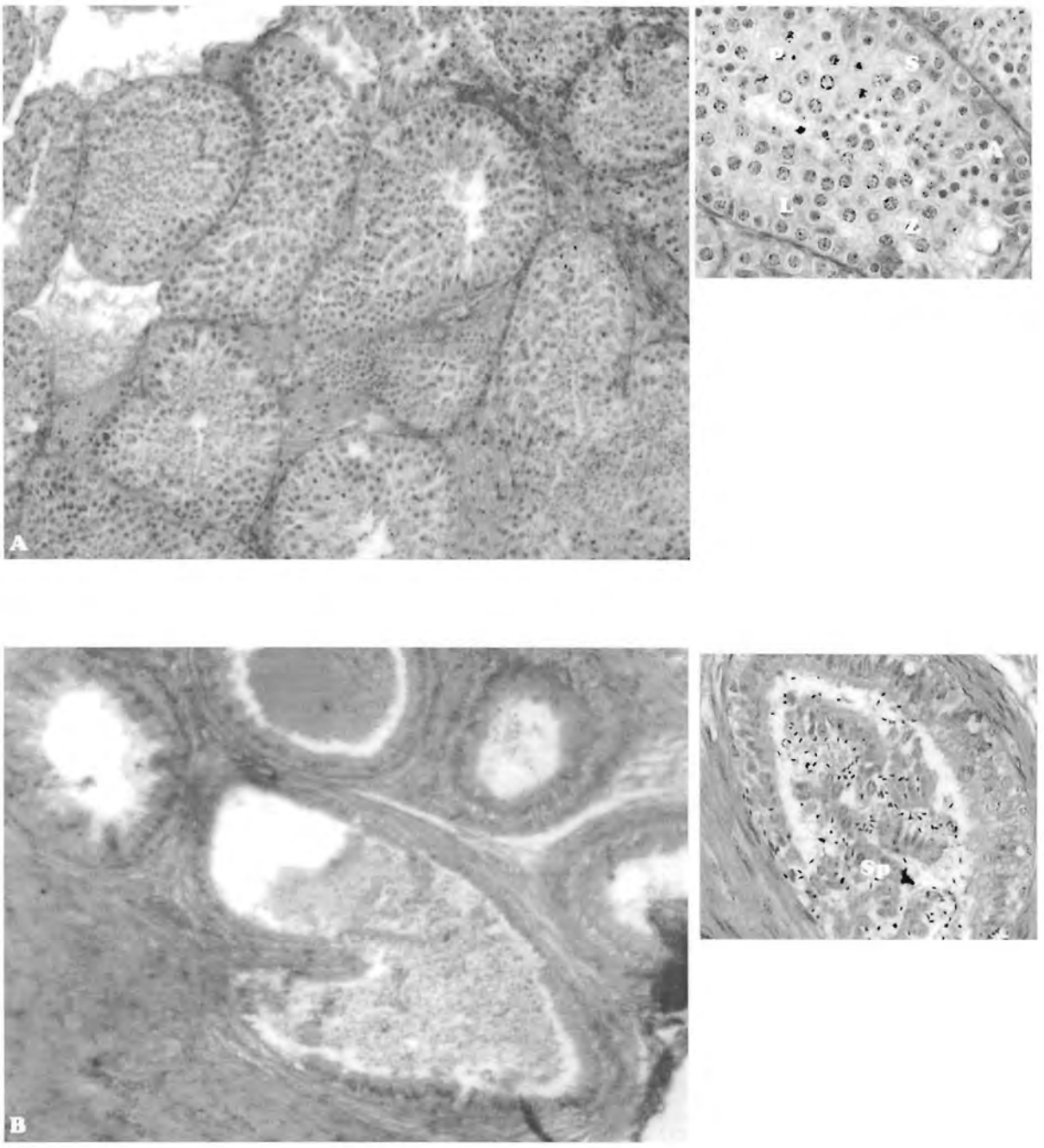


Plate 6(c) simulated winter SD 18°C

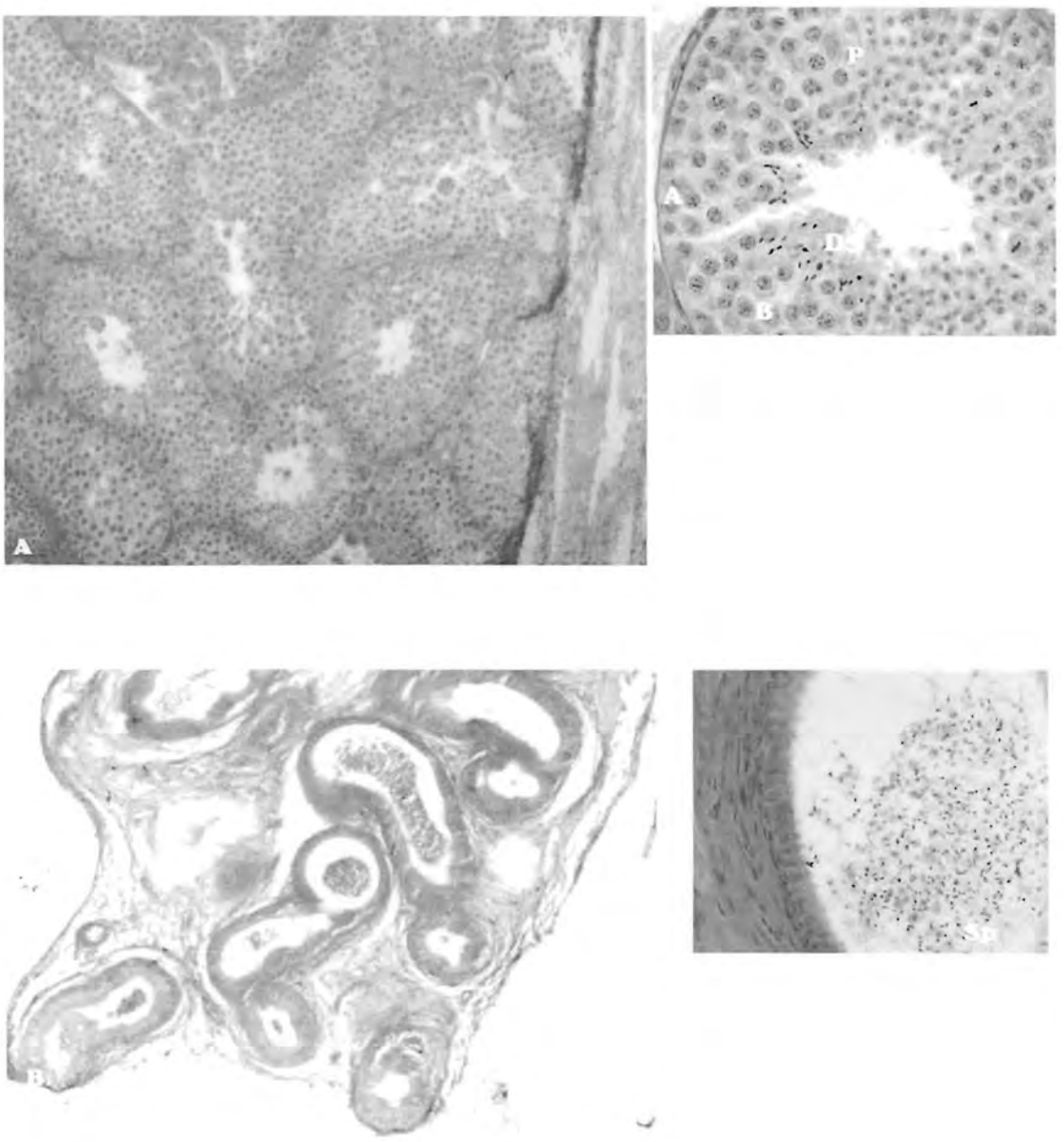


Plate 6(d) simulated spring LD 18°C

Comparative sperm motility and vitality parameters:

Overall there was no significant difference in the sperm motility parameters during the different regimes (GLM: F = 1.07, P = 0.302). However when the sperm parameters were looked at in an individual context, most of the motility parameters were affected by a reduction in ambient temperature (Table 13). During high ambient temperatures very few of the parameters were affected by the length of the day. The most important parameter straight-line velocity was not affected by either temperature or photoperiod (GLM: F = 1.01, P = 0.315 and GLM: F = 0.02, P = 0.891, respectively, Table 13).

A comparison of live to dead spermatozoa revealed that there was an increase in the percentage of live spermatozoa from autumn peaking in the winter. A gradual decline in the percentage of live sperm occurred in simulated spring with the lowest numbers in summer (Fig 15). When the effect of potential season was investigated with respect to the types of abnormalities found during the different regimes an increase in the number of sperm precursors and a reduction in other abnormalities occurred with the onset of the simulated summer period (Fig 16a).

During simulated autumn sperm precursors are reduced, whereas other sperm abnormalities showed an increase (Fig 16b). In winter a higher percentage of sperm with tail and neck defects and increased numbers of sperm precursors are found indicative of a lowering of sperm motility (Fig 16c). However, in spring when considering ambient temperature there is an equal distribution of the sperm abnormalities (Fig 16d). The types of spermatozoa abnormalities were uniform across all seasons with sperm precursors found in all samples (Plates 7 & 8).

Table 13: Comparative sperm motility characteristics for *C.h.pretoriae* during different combinations of temperature and photoperiod (* = significant).

Variable	Temperature		Photoperiod	
	F-value	P > fr	F-value	P > fr
VCL	44.82	0.0001*	3.54	0.061
VSL	1.01	0.315	0.02	0.891
LIN	4.37	0.037*	0.39	0.534
MnALH	24.92	0.0001*	0.01	0.943
MxALH	24.87	0.0001*	1.49	0.229
BCF	6.4	0.012*	3.92	0.04*
DNC	18.04	0.0001*	0.01	0.907
DNCmn	38.25	0.0001*	16.01	0.0001*
VAP	3.03	0.083	0	1
WOB	4.49	0.035*	0.78	0.378
STR	0.01	0.934	0	0.97
RAD	0.08	0.773	0.34	0.563
CURV	2.05	0.154	0.35	0.555

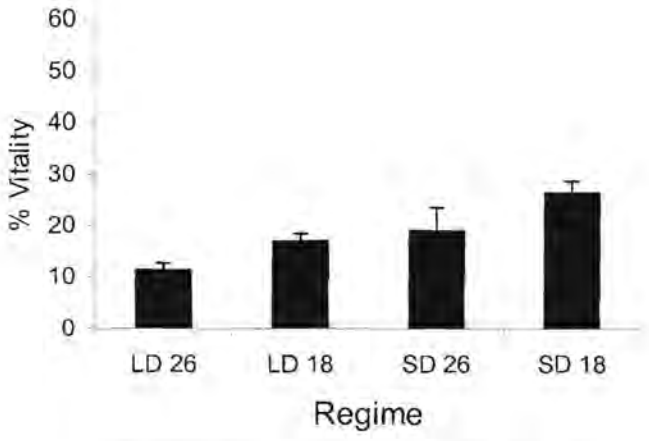


Fig 15: Comparative vitality for *C.h.pretoriae* during different lighting and temperature regimes (X ± SD).

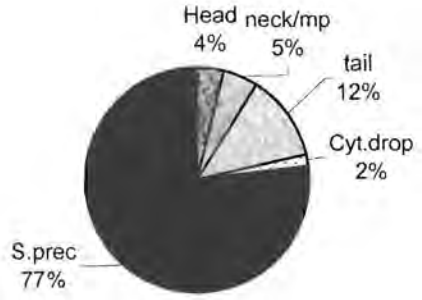


Fig 16(a) simulated summer

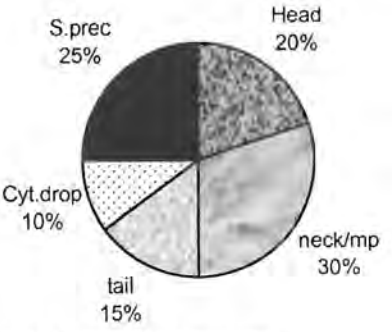


Fig 16(b) simulated autumn

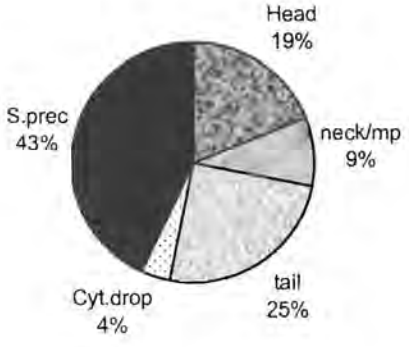


Fig 16(c) simulated winter

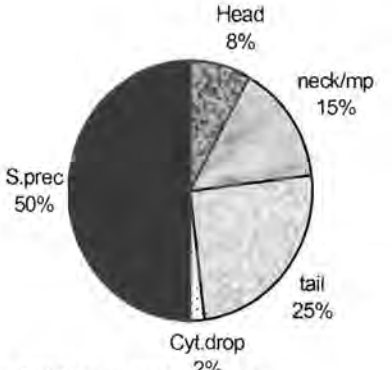


Fig 16(d) simulated spring

Figure 16(a-d): Percentage of abnormal spermatozoa types found in the epididyme of *C.h.pretoriae* during simulated summer, autumn, winter and spring.

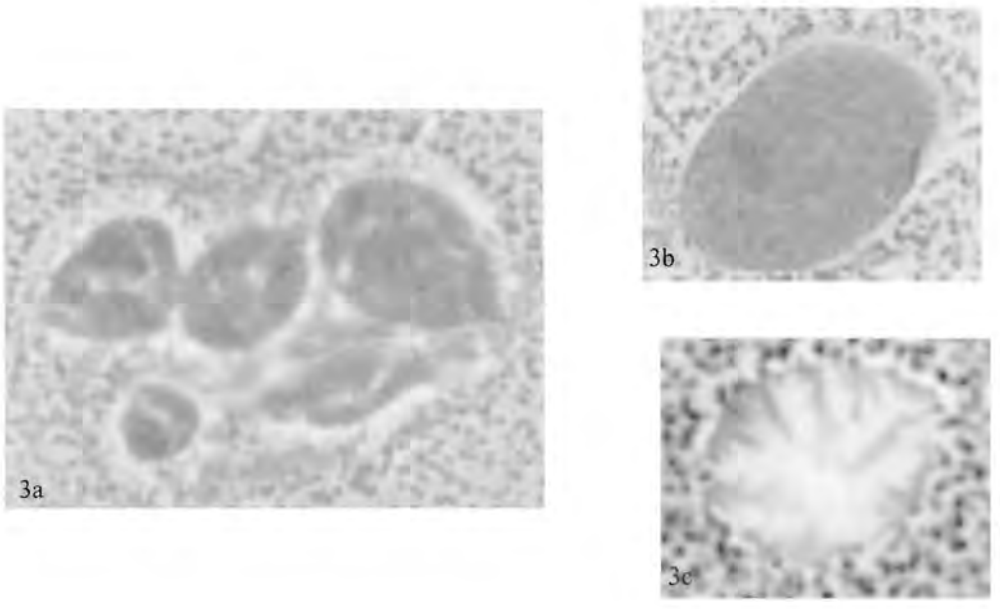


Plate 7: Photomicrographs of the different types of sperm precursors found in the epididymal aspirate in *C.h.pretoriae* under light microscopy x20, 3(a) giant cells, 3(b) type a spermatogonia and 3(c) zygotene stage.

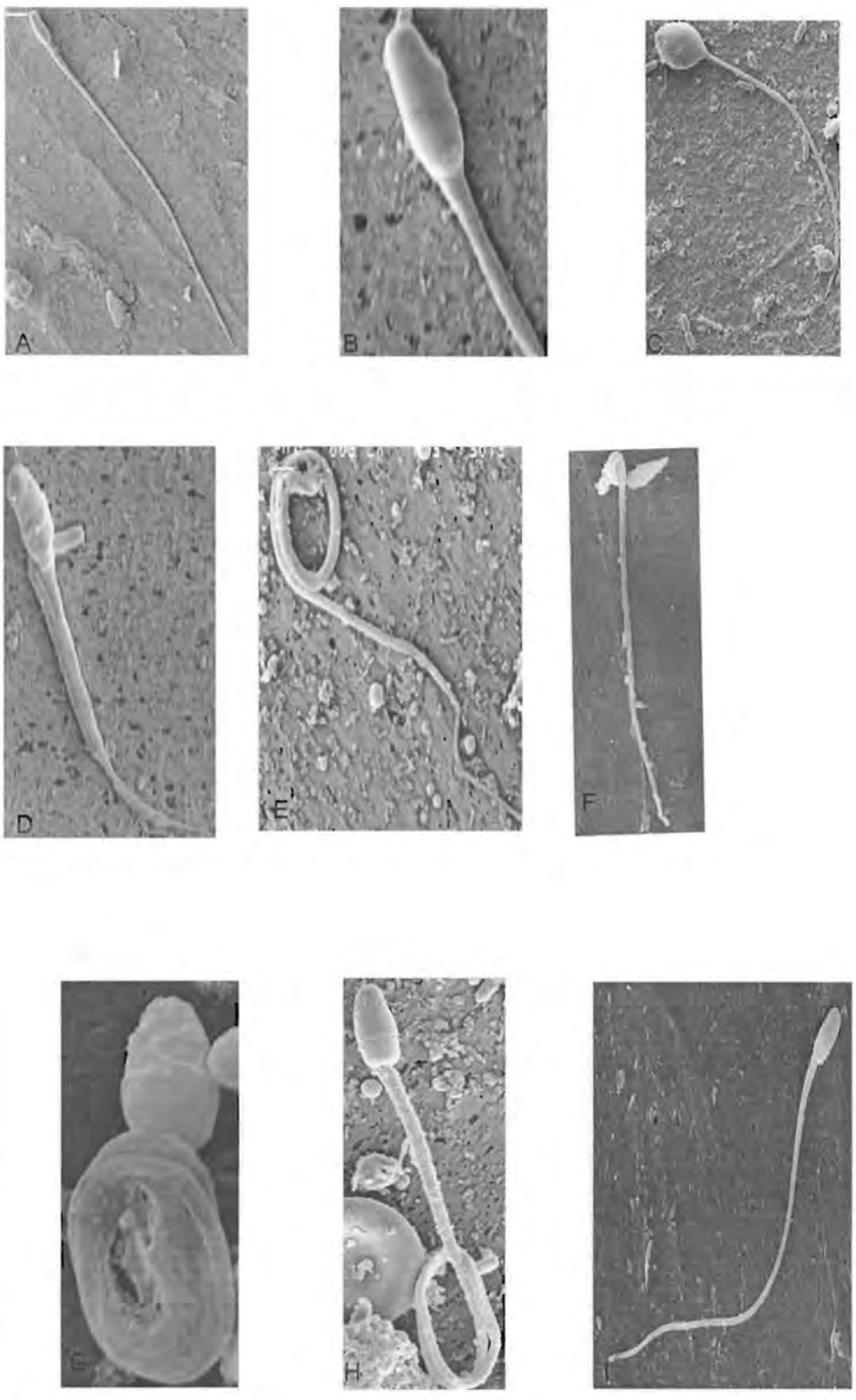


Plate 8: Scanning electron microscopy (Jeol 840) of aspirated epididymal spermatozoa in *C.h.pretoriae* showing the normal morphology and the types of abnormal spermatozoa prevalent in the tubules, (a) normal, (b) head region, (c) macrocephalic head, (d) bent principal piece, (e) headless, (f) bent midpiece, (g-h) coiled tails.

Comparative endocrinology:

Plasma testosterone concentrations:

There were no significant differences in the amount of plasma testosterone secreted over the four simulated seasons. Photoperiod did not have any effect on testosterone concentration (GLM: F = 0.32, P = 0.575, fig 17). Likewise temperature had no effect on circulating testosterone concentration (GLM: F = 0.05, P = 0.828). Not surprisingly there was no interaction between the two parameters (GLM: F =1.6, P = 0.217, Table 14).

Table 14: Testosterone profiles during different combinations of photoperiod and temperature.

Variable	F-value	P>f
Photoperiod	0.32	0.575
Temperature	0.05	0.828
Interaction P & T	1.6	0.217

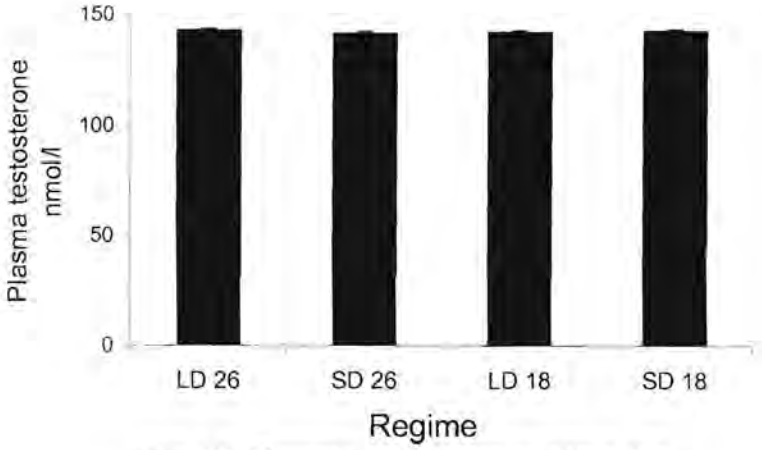


Fig 17: Plasma testosterone titres for *C.h.pretoriae* during different light and temperature regimes (n = 30).

Urinary melatonin concentrations:

Melatonin secretion was not affected by either temperature (GLM: F = 0.34, P = 0.561), or photoperiod (GLM: F = 0.52, P = 0.475). There was no significant interaction between the two parameters (GLM: F = 0.43, P = 0.512).

During LD 26°C (simulated summer) there was a clear diurnal rhythm of urinary melatonin concentration with secretion rising from daytime levels and peaking at T3 through to T9. There is a significant difference between daytime and nighttime concentrations of urinary melatonin, T15 vs. T3 (GLM: F = 9.15, P = 0.01), T15 vs. T9 (GLM: F = 4.68, P = 0.014).

There was a significant difference in the amount of melatonin secreted during T21 vs. T3 (GLM: F = 5.8, P = 0.033) and at T21 vs. T9 (GLM: F = 6.58, P = 0.0289). There was no significant difference in secretion between T15 and T21 (GLM: F = 0.36, P = 0.557). Likewise, there was no significant difference in the urinary melatonin concentrations produced at night and subjective dawn T 3 vs. T9 (GLM: F = 0.06, P = 0.817, Fig 18a).

During SD 26°C (simulated autumn) urinary melatonin concentration rose steadily from T 15 through T21 and peaked at T3, with a decline at T9. There was no significant difference in the urinary melatonin concentrations during T15 vs. T21 (GLM: F = 0.15, P = 0.703) or between T3 vs. T9 (GLM: F = 2.61, P = 0.125). There was a significant difference in secretion at T15 vs. T3 (GLM: F = 6.71, P = 0.019) and T21 vs. T3 (GLM: F = 3.49, P = 0.05, Fig 18b).

During SD18°C (simulated winter) urinary melatonin concentration rose steadily from day to night with a peak at T3 and a slight lowering in concentration at T9. There was no significant difference in the concentration of melatonin during the day T15 vs. T21 (GLM: F = 3.15, P = 0.103) and no difference at night T3 vs. T9 (GLM: F = 0.04, P = 0.847). There was a significant difference in the concentration of melatonin between the day and night T15 vs. T3 (GLM: F = 7.12, P = 0.021) and T15 vs. T9 (GLM: F = 6.16, P = 0.03, Fig 18c).

During LD18°C (simulated spring) there was a slight increase in the concentration of melatonin from T15 through to T3, with a marginally significant difference in concentration during the day and night. There was no significant difference in urinary melatonin concentration at T 15 vs. T21 (GLM: $F = 1.77$, $P = 0.202$), T21 vs. T3 (GLM: $F = 1.64$, $P = 0.22$), T21 vs. T9 (GLM: $F = 1.2$, $P = 0.291$) or at T3 vs. T9 (GLM: $F = 0.02$, $P = 0.879$). There was a slight difference in the concentration of urinary melatonin during T15 and T3 (GLM: $F = 3.83$, $P = 0.057$, Fig 18d).

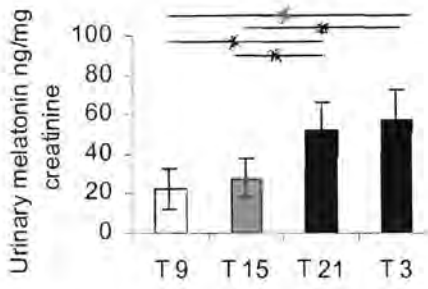


Fig 18(a) simulated summer

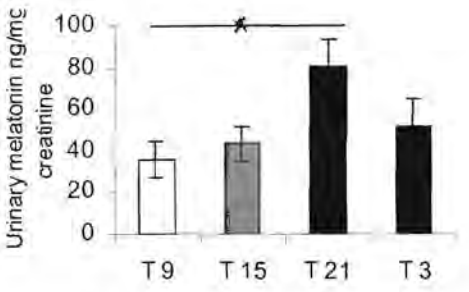


Fig 18(b) simulated autumn

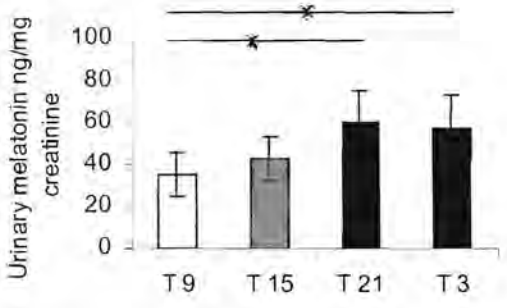


Fig 18(c) simulated winter

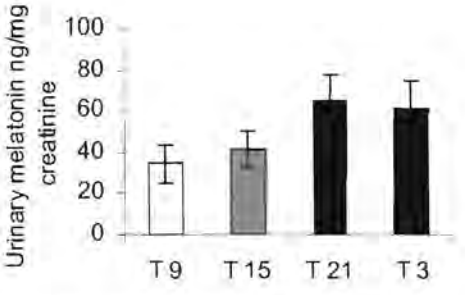


Fig 18(d) simulated spring

Figure 18(a-d): Melatonin titres of male highveld mole-rats kept under the different simulated seasons (ng/mg creatinine, * = significant $p<0.05$).

Chapter 4

Discussion

The environment is an important determinant of the behavioural and physiological traits of an animal. Environmental forces that act upon reproduction operate at two levels, the ultimate and proximate level. Ultimate factors are important in the long-term evolutionary sense whilst proximate factors provide immediate cuing for the onset and cessation of reproductive activity (Lofts, 1970). Rainfall and temperature determine the amount of resources available, which in turn impacts directly on reproductive function (Lofts, 1970). Reproduction is important to the biology of all organisms and is the means by which an individual perpetuates copies of their genes (Bennett and Faulkes, 2000).

Subterranean mammals inhabit an environment that is normally devoid of large environmental variations and are therefore deprived of the cues that are important in above ground mammals to time life history events to the optimum (Bennett and Faulkes, 2000). Although most mole-rat species inhabit seasonal habitats their reproduction is not restricted to a particular time of the year (Spinks *et al.*, 1997, 1999, Janse van Rensburg *et al.*, 2002). Most phyletic lineages of subterranean rodents have undergone convergent evolution with regard to environmentally related selection pressures, resulting in species choosing the type of cue/cues that are most important for their particular habitat that they inhabit (Nevo, 1979). In some subterranean habitats where regulating reproduction is advantageous, photoperiodic signal might be inappropriate or deficient (Heideman and Bronson, 1994).

Only two types of environmental cues have been implicated in the control of seasonal cycles in subterranean mammals via the interaction with some type of internal time keeping mechanism, these are photoperiod and ambient temperature (Goldman, 1993). While temperature may have a very direct and obvious relationship to certain types of seasonal adaptations, e.g. pelage, choice of time to reproduce, etc, photoperiod would appear to have little direct importance for most organisms (Wehr, 1991, Bittman *et al.*, 1983). However, photoperiod is a more reliable source of cue in anticipating future environmental conditions compared to temperature, which can fluctuate very steeply from year to year (Goldman, 1993). In many regions harsh climatic conditions prevail on a seasonal basis and as a consequence animals restrict reproduction to the favourable periods that are more likely to be optimal for maximizing reproductive success.

The ideal method of evaluating the fertility of a sexually active male, other than the ability to produce pregnancy, is to examine the semen quality. Semen from most males contains a proportion of abnormally formed sperm; usually this is not associated with lower fertility rates until the proportion of abnormal sperm exceeds 20%. Sperm motility is essential for fertility, but not necessarily an indicator of fertilising capacity (Hafez, 1987). The evaluation of sperm cell motility and morphology are essential parameters in the examination of sperm quality and in the establishment of correlations between sperm quality and fertility (Verstegen *et al.*, 2002).

General findings in light of the reproductive biology of the three species

The Damaraland mole-rat is an aseasonal breeder. The reproductive female within the colony has the capacity to have and rear three litters of offspring per annum (Bennett and Jarvis, 1988; Bennett *et al.*, 1991, Bennett and Faulkes, 2000). Litters can range from 1 to 6 pups. Female Damaraland mole-rats have been shown to increase body size and sensitivity to GnRH rapidly after good rains in anticipation of dispersal. These dispersing morphs have mean body sizes that are larger than the population mean (Molteno, 1999). It has been shown that reproductive males produce by far a greater number of sperm than their non-reproductive counterparts (Maswanganye *et al.*, 1999). This species was used as a form of control baseline species with which to monitor a number of morphological, histological and spermatological variables in comparison with those from two phylogenetically closely related sister taxa that exhibit marked seasonal breeding.
In this study the mole rats caught during summer exhibited larger body masses and the winter group had lower body masses. This might be an indication of availability of food resources during particular seasons with the mole-rats adjusting their physiology to suite the environmental conditions.

Interestingly, the mean relative testes mass of male Damaraland mole-rats was shown to be influenced by temperature, but not the photoperiod, low temperatures resulting in an increased testicular mass.

Testis volume is also affected by temperature with an increase in volume when temperatures are high and a concomitant reduction when the temperatures are low. Testis volume and mass may vary concomitantly or in opposition. An increase in testicular mass during low temperature necessitates an increase in volume for compensation with no overall effect on testicular function. . Mean seminiferous tubule and epididyme areas did not, however, appear to be affected by the different photo and thermoperiods presented. As would be expected the sperm motility parameters and sperm vitality of the mole-rats housed under the four lighting regimes failed to show a seasonality. But when the parameters were considered individually, temperature did appear to have some role on some motility parameters, but not the most important parameter, namely straight line velocity. Temperature again was found to have some effect on both the numbers and abnormalities of sperm. On those regimes with low temperatures (LD LT and SD LT) there were fewer abnormalities and enhanced sperm production that correlates nicely with the anatomical finding of a recrudescence of testicular mass.

The common mole-rat, *Cryptomys hottentotus hottentotus*, exhibits marked seasonal activity producing young during the austral spring and summer (October – January). The common mole-rat has the reproductive capacity to produce two litters per annum with litters varying from 1-6 pups with a mean of 3 (Bennett, 1989; Bennett *et al.*, 1991; Bennett and Faulkes, 2000). Spinks *et al.*, (1997) found that there was no apparent manifestation of season on testicular activity, spermatogenesis and sperm quality (motility and percentage normal morphology) in the reproductively active and inactive periods. Interestingly, from this study, the body masses of males caught during summer were greater than males caught in winter. The testicular mass and volume were not significantly affected by changes in temperature, but were affected by photoperiodic changes. The seminiferous tubule area remained unaffected by both variables. In contrast, the epididymal areas were enhanced by photoperiodic changes with the area increasing when the days are short and decreasing during long days.

Given that the gestation period of *C. h. hottentotus* is 59-66 days (approximately two months), if this is considered in light of the time when the young are born, it tends to suggest that female common mole-rats are inseminated around June-July (shortest days in the Cape Province). This finding intimates that photoperiod may potentially be important for timing reproduction in the common mole-rat.

The highveld mole-rat, *Cryptomys hottentotus pretoriae*, also exhibits a marked seasonal activity producing young during the austral spring and early summer (July-November). The highveld mole-rat can produce two litters per annum varying from 1-3 pups with an average of 2 (Janse van Rensburg *et al.*, 2002; Malherbe *et al.*, in press). Janse van Rensburg *et al.*, (in press) found that there was no apparent difference in sperm motility between reproductive and non-reproductive colony members throughout the year. The relative mean testis mass appears to be affected by temperature, testis mass increasing on a low temperature. Seminiferous tubule and epididymal cross sectional area interestingly was affected by a reduction in daylength. Sperm motility was similarly affected by a reduction in ambient temperature. There was an increase in the vitality of the spermatozoa brought about by a lowering of the ambient temperature. Interestingly, sperm numbers were greatest in the winter and declined towards the summer. Again, assuming a gestation period of 66 days (two months), if this is considered in light of the time when the young are born, it tends to suggest that female highveld mole-rats are inseminated around May to June (the shortest days and lowest ambient temperatures in the highveld of South Africa. Again increased testicular mass, seminiferous tubule diameter and associated production of spermatozoa appeared to be triggered by a reduced ambient temperature and photoperiod. The two variables temperature and photoperiod interact to affect reproductive function and the GnRH neuronal system in Prairie voles. The voles housed under short days and low temperature exhibit a regressed reproductive system. This suggests that voles in their natural environment not only evaluate the costs of maintaining reproduction based upon photoperiodic information but also several proximate factors to weigh the cost plus benefits of maintaining or inhibiting reproduction during winter (Kriegsfield *et al.*, 2000).

In the present study one pattern that appears to stand out is that of sustained sperm production throughout the year with an absence of testicular regression during the non-reproductive period. In seasonally breeding mammals in which cyclicity of male reproductive characteristics has been investigated, testicular regression and an associated reduction in sperm numbers is the norm (Gottreich, *et al.*, 1995, 2000). The observed pattern of reproductive cyclicity is not reflected in testicular activity. The maintenance of reproductive activity outside of the breeding season is uncommon amongst seasonally breeding mammals. This is in marked contrast to the solitary, seasonal breeding Cape dune mole-rat (*Bathyergus suillus*) and the Cape mole-rat (*Georychus capensis*) that both exhibit a marked seasonality in male reproductive characteristics (Van der Horst, 1972; Bennett and Jarvis, 1988). In both species a cessation of spermatogenesis and testicular regression occur during the non-active period.

There were no significant differences found in the plasma testosterone concentrations of the males of the three social species maintained on the four simulated seasons. However Janse van Rensburg, (2000) noted two peaks in testosterone concentration as well as a gradual increase in testicular mass and volume towards and during the breeding season, which reaches a peak within the month of July. The second peak coincides with the peak reproductive month of the females. In my study there were no differences in testosterone secretion since there is no aggression and competition between males for mating rights. This would be predicted in animals that lack a reproductive cessation of both gonadal size and activity (Maswanganye *et al.*, 1999; Spinks *et al.*, 1999; Janse van Rensburg *et al.*, 2002). This is in marked contrast to the findings found in the solitary seasonally breeding male Cape blesmol, *G. capensis* and Namaqua dune mole-rat, *B. janetta* where the testosterone concentrations are highest in the breeding season and lowest or non-detectable out of the breeding phase (Bennett and Jarvis, 1988; Herbst, 2002).

Sperm motility parameters

There is variability in the yearly onset and offset of the breeding season in nature. In most mammals that are seasonal, the non-reproductive period is characterized by testicular regression and cessation of spermatogenesis with recrudescence occurring during the reproductive season. Seasonal differences in testicular, anatomical and histological features did not reflect changes in spermatogenic activity and sperm motility in all three species. For example in some species like the rock elephant shrew, *Elephantus myurus,* there is no apparent manifestation of season. They show active spermatogenesis all year round, but have significant changes in testis and prostate size, proportion of interstitial cells from April – June. These changes are not associated with changes in body mass (Woodwall *et al.*, 1989).

The male Damaraland mole-rats exhibited no significant differences in sperm motility under the different regimes. With equal amounts of sperm showing progressive motility. A few of the parameters were affected by temperature but not photoperiod. VSL was not affected by either temperature or photoperiod. The common mole-rat exhibits no apparent manifestation of season on testicular activity. Spinks *et al.*, (1997), have shown that in the reproductive males, spermatogenesis and sperm quality (motility, vitality and normal morphology) were similar in the reproductively active and inactive periods. Although there were no differences in the motility between the different seasons, straight-line velocity (VSL), VAP and LIN were affected by changes in temperature with an increase in the forward progression of the spermatozoa.

Male Highveld mole-rat displayed no differences in motility between different seasons. A few of the parameters were affected by a reduction in temperature. Straight-line velocity and VAP were not affected by both parameters. Outbreeding opportunities maybe important determinants of reproductive activity moderating seasonal effects. The reproductive periodicity evident in the common mole-rat is typical of both subterranean and surface dwelling mammals inhabiting seasonal environments (Gottreich *et al.*, 1996).

Sperm abnormality and vitality

The presence of epididymal spermatozoa exhibiting abnormal morphology was characteristic for all three mole-rat species and during all simulated seasons. There was a high incidence of sperm with cytoplasmic droplets and sperm precursors. Spermatozoa exhibit a vast spectrum of abnormalities even within a single ejaculate. In most mammals, spermatozoa are not mature when released from the seminiferous tubules. Each contains a cytoplasmic droplet on its midpiece and must undergo maturation in the epididyme to gain progressive motility, structural stability and fertilizing ability (Johnson *et al.*, 1997, Axner *et al.*, 1999). For example some domestic cats and many species of wild felids have high proportions of abnormal spermatozoa in their ejaculates. In the domestic cat the proportion of sperm abnormalities originating in the testes decreases during epididymal transport, while some sperm tail abnormalities may actually originate in the epididymis (Axner *et al.*, 1999).

The number of viable sperm throughout the four seasons in the aseasonally breeding Damaraland mole-rat was not significantly different, again supporting the lack of seasonality in this rodent mole. A similar trend was found in the common mole-rat, However, in the highveld mole-rat the number of viable sperm was found to increase dramatically in males maintained on a short day and particularly when this was linked with an lower ambient temperature.

The proportions of abnormally formed spermatozoa found in all three species were not of importance since most of them could not reduce fertility. There is a very small percentage of primary and secondary deformities. Most deformities observed are of a tertiary nature indicating that the sperm might have been damaged during passage through the epididyme or through handling during sample preparation. Morphology of live spermatozoa correlates better with fertility potential of a male than with the overall sperm morphology of an ejaculate (Hafez, 1987).

The types of spermatogonia and spermatocytes found within the seminiferous tubules are indicative of whether spermatogenesis is occurring or not. Multi-nucleated giant cells are often seen in semen after temperature elevation or decreases, they are usually formed by a coalition of spermatocytes and spermatids, which are arrested at some stage of development within the seminiferous tubules (Courtens, 1999, Clermont Y 1962, Johnson *et al.*, 1997).

Melatonin secretion within simulated seasons

Subterranean mammals are active day and night; their activity patterns are linked with feeding periods. Activity patterns vary with individual, species, day, season and habitat (Nevo, 1979; Nevo *et al.*, 1982). Seasonal changes in melatonin secretion may not be as dramatic in laboratory-housed animals as compared to their outdoor counterparts. The laboratory-induced square wave photoperiods only approximate the natural photoperiods, the onset of darkness and light shifts albeit, slightly every day in the natural environment. In addition, light increases gradually at dawn and decreases gradually at dusk. Some animals use non-photic cues, whilst others use photic cues to time their reproduction (Bartness and Goldman, 1989, Wehr, 1997). In *Spalax ehrenbergi* the structural and molecular investigations of the atrophied, subcutaneous eye suggest a functional role of the retina in light perception. The entrainment of circadian locomotor and thermoregulatory rhythms by ambient light demonstrates the capacity for photoperiodic detection (Ben-Shlomo *et al.*, 1996). The photoperiodic system, sustaining appropriate reproductive and thermoregulatory responses have been selectively expanded whereas the acute metabolic burden of maintaining a large eye and non functional image forming visual system provides the underlying evolutionary impetus for their morphological regression. This represents one aspect of the unique range of adaptation that subterranean mammals have evolved to cope with environmental constraints imposed by their underground niche (Cooper *et al.* 1993). Melatonin secretion rises at night and lowers just before light onset (Ben-Shlomo *et al.*, 1996).

Circadian melatonin secretion can be entrained to photoperiod and can therefore be used as an index of season (Richter *et al.*, in press). Subterranean mammals inhabit an environment that is completely devoid of light and thus deprived of photoperiodic information. Richter *et al.* (in press) and Miethe *et al.* (submitted) have found that in both the Damaraland and highveld mole-rats respectively, a diurnal rhythm of melatonin secretion that is not readily inhibited by exposure to light. Furthermore, they found the phase of the melatonin rhythm does not change in response to changes in photoperiod. This suggests that although mole-rats possess a circadian melatonin rhythm, it is unlikely that the phase of the melatonin rhythm can be physiologically modulated to photoperiod. There was a distinct difference in the urinary melatonin collected during the day and night. Mole-rats exposed to the four different seasonal protocols failed to show differences with respect to temperature, but exhibited different patterns of secretion on the respective photoperiodic regimes. On long nights (winter) melatonin concentrations were highest during the early morning collection periods (T3 & T9) in all three species. However, all simulated seasons showed much the same pattern of melatonin manufacture with samples collected under T15 and T21 having much lower concentrations than those collected under T3 and T9. In the common mole-rat, however, melatonin concentration exhibited an enhanced productivity under the winter regime and a reduced concentration in the summer regime. Indeed, the common mole-rat appeared to be the most photo-responsive of the three species studied.

There is very little knowledge regarding the regulation of reproduction in both aseasonal and seasonally breeding mole-rats (Bennett and Faulkes, 2000). In habitats that are highly variable, adaptations that maximize reproductive output without taking timing into account (obligate or opportunistic adaptations) are of greater importance i.e. the animals have a greater probability of successfully raising some offspring. My goal was to determine whether or not reproduction is regulated by photoperiod or temperature changes. Photoperiod and temperature were chosen as the variables to be investigated since they are commonly used by mammals which breed seasonally.

The Damaraland mole-rat was selected as a control animal for the study, since it is an aseasonally breeding species and it is expected to be reproductively competent throughout the year. My first goal was to place the animals under different combinations of light and temperature regimes to discern if these animals respond to changes in environmental factors. I found that the Damaraland mole-rat is indeed aseasonal with temperature and light having little affect on reproductive function and the ability to produce viable motile spermatozoa throughout the different simulated seasons. The testosterone assay augmented these results with no differences in its concentration throughout. The next step was to try and find out if the pineal melatonin system is intact and whether these animals are using it to fine tune their time of reproducing maximally. I did find a rhythm of melatonin secretion with a nadir at night, but the animals were unable to detect differences between the simulated seasons. These results elegantly illustrate that the Damaraland mole-rat does not rely on the pineal melatonin system to cue reproduction.

I furthered my investigation on two social species known to seasonally breed, these being the common and the highveld mole-rats. The common mole-rat inhabits a winter rainfall region, whilst the highveld mole-rat inhabits a summer rainfall region. These two species although termed seasonal are reproductively primed throughout the year. They are able to produce spermatozoa that are viable and there is no regression or cessation of spermatogenesis. Testosterone concentrations are also similar during the different simulated seasons. Both of these species have a rhythmic secretion of melatonin secretion although in the highveld mole-rat it is not as marked as the one expressed by the common mole-rat.

It is very difficult to tease apart the potential zeitgebers that influence reproduction within subterranean mammals. Even in the area of reproduction itself, much diversity exists among the various mammalian species with respect to the degree of endogenous control of seasonal rhythmicity, the types of environmental cues employed to synchronize the rhythms, and the ways in which these cues are used (Hing-Sing Yu *et al.*, 1993).

It would appear that in the seasonally breeding common mole-rat, that light might be an important component for the timing of the onset of reproduction. In contrast, within the highveld mole-rat it would appear that both photoperiod and temperature are important to varying degrees. Rainfall is inexplicitly linked to the reproduction of both species. In the common mole-rat a reduction in photoperiod and drop in temperature is linked to rainfall events in the Cape Province (winter rainfall region) whereas in the highveld mole-rat, an increased photoperiod and raised ambient temperature is linked to rainfall events in the Gauteng Province (summer rainfall). Rainfall is not easy to simulate in the laboratory, but it could be an important component to triggering reproduction. Hence, the results of this thesis should be interpreted with some caution since rainfall is closely linked to the other two environmental variables investigated within this thesis.

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