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Pre- and postcopulatory competition affect testes mass and organization differently in two monophyletic mole-rat species, *Georychus capensis* and *Fukomys damarensis*

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Sperm competition results from postcopulatory continuation of male–male competition for paternity. The level of sperm competition is predicted to be highest in species with greater polyandry and weakest in monogamous pairs. Sperm competition levels can be indexed using traits that reflect male investment in fertilization, particularly relative testes mass (RTM). However, the relationship between RTM and levels of sperm competition may also be influenced by precopulatory competition selecting for higher levels of testosterone, also produced by the testes. To test the relationship between RTM and both pre- and postcopulatory male–male competition we compared two bathyergid mole-rat species, the promiscuous *Georychus capensis* and the monogamous eusocial *Fukomys damarensis*. The promiscuous species had not only larger RTM, but also a greater proportion of spermatogenic tissue, maximizing germ cell production as well. Conversely, the eusocial species had smaller testes, but a higher proportion of interstitial tissue (which contains the androgenic Leydig cells) and higher levels of testosterone. Consequently, testicular traits as well as testes mass may be under selection, but these are not normally measured. More research is required on relative investment in different testicular traits in relation to both pre- and postcopulatory selection pressures.

Key words: Bathyergidae, eusocial, monogamous, promiscuous, RTM, spermatogenic tissue

Postcopulatory sperm competition represents hidden, but biologically important, intermale competition, whereby sperm of rival males compete for fertilization in the female reproductive tract (Parker 1970). The risk of sperm competition is predicted to be lowest among monogamously breeding species, and to increase with the degree of polyandry (e.g., Kvarnemo and Simmons 2013). The prediction can be tested in divergent social systems, provided a reliable indicator of sperm competitiveness can be measured (Kenagy and Trombulak 1986). Potential candidate measures represent male investment in fertilization success–including ejaculate and sperm traits, penis traits, and testes size (Møller 1998)–although each measure relies on key assumptions about their link to sperm competitiveness.

Intense sperm competition should select for traits that increase competitiveness, including increased spermatozoa size, velocity, and motility, or even the rate and type of spermatozoa defects (Breed and Taylor 2000; Lemaitre et al. 2012a). However, the sperm traits under selection are hard to predict as they vary within and between species (Fitzpatrick and Lüpold 2014). Other potential indicators of sperm competition are penis traits (Lemaitre et al. 2012b) which may be confounded by precopulatory selection pressures, particularly female choice (Mautz et al. 2013).

Testes size is therefore the most commonly used indicator of sperm competition risk (Kenagy and Trombulak 1986; Ramm and Schärer 2014). Given that testes primarily produce sperm, testes mass should increase with higher sperm competition to enable a competitive advantage through the production of more sperm or more competitive sperm (Ramm and Schärer 2014). Furthermore, testes have few other functions, so testes mass is presumed to be unaffected by other selection pressures (Birkhead and Pizzari 2002), except the production of

androgens, with almost all male sex hormone production occurring in the testes interstitium (Stocco and McPhaul 2006). As with most mammalian sexually selected traits, testes mass has a positive allometric relationship with body size, which can be accounted for by calculating relative testes mass (RTM; Kenagy and Trombulak 1986). Positive relationships between RTM and degree of polyandry have consistently been demonstrated in multispecies comparisons (Harcourt et al. 1981; Kenagy and Trombulak 1986; Breed and Taylor 2000; Montoto et al. 2012), confirming RTM as a reliable indicator of sperm competition risk. However, despite the confidence regularly placed in RTM, there are some potentially confounding variables (Ramm and Schärer 2014)–particularly androgen production–that may be associated with sperm traits, but is also linked to precopulatory competition through factors such as aggressive behavior (Ramm and Schärer 2014).

Even when increased RTM is selected by sperm competition, there is still potential for confounding variables to have an influence as a result of coevolved traits. As with sperm traits, there are several alternative testicular traits that could be selected in addition to, or independent of, testes size. Testes are composed of many seminiferous tubules, each comprising spermatogenic epithelium for spermatogenesis, and internal lumen space for storage and transport of spermatozoa, as well as interstitial tissue that surrounds the tubules and contains Leydig cells for testosterone production (Ramm and Schärer 2014). Increases to the rate of spermatogenesis through an increased rate of meiosis (delBarco-Trillo et al. 2013), as well as large RTM, have been reported in many promiscuous mammals (Parapanov et al. 2007; Ramm and Stockley 2010). Emerging evidence, however, suggests that testicular tissue organization may be selected independent of RTM (Ramm and Schärer 2014; Firman et al. 2015), because different testicular areas are used for spermatogenesis and androgenesis (Stocco and McPhaul 2006). RTM may consequently underestimate or overestimate the level of sperm competition.

In birds, the ratio of spermatogenic to androgenic testicular tissue is related to sperm competition (Lüpold et al. 2009), but in mammals, histological work has focused on seasonality and maturation (e.g., Balarini et al. 2012) rather than sperm competition (Harcourt et al. 1981; Montoto et al. 2012). However, the ratio of spermatogenic to interstitial tissue does vary (interspecific–Russell et al. 1990; Ramm and Schärer 2014; intraspecific–Firman et al. 2015; Peirce et al. 2022). The proportion of nonspermatogenic tissue is highly variable (e.g., 67% in *Marmota monax* to 7% in *Octodon degus*; Russell et al. 1990), with androgenic Leydig cells composing up to 60% of testes volume (Fawcett et al. 1973). Precopulation competition increasing aggression or development of secondary sexual characteristics may influence the proportion of androgenic tissue more than postcopulatory competition, confounding the influence of sperm competition on testes size, if testes tissue architecture is not also considered. For example, in capybaras (*Hydrochoerus hydrochoerus*), testes size is independent of sperm traits or sperm competition, but associated with both dominance (Moreira et al. 1997a, 1997b) and scent gland size (Costa and Paula 2006). Thus, the amount of interstitial tissue may indicate precopulatory competition, whereas increased spermatogenic tissue may only occur with high postcopulatory competition.

Furthermore, an intrinsic problem with large, multispecies comparisons that have been performed is a lack of phylogenetic control (Harcourt et al. 1981; Kenagy and Trombulak 1986), although later studies have performed analyses controlling for phylogenetic effects (e.g., Baker et al. 2020; Firman et al. 2022). delBarco-Trillo et al. (2013) showed that spermatogenic testicular tissue increased with relative testes size and was sensitive to sperm competition after accounting for phylogeny, but may be constrained by metabolic rate. Meta-analyses are limited due to variances in study design and methodology, and so there is a pressing need for a controlled investigation into the possibility of selection of testicular traits beyond just testes size (Ramm and Schärer 2014), and the relationship of these traits to RTM.

Controlling for the confounding effects of potential selection pressures arising from environmental variation of female choice, while simultaneously controlling for phylogeny, requires RTM comparisons within a family with nonvisible internal testes, and yet a variety of mating systems, with different precopulatory and postcopulatory competition. The Bathyergidae–a family of subterranean African mole-rats–are ideal to test sperm competition predictions, as they have both internal testes (eliminating female preference), and considerable diversity of sociality (Bennett 2009), enabling us to test closely related species. Here we test whether interspecific differences in testicular tissue organization and RTM are related to precopulatory intermale competition and postcopulatory sperm competition. We predict that both precopulatory and postcopulatory competition will influence testes size but will influence testicular tissue organization differently through effects on androgenic (precopulatory competition) and spermatogenic (postcopulatory) regions of the testes.

Materials and Methods

Cape mole-rats (CMR), *Georychus capensis*, are solitary, territorial, and aggressive toward conspecifics outside the breeding season (Bennett and Jarvis 1988a). Territoriality and aggression stop during a short breeding season in May–June (Bennett and Jarvis 1988a; Oosthuizen and Bennett 2009) when males signal to females by drumming with their hind feet (Bennett and Jarvis 1988a)–both males and females may mate with multiple partners (Oosthuizen and Bennett 2009)–and females are induced ovulators (van Sandwyck and Bennett 2005). There is therefore a high rate of female multiple mating, with female-biased operational sex ratios in populations across different geographic areas (Visser et al. 2017), reducing male competition for females and precopulatory aggression. Testes size is correlated with the number of females in the population, suggesting that males mate multiply (Visser et al. 2017), and females mate multiply with a series of brief copulations (Bennett 1988; Bennet et al. 2006). Low male precopulatory

competition is further supported by either a lack of sexual size dimorphism (Visser et al. 2017), or even females reported as larger (Thomas et al. 2012), since males are normally larger in mole-rat species with high male–male competition for mating (Thomas et al. 2012; Visser et al. 2017). Males also lack fat padding on their neck, another trait associated with high rates of precopulatory fighting (Scantlebury et al. 2006; Visser et al. 2017). Instead, females have a larger zygomatic arch (Thomas et al. 2012) which may be linked to female–female competition over mates, as in the Cape Dune mole-rat (Thomas et al. 2009). Postcopulatory competition is suggested by sharpened penile spines in males, which is associated with species in which females mate multiply (Parag et al. 2006).

Damaraland mole-rats (DMR), *Fukomys damarensis*, live in familial colonies of up to 40 individuals (Bennett and Jarvis 1988b; Jarvis and Bennett 1993), consisting of a single reproductive female (with induced ovulation; Voight et al. 2021) who controls a strict breeding hierarchy (Bennett and Jarvis 1988b; Bennett and Navarro 1997). All subordinate members of DMR colonies are offspring of the dominant pair (Bennett and Jarvis 1988b). Breeding males are identified by brown-stained mouth bristles (Bennett and Jarvis 2004), and a larger body size (Bennett and Jarvis 1988b). Thus, DMRs are either monogamous (breeders) or nonbreeding, but there can be competition between males before copulation as more than one male may exhibit spermatogenesis and the reproductive female may show interest in more than one male, but will then only mate with one male (Bennett and Jarvis 1988b; Bennett 1994). Furthermore, the DMR dispersal strategy may generate male–male competition, since male breeders face a higher turnover in tenure caused by challenges by males from outside the colony (Torrents-Ticó et al. 2018). Breeding occurs year-round when conditions are favorable, and recent evidence suggests that this species is an induced ovulator (Voight et al. 2021).

All mole-rats were captured with modified Hickman live traps (Hickman 1979) in Darling, Western Cape Province (CMR, $n = 8$; 33°22′S, 15°25′E), and in Kathu, Northern Cape Province (DMR, $n = 28$, 10 breeders, 16 nonbreeders, and two juveniles, excluded from further analysis; 27°42′S, 23°03′E), between May and June 2015, which encompasses the breeding season for CMR (van Sandwyk and Bennett 2005). Animals were sacrificed by decapitation; due to restraint difficulties and to avoid stress, animals were anesthetized with halothane inhalation immediately prior to decapitation. To minimize effects of circadian rhythms, all animals were euthanized in the morning, although mole-rats generally lack robust circadian rhythms due to their fossorial lifestyle (Oosthuizen and Bennett 2022). Post death, 500 µl blood was collected into heparin-coated test tubes, centrifuged, and separated. Serum was frozen at −40°C for testosterone assays. Physical measurements taken included body mass, and (using digital calipers) anogenital distance (AGD), hind right pes length, and hind right digit lengths (Dressler and Voracek 2011). Each testis was removed, cleaned of connective tissues, weighed, measured, and identified with a blind ID.

Frozen plasma samples were thawed and testosterone assayed using a commercially available coated-tube assay kit (Coat a-Count, Diagnostic Products Corporation, Los Angeles, California). All samples were run in one assay, and validated using serial doubling dilutions of unextracted plasma over the dilution range (1:1 to 1:64). The assay has been previously validated for use in the DMR (Lutermann et al. 2013) and was validated for the CMR following a logit transformation, with no difference in slope of the lines (ANCOVA; $F_{1,9} = 2.32$, $P = 0.62$). Sensitivity of the assay (90% binding) was 63.4 ng/dl, with a detection limit of 0.5 ng/dl, resulting in values for eight CMRs and 21 DMRs, with eight breeders and 13 nonbreeders. The antiserum is highly specific for testosterone and has a low cross-reactivity with other naturally occurring steroids except dihydrotestosterone which is 5.1%. All samples were assayed in duplicate and the intra-assay coefficient of variation was 4.3%.

The longitudinal ends of each testis were incised to ensure complete permeation with Bouin's fixative solution. Tissues were soaked in 12 ml fixative for 12–24 h depending on the size of the testis, and then multiply rinsed in four ml of 70% ethanol to remove picric acid, which interferes with histological staining (Smith and Bruton 1977) with both species treated equivalently so any effects of Bouin's fixative on testicular architecture would be consistent. Specimen embedding and sectioning was conducted by Royal Hobart Pathology Services, Tasmania. Tissues were embedded in paraffin wax and sectioned transversally by automatic microtome. Five nonserial mid-section five-µm-thick slices from each left testis were mounted on glass slides and labeled with the blind identifier. Tissues were stained with hematoxylin and eosin prior to imaging. Images were taken using a Nikon DS-Fi2 camera mounted on a calibrated compound microscope fitted with a 1× magnification scope tube. Four images were taken per slide, one at 4× magnification and three at 10× magnification. Areas to be photographed were selected randomly. Absolute and proportional tissue measurements were obtained using ImageJ v1.49 software (Schneider et al. 2012), calibrated for each image according to magnification level.

Absolute seminiferous tubule measurements were obtained from the 4× magnification images. Four tubules per image ($n = 20$ per testis) were selected randomly by overlaying a 50,000-µm grid and systematically selecting histological cross sections rather than longitudinal sections located on grid crosses. Measurements included tubule and lumen circumference, area, length, and width. Epithelium height was calculated by, respectively, subtracting lumen length or width from tubule length or width, and dividing by four. Proportional measurements of testicular tissue types were obtained from the 10× magnification images. A 50,000-µm grid was projected onto each image and one grid square per image was randomly selected to measure ($n = 15$ per testis) with grid area, interstitial area, and lumen area recorded. Tubule area was calculated by subtracting interstitial area from grid area, and epithelial area was calculated by subtracting lumen area from tubule area. The proportion of spermatogenic tissue was calculated by dividing epithelial area with grid area, and the proportion of interstitial area by dividing interstitial area with grid area. The median value of each

measure per testis was used for statistical analysis as there was high within-individual variability, and the median values followed a normal distribution.

The 2D:4D digit ratio is an indication of prenatal testosterone exposure, and calculated by dividing the second and fourth digit (Dressler and Voracek 2011). Anogenital index (AGI) was calculated by dividing AGD with the cube root of body mass to account for body size (Mitchell et al. 2015). Body condition was calculated from the residuals of an ordinary least squares regression of body mass (dependent variable) and pes length (independent variable; Schulte-Hostedde et al. 2005b), using the laboratory body mass. RTM was calculated to account for allometric relationships between testes mass and body mass (Kenagy and Trombulak 1986).

Data analysis.—All statistics were performed using Rv3.1.3 software (R Core Team 2014). One adult DMR was excluded due to incomplete data.

Q–Q plots of numeric variables were visually inspected for normality, which was deemed the most appropriate test due to small sample sizes. All testes mass, length, and width variables were log transformed, and testosterone level was square-root transformed. Numeric variables were centered to a mean of zero, and Pearson's correlation was used to exclude co-predictor variables when $r \geq 0.7$, based on sample sizes. Testosterone analyses used data sets that excluded values which had low confidence due to high variance between replicates. Two data subsets were created: 'sperm competition data' that included only the breeders of both species ($n = 18$) to test sperm competition hypotheses, and 'Damaraland data' that included the breeding and nonbreeding DMRs only ($n = 26$) to test the effect of social role on testicular development.

We used multiple linear regression models using the packages 'lmerTest' (Kuznetsova et al. 2017) and 'lme4' (Bates et al. 2015). We tested sperm competition hypotheses using breeder's-only data, and social role hypotheses using DMR-only data with colony identification as a random variable. The models tested for predictors of variation in the response variable, particularly effects due to species or breeding status. In each case, a single response variable was tested against several biologically meaningful predictor variables. We excluded interaction terms due to small sample sizes (Bolker et al. 2009). We reduced models by removing all nonsignificant variables ($P > 0.05$), and comparing the full and reduced model via analysis of variance (ANOVA). When the difference was statistically nonsignificant we accepted the reduced model, otherwise we reduced the full model by removing individual variables that contributed the least to response variation ($P \geq 0.09$) in the full model. We sequentially compared reduced models using ANOVA to find the model of best fit, one step prior to a significant ANOVA result. Additional statistics, including *t*-tests and correlations, have been reported where relevant.

Ethical statement.—All procedures were approved by the University of Pretoria in South Africa, under ethics approval EC070-14, acknowledged by University of Tasmania A0014519. The protocols follow ASM guidelines (Sikes et al. 2016), and the legal requirements in both Australia and South Africa.

Results

CMRs had significantly greater RTM (4.95 ± 0.55 mg/g body mass) than DMR breeders (1.25 ± 0.28 mg/g body mass; Fig. 1; Table 1; $F_{8,10} = 256.61$, $P < 0.01$), which was positively influenced by body condition ($F_{8,10} = 11.85$, $P < 0.01$). Neither AGI ($F_{8,10} = 0.19$, $P = 0.67$) or digit ratio ($F_{8,10} = 0.05$, $P = 0.83$) were related to RTM.

CMRs had a higher median proportion of seminiferous epithelium in the testes (0.83 ± 0.01) than did DMR breeders (0.71 ± 0.01; Fig. 2; Table 1; $F_{8,10} = 43.39$, $P < 0.01$), which was also positively influenced by body condition ($F_{8,10} = 4.83$, $P = 0.04$), although body mass was not related ($F_{8,10} = 2.6$, $P = 0.13$). AGI ($F_{8,10} = 0.68$, $P = 0.43$) and digit ratio ($F_{8,10} = 1.34$, $P = 0.27$) also had no explanatory power for median proportion of seminiferous epithelium. The absolute size of seminiferous tubules did not differ by species when measured as surface area ($F_{8,10} = 0.001$, $P = 0.97$), circumference ($F = 0.002$, $P = 0.96$), or epithelial height ($F_{8,10} = 0.32$, $P = 0.58$). However, absolute testes mass is positively, albeit weakly in one case, associated with surface area ($F_{8,10} = 4.04$, $P = 0.06$), circumference ($F_{8,10} = 4.49$, $P = 0.05$), and epithelial height ($F_{8,10} = 10.33$, $P = 0.01$).

Mean testosterone levels of CMRs were very low (2.53 ± 1.37 ng/dl), and not detectable for three individuals (<0.5 ng/dl) (Table 1). Furthermore, CMR testosterone levels were significantly lower ($F_{8,8} = 40.91$, $P < 0.01$) with considerably less variance than that of DMR breeders (36.98 ± 13.07 ng/dl). There was a strong interaction between proportion of interstitial area and species when predicting plasma testosterone levels, with a strong positive relationship for DMR breeders and no relationship for CMRs (Fig. 3; $F_{8,8} = 8.83$, $P = 0.02$), although interstitial area and testosterone were related for the whole data set ($F_{8,8} = 17.0$, $P < 0.01$). Body condition ($F_{8,8} = 0.07$, $P = 0.80$), AGI ($F_{8,8} = 3.58$, $P = 0.09$), and digit ratio ($F_{8,8} = 0.25$, $P = 0.63$) were not related to plasma testosterone levels.

Breeding DMRs have significantly greater RTM (1.25 ± 0.28 mg/g body mass) than helpers (0.99 ± 0.24 mg/g body mass; Fig. 4; $F_{10,16} = 12.98$, $P < 0.01$). Body condition ($F_{10,16} = 0.62$, $P = 0.44$), colony size ($F_{10,16} = 0.95$, $P = 0.34$), AGI ($F_{10,16} = 1.47$, $P = 0.24$) and digit ratio ($F_{10,16} = 0.11$, $P = 0.74$) had no explanatory power on RTM.

There is no significant difference in the median proportion of seminiferous epithelium in testes of breeding (0.71 ± 0.01%) and helper (0.71 ± 0.02%; $F_{10,16} = 1.65$, $P = 0.22$) DMRs. Absolute testes mass ($F_{10,16} = 2.17$, $P = 0.16$), body condition ($F_{10,16} = 0.32$, $P = 0.58$), body mass ($F_{10,16} = 0.003$, $P = 0.96$), colony size ($F_{10,16} = 0.37$, $P = 0.55$), AGI ($F_{10,16} = 0.32$, $P = 0.58$), and digit ratio ($F_{10,16} = 0.04$, $P = 0.84$) also had no explanatory power. The absolute size of seminiferous tubules does not differ by breeding status when measured as surface area ($F_{10,16} = 0.76$, $P = 0.39$), circumference ($F_{10,16} = 0.66$, $P = 0.43$), or epithelial height ($F_{10,16} = 0.95$, $P = 0.33$). However, absolute testes mass is

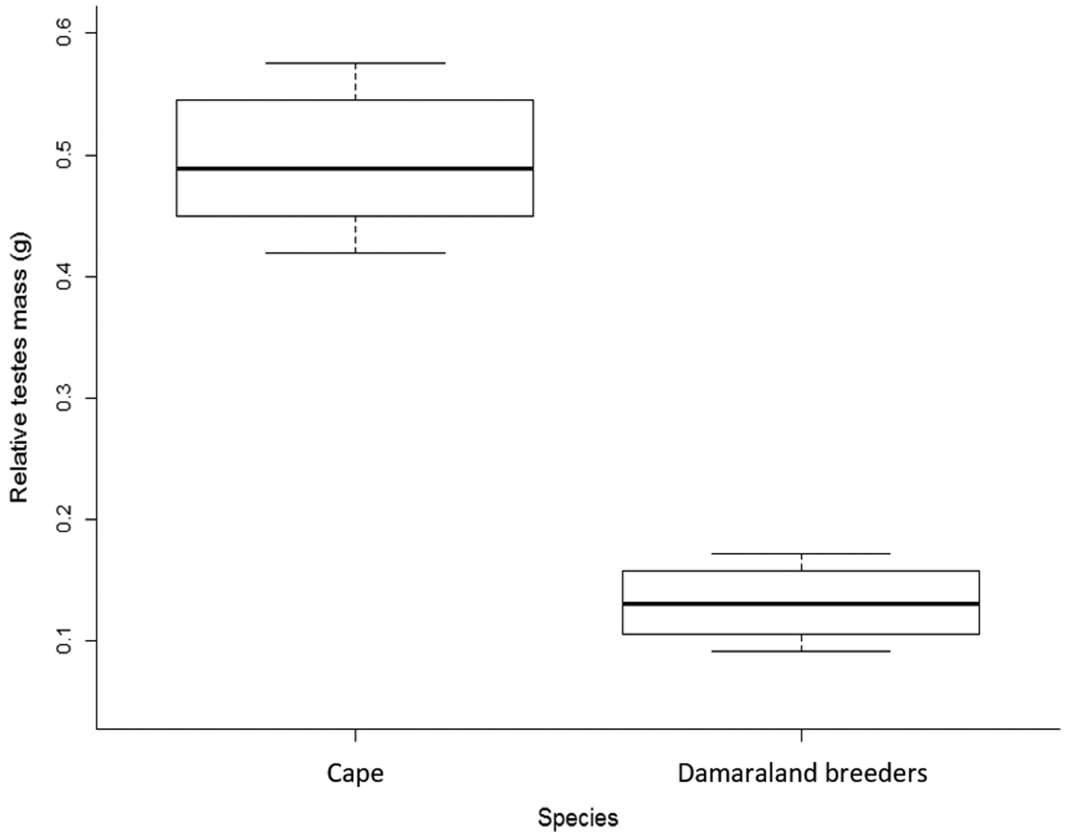

Fig. 1.—Promiscuous Cape mole-rats ($n = 8$) have significantly larger relative testes mass than eusocial breeding Damaraland mole-rats ($n = 10$).

an important positive predictor of surface area ($F_{10,16} = 23.48$, $P < 0.01$), circumference ($F_{10,16} = 25.14$, $P < 0.01$), and epithelial height ($F_{10,16} = 26.18$, $P < 0.01$). There was no significant difference between mean plasma testosterone levels of breeding (36.98 ± 13.07 ng/dl, $n = 8$) and helper (30.00 ± 5.41 ng/dl, $n = 13$; $F_{10,13} = 3.08$, $P = 0.12$) DMRs. When both breeders and helpers were considered, there was also no significant relationships between plasma testosterone level and interstitial tissue ($F_{10,16} = 0.05$, $P = 0.82$), absolute testes mass ($F_{10,16} = 1.65$, $P = 0.24$), body condition ($F_{10,16} = 0.01$, $P = 0.93$), colony size ($F_{10,16} = 0.71$, $P = 0.66$), AGI ($F_{10,16} = 0.04$, $P = 0.85$), and digit ratio ($F_{10,16} = 0.05$, $P = 0.82$).

Indices of prenatal androgen measures do not differ significantly between DMR breeders and helpers when measured as digit ratio ($t_{24} = 0.08$, $P = 0.94$) or AGI ($t_{23} = 0.18$, $P = 0.86$). There is a significant difference between digit ratios of CMRs and breeding DMRs ($t_{18} = 2.62$, $P = 0.02$), but not AGI ($t_{12} = 0.06$, $P = 0.95$).

Discussion

Our results support our prediction that higher sperm competition is associated with larger RTM, and more testicular spermatogenic tissue. Where there is very low potential for sperm competition in the cooperatively breeding DMR, there was a smaller overall RTM, but a higher ratio of interstitial tissue which contains the androgenic Leydig cells and is associated with levels of testosterone in the blood, indicating higher production of testosterone in the testes, likely due to high precopulatory aggression and competition. Although we were not able to directly measure testosterone production in the testes or the proportion of Leydig cells in this study, a similarly high proportion of interstitial tissue, with a high density of Leydig cells, has been reported on a small sample of eusocial naked mole-rats (*Heterocephalus glaber*; Fawcett et al. 1973), mirroring our results here. These findings have implications for current research practices, whereby the level of sperm competition is often inferred from RTM without testing the existence of a causal relationship, and without considering the androgenic role of interstitial tissue (Ramm and Schärer 2014). Furthermore, we have detected few morphological or physiological differences between male reproductive groupings in eusocial DMRs, consistent with recent works showing a lack of individual specialization in cooperative activities (e.g., Thorley et al. 2018).

The CMRs had significantly greater RTM compared to the breeding DMRs. Greater testes investment under higher polyandry relative to monogamy is predicted and previously reported (Kenagy and Trombulak 1986; Harcourt et al. 1995; Montoto et al. 2012). The low rates of aggression and precopulatory competition indicated by female-biased operational sex ratios and promiscuous breeding (Visser et al. 2017) suggest that the main influence on RTM in CMRs is sperm competition, supported by the low ratio of interstitial:spermatogenic tissue. Our results support using RTM (Kenagy and Trombulak 1986; Ramm and Schärer 2014) as an index of the level of sperm competition where other influences are minimal.

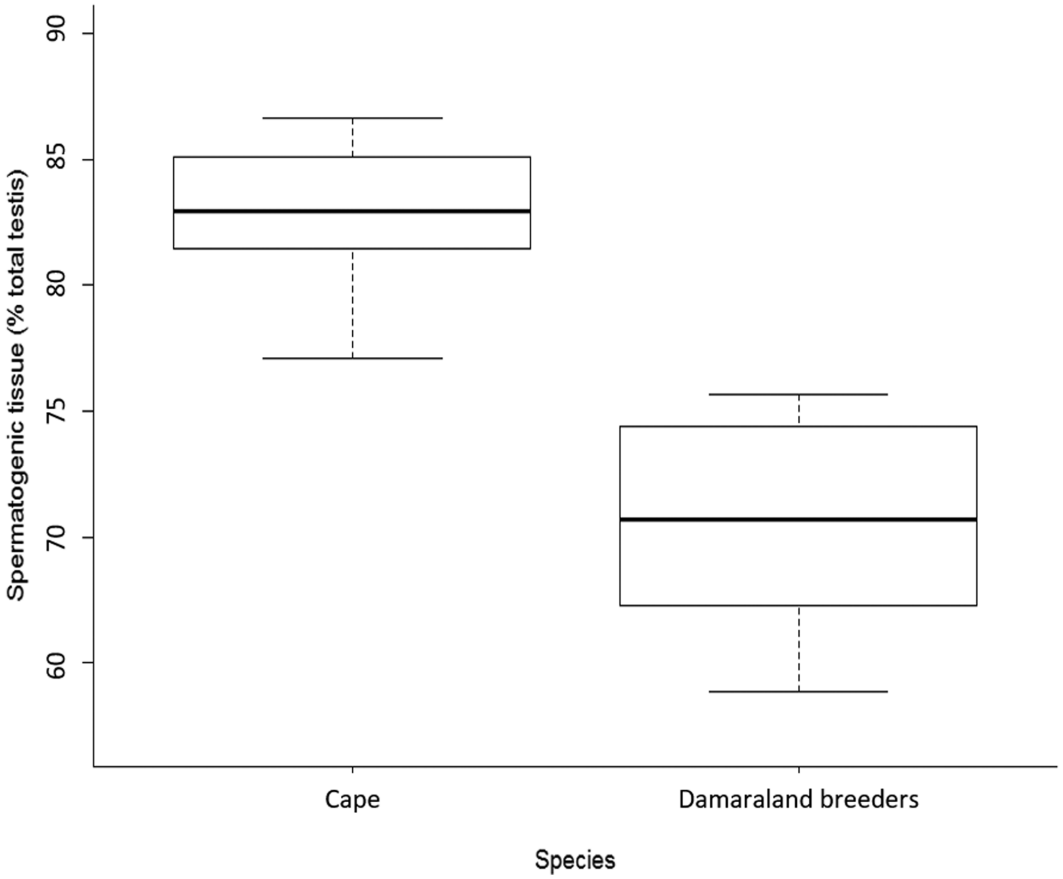

Fig. 2.—Promiscuous Cape mole-rats ($n = 8$) have a significantly higher proportion of seminiferous epithelium than eusocial breeding Damaraland mole-rats ($n = 10$).

Table 1.—Breeding season measurements of testes in Cape mole-rats (CMR), Damaraland mole-rat breeders (DMR-breed) and nonbreeders (DMR-nonbreed). Values are expressed as mean ± standard error.

	CMR	DMR-breed	DMR-nonbreed
Absolute testes mass (g)	6.93 ± 0.60	2.11 ± 0.19*	1.24 ± 0.14^
Relative testes mass (g)	4.95 ± 0.55	1.25 ± 0.28*	0.99 ± 0.24^
Proportion of seminiferous epithelium	0.83 ± 0.01	0.71 ± 0.01*	0.71 ± 0.02
Proportion interstitial tissue	0.12 ± 0.01	0.20 ± 0.02*	0.79 ± 0.02^
Proportion spermatogenic tissue	0.88 ± 0.01	0.80 ± 0.02*	0.21 ± 0.02^
Seminiferous tubule area (μm^2)	38167 ± 3416	31340 ± 2395	22470 ± 2028^
Seminiferous tubule circumference (μm)	695 ± 31	627 ± 24	533 ± 24^
Seminiferous epithelial height (μm)	82.64 ± 4.57	70.20 ± 2.78	64.12 ± 3.32
Testosterone (ng/dl)	2.53 ± 1.37	36.98 ± 13.07*	26.05 ± 4.58

*Significant difference between CMR and DMR breeders.
^Significant difference between DMR breeders and nonbreeders.

CMRs had proportionally more testicular seminiferous epithelium, lower variance within individuals and between individuals, and more homogeneous tubule shape and tissue structure compared to breeding DMRs. These factors signal greater optimization of testicular tissues, which would further increase spermatogenic potential (Ramm et al. 2014). Interestingly, both previous studies that explicitly test the effects of sperm competition on mammalian testicular histology show similar results (delBarco-Trillo et al. 2013; Firman et al. 2015). Thus, selection for more optimized tissues may be widespread in mammals, which would result in RTM underestimating the level of sperm competition (Ramm and Schärer 2014).

Both RTM and epithelial proportions of CMRs and breeding DMRs were influenced by the level of sperm competition and individual body condition. The positive relationship with body condition was expected because it measures excess energy resources available for nonessential growth, including reproduction (Schulte-Hostedde et al. 2005a). Body condition has been shown to be an important

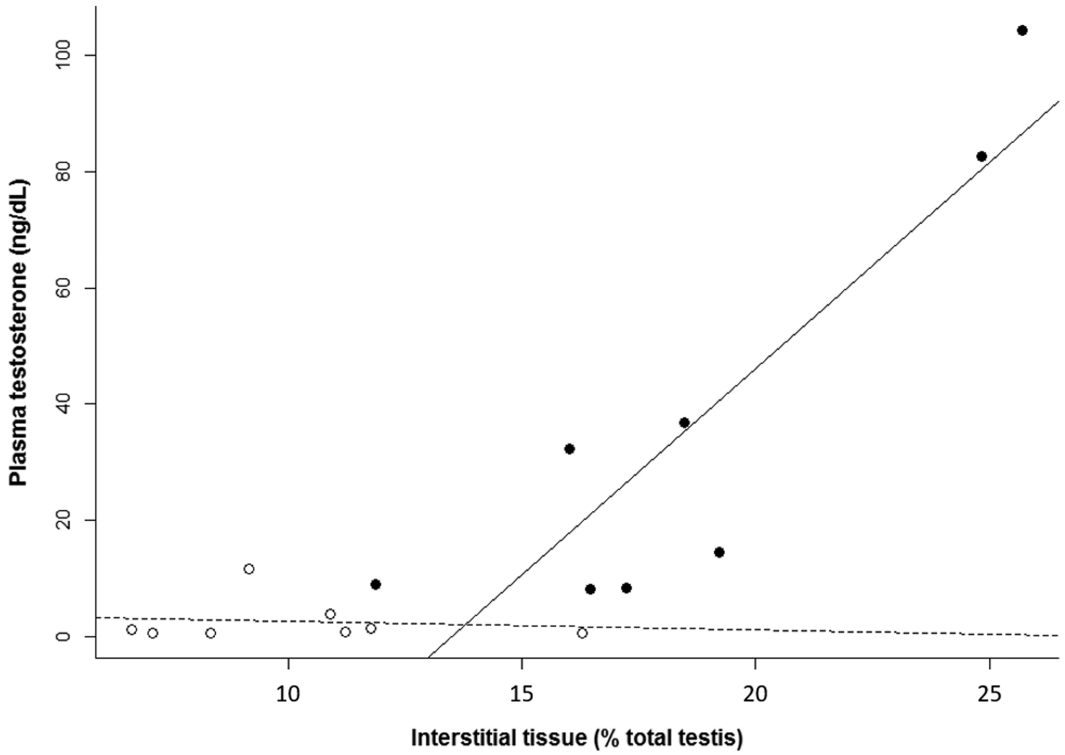

Fig. 3.—There is a positive relationship between median proportions of interstitial tissue and plasma testosterone levels for breeding Damaraland mole-rats (solid dots/line, $n = 10$), but not Cape mole-rats (open dots/line, $n = 8$).

determinant of testes size in many mammals (Schulte-Hostedde et al. 2005a; Brito et al. 2007; Sarasa et al. 2010) and evidently, condition also influences investment in testicular tissues. Also implicated in mammalian testicular development is prenatal androgen exposure (e.g., Herman et al. 2000). However, there was no effect of androgens on RTM or epithelial proportions, despite a significant difference in digit ratio—but not AGI—between the species, which are usual markers of prenatal androgen exposure (Hurd et al. 2008). Likewise, adult testosterone levels differed between species, but not in the intuitive direction, and with variable effects on RTM and epithelial proportions.

Larger RTM is often associated with higher levels of testosterone (e.g., Malo et al. 2009; Preston et al. 2012), but instead CMRs have exceedingly low testosterone levels. In contrast, breeding DMRs have high testosterone and small RTM, with no apparent selection for epithelial tissue proportions or tissue structure—lack of selection was expected due to reproductive monogamy and oligospermy in this species (Faulkes et al. 1994; Maswanganye et al. 1999). Interestingly, there was a positive relationship between testosterone levels and the proportion of interstitial tissue, not epithelial tissue. It seems that the absence of sperm competition has freed breeders to increase hierarchical control via investment in testosterone-producing tissues, without negative effects from reduced spermatogenic investment. This would be beneficial for breeders because they engage in dominant and aggressive behaviors (Bennett and Jarvis 1988b), which are usually driven by high testosterone (e.g., Malo et al. 2009). Therefore, selection for interstitial tissue in breeding DMRs appears to be related to social testosterone requirements, whereas selection for spermatogenic tissue in CMRs is driven by sperm competition.

Breeding DMRs had significantly larger RTM than helpers. This contrasts with sperm competition predictions, whereby subordinates should invest in greater RTM to enable mating when, and if, opportunities arise (Parker 1990; Neff et al. 2003; Sarasa et al. 2010). However, unlike other breeding systems, eusociality and cooperative breeding enforces a strict breeding hierarchy where there is an almost complete absence of mating opportunities for male helpers. Thus, low RTM of helpers likely represents resource conservation in the face of negligible reproductive opportunities. The proportion of epithelial tissue in the testes did not differ between reproductive groupings, although the seminiferous tubules of breeding males were more compact and regular in shape than the more variable helpers. Additionally, there were no intraspecific testosterone differences between breeders and helpers that both had high levels, similar to previous studies (e.g., Maswanganye et al. 1999; Voigt et al. 2016), and no differences in AGI or digit ratio.

We have shown that the degree of polyandry predicts RTM in African mole-rats, which supports our predictions based on sperm competition (Parker 1990). Critically, we have also demonstrated an effect beyond testes size, with the proportion of spermatogenic tissue in testes of the promiscuous species being greater than that of the eusocial species in which sperm competition was absent. The significance of these key findings is 2-fold. Our study first supports the

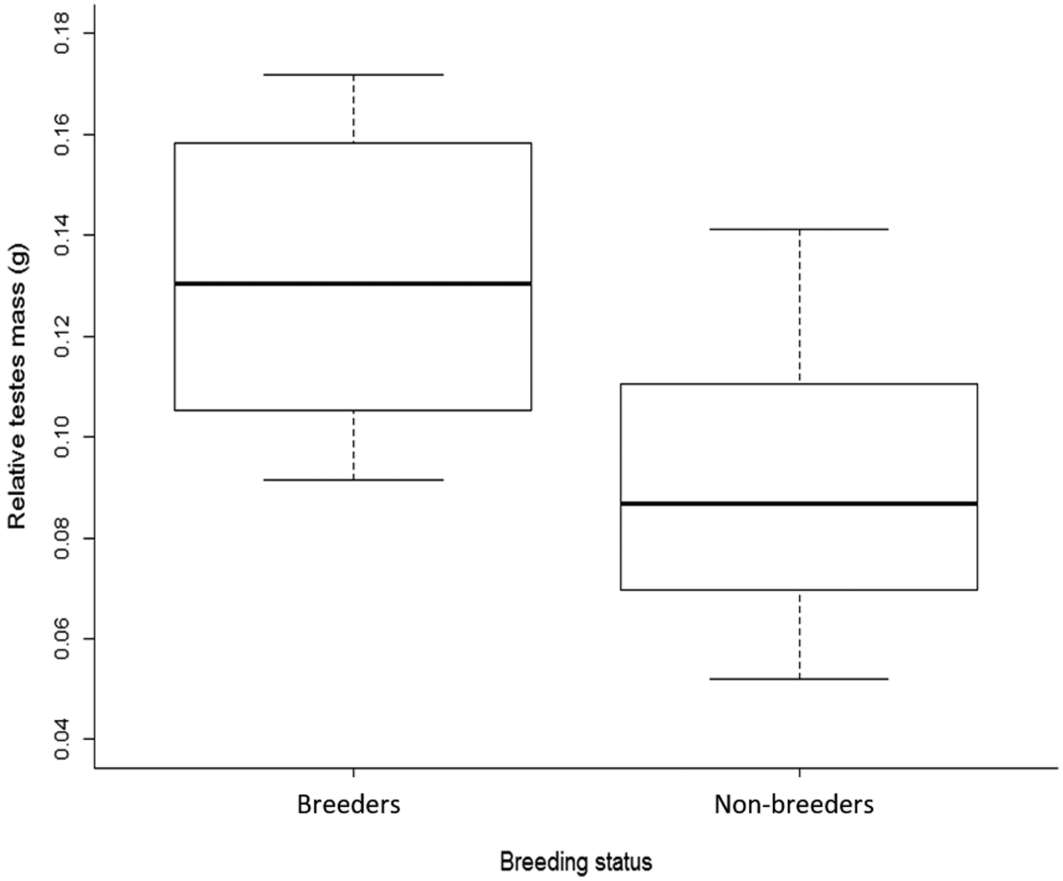

Fig. 4.—Breeding Damaraland mole-rats (n = 10) have significantly greater relative testes mass than non-breeders (n = 16).

widespread use of RTM as a proxy measure of the level of sperm competition, and secondly reiterates Ramm and Schärer (2014) by emphasizing the need to be cautious when using RTM alone to infer the amount of sperm competition. Finally, we show that different types precopulatory and post-copulatory competition both influence testes organization, which may complicate a simple measure of RTM as an index of sperm competition.

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Literature Cited

Baker J., Humphries S., Ferguson-Gow H., Meade A., Vendetti C. 2020. Rapid decreases in relative testes mass among monogamous birds but not in other vertebrates. Ecology Letters 23:283–292.

Balarini M.K., de Paula T.A.R., da Matta S.L.P., Peixoto J.V., Guião-Leite F.L., Júnior J.L.R., Junior A.C.C., Walker N.J. 2012. Stages and duration of the cycle of the seminiferous epithelium in oncilla (*Leopardus tigrinus*, Schreber, 1775). Theriogenology 77:873–880.

Bates D., Mächler M., Bolker B., Walker S. 2015. Fitting linear mixed-effects models using lme4. Journal of Statistical Software 67:1–48.

Bennett N.C. 1988. The trend towards sociality in three species of southern African mole-rats Bathyergidae: causes and consequences. Dissertation, University of Cape Town, South Africa.

Bennett N.C. 1994. Reproductive suppression in social *Cryptomys damarensis* colonies—a lifetime of socially-induced sterility in males and females (Rodentia: Bathyergidae). Journal of Zoology 234:25–39.

Bennett N.C. 2009. African mole-rats (family Bathyergidae): models for studies in animal physiology. African Zoology 44:263–270.

Bennett N.C., Jarvis J.U.M. 1988a. The reproductive biology of the Cape mole-rat, *Georychus capensis* (Rodentia, Bathyergidae). Journal of Zoology 214:95–106.

Bennett N.C., Jarvis J.U.M. 1988b. The social structure and reproductive biology of colonies of the mole-rat, *Cryptomys damarensis* (Rodentia, Bathyergidae). Journal of Mammalogy 69:293–302.

Bennett N.C., Jarvis J.U.M. 2004. *Cryptomys damarensis*. Mammalian Species 756:1.

Bennett N.C., Maree S., Faulkes C.G. 2006. *Georychus capensis*. Mammalian Species 799:1–4.

Bennett N.C., Navarro R. 1997. Differential growth patterns between successive litters of the eusocial Damaraland mole-rat, *Cryptomys damarensis*, from Namibia. Journal of Zoology 241:465–473.

Birkhead T.R., Pizzari T. 2002. Postcopulatory sexual selection. Nature Reviews Genetics 3:262–273.

Bolker B.M., Brooks M.E., Clark C.J., Geange S.W., Poulsen J.R., Stevens M.H.H., White J.-S.S. 2009. Generalized linear mixed models: a practical guide for ecology and evolution. Trends in Ecology and Evolution 24:127–135.

Breed W.G., Taylor J. 2000. Body mass, testes mass, and sperm size in murine rodents. Journal of Mammalogy 81:758–768.

Brito L.F.C., Barth A.D., Rawlings N.C., Wilde R.E., Crews D.H. Jr., Boisclair Y.R., Ehrhardt R.A., Kastelic J.P. 2007. Effect of feed restriction during calfhood on serum concentrations of metabolic hormones, gonadotropins, testosterone, and on sexual development in bulls. Reproduction 134:171–181.

Costa D.S., Paula T.A.R. 2006. Testosterone level, nasal gland volume and Leydig cell morphometry in capybaras (*Hydrochoerus hydrochaeris*). Arquivo Brasileiro de Medicina Veterinária e Zootecnia 58:157–166.

delBarco-Trillo J., Tourmente M., Roldan E.R.S. 2013. Metabolic rate limits the effect of sperm competition on mammalian spermatogenesis. PLoS One 8:e76510.

Dressler S.G., Voracek M. 2011. No association between two candidate markers of prenatal sex hormones: digit ratios (2D:4D and other) and finger-ridge counts. Developmental Psychobiology 53:69–78.

Faulkes C.G., Trowell S.N., Jarvis J.U.M., Bennett N.C. 1994. Investigation of numbers and motility of spermatozoa in reproductively active and socially-suppressed males of two eusocial African mole-rats, the naked mole-rat (*Heterocephalus-glaber*), and the Damaraland mole-rat (*Cryptomys damarensis*). Journal of Reproduction and Fertility 100:411–416.

Fawcett D.W., Neaves W.B., Flores M.N. 1973. Comparative observations on intertubular lymphatics and the organization of the interstitial tissue of the mammalian testis. Biology of Reproduction 9:500–532.

Firman R.C., Garcia-Gonzalez F., Thyer E., Wheeler S., Yamin Z., Yuan M., Simmons L.W. 2015. Evolutionary change in testes tissue composition among experimental populations of house mice. Evolution 69:848–855.

Firman R.C., Rubenstein D.R., Buzatto B.A. 2022. The spatial and temporal distribution of females influence the evolution of testes size in Australian rodents. Biology Letters 18:20220058.

Fitzpatrick J.L., Lüpold S. 2014. Sexual selection and the evolution of sperm quality. Molecular Human Reproduction 20:1180–1189.

Harcourt A.H., Harvey P.H., Larson S.G., Short R.V. 1981. Testis weight, body weight and breeding system in primates. Nature 293:55–57.

Harcourt A.H., Purvis A., Liles L. 1995. Sperm competition: mating system, not breeding season, affects testes size of primates. Functional Ecology 9:468–476.

Herman R.A., Jones B., Mann D.R., Wallen K. 2000. Timing of prenatal androgen exposure: anatomical and endocrine effects on juvenile male and female rhesus monkeys. Hormones and Behavior 38:52–66.

Hickman G.C. 1979. A live-trap and trapping technique for fossorial mammals. South African Journal of Zoology 14:9–12.

Hurd P.L., Bailey A.A., Gongal P.A., Yan R.H., Greer J.J., Pagliardini S. 2008. Intrauterine position effects on anogenital distance and digit ratio in male and female mice. Archives of Sexual Behavior 37:9–18.

Jarvis J.U.M., Bennett N.C. 1993. Eusociality has evolved independently in two genera of Bathyergid mole-rats, but occurs in no other subterranean mammal. Behavioral Ecology and Sociobiology 33:253–260.

Kenagy G.J., Trombulak S.C. 1986. Size and function of mammalian testes in relation to body size. Journal of Mammalogy 67:1–22.

Kuznetsova A., Brockhoff P., Christensen R. 2017. lmerTest package: tests in linear mixed effects models. Journal of Statistical Software, Articles, 82:1–26.

Kvarnemo C., Simmons L.W. 2013. Polyandry as a mediator of sexual selection before and after mating. Philosophical Transactions of the Royal Society of London, B: Biological Sciences 368:20120042.

Lemaitre J.F., Ramm S.A., Hurst J.L., Stockley P. 2012a. Sperm competition roles and ejaculate investment in a promiscuous mammal. Journal of Evolutionary Biology 25:1216–1225.

Lemaitre J.F., Ramm S.A., Jennings N., Stockley P. 2012b. Genital morphology linked to social status in the bank vole (*Myodes glareolus*). Behavioral Ecology and Sociobiology 66:97–105.

Lüpold S., Linz G.M., Rivers J.W., Westneat D.F., Birkhead T.R. 2009. Sperm competition selects beyond relative testes size in birds. Evolution 63:391–402.

Lutermann H., Young A.J., Bennett N.C. 2013. Reproductive status and testosterone among females in cooperative mole-rat societies. General and Comparative Ecdocrinology 187:60–65.

Malo A.F., Roldan E.R.S., Garde J.J., Soler A.J., Vicente J., Gortazar C., Gomendio M. 2009. What does testosterone do for red deer males? Proceedings of the Royal Society of London, B: Biological Sciences 276:971–980.

Maswanganye K.A., Bennett N.C., Brinders J., Cooney R. 1999. Oligospermia and azoospermia in non-reproductive male Damaraland mole-rats *Cryptomys damarensis* (Rodentia: Bathyergidae). Journal of Zoology 248:411–418.

Mautz B.S., Wong B.B.M., Peters R.A., Jennions M.D. 2013. Penis size interacts with body shape and height to influence male attractiveness. Proceedings of the National Academy of Sciences of the United States of America 110:6925–6930.

Mitchell R.T., Mungall W., McKinnell C., Sharpe R.M., Cruickshanks L., Milne L., Smith L.B. 2015. Anogenital distance plasticity in adulthood: implications for its use as a biomarker of fetal androgen action. Endocrinology 156:24–31.

Møller A.P. 1998. Sperm competition and sexual selection. In: Birkhead T.R., Møller A.P., editors. Sperm competition and sexual selection. Academic Press, London, United Kingdom; p. 55–90.

Montoto L.G., Arregui L., Sanchez N.M., Gomendio M., Roldan E.R.S. 2012. Postnatal testicular development in mouse species with different levels of sperm competition. Reproduction 143:333–346.

Moreira J.R., Clarke J.R., MacDonald D.W. 1997a. The testis of capybaras (*Hydrochoerus hydrochaeris*). Journal of Mammalogy 78:1096–1100.

Moreira J.R., MacDonald D.W., Clarke J.R. 1997b. Correlates of testis mass in capybaras (*Hydrochaeris hydrochaeris*): dominance assurance or sperm production? Journal of Zoology 241:457–463.

Neff B.D., Fu P., Gross M.R. 2003. Sperm investment and alternative mating tactics in bluegill sunfish (*Lepomis macrochirus*). Behavioral Ecology 14:634–641.

Oosthuizen M.K., Bennett N.C. 2009. Seasonal variation in gonadal steroids of males and females in the Cape mole-rat (*Georychus capensis*): the potential for opportunistic breeding. African Zoology 44:117–122.

Oosthuizen M.K., Bennett N.C. 2022. Clocks ticking in the dark: a review of biological rhythms in subterranean African mole-rats. Frontiers in Ecology and Evolution 10:878533.

Parag A., Bennett N.C., Faulkes C.G., Bateman P.W. 2006. Penile morphology of Africa mole rats (Bathyergidae): structural modification in relation to mode of ovulation and degree of sociality. Journal of Zoology 270:323–329.

Parapanov R., Nussle S., Vogel P. 2007. Cycle length of spermatogenesis in shrews (Mammalia: Soricidae) with high and low metabolic rates and different mating systems. Biology of Reproduction 76:833–840.

Parker G.A. 1970. Sperm competition and its evolutionary consequences in the insects. Biological Reviews 45:525–567.

Parker G.A. 1990. Sperm competition games: raffles and roles. Proceedings of the Royal Society of London, B: Biological Sciences 242:120–126.

Peirce E., Moya-Smith T., Tuke J., Leigh C., Breed W. 2022. Intraspecific variation in testis organisation and sperm head morphology of the delicate mouse (*Pseudomys delicatulus*): its possible causes and consequences. Australian Mammalogy 44:76–80.

Preston B.T., Stevenson I.R., Lincoln G.A., Monfort S.L., Pilkington J.G., Wilson K. 2012. Testes size, testosterone production and reproductive behaviour in a natural mammalian mating system. Journal of Animal Ecology 81:296–305.

R Core Team. 2014. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Ramm S.A., Schärer L. 2014. The evolutionary ecology of testicular function: size isn't everything. Biological Reviews 89:874–888.

Ramm S.A., Schärer L., Ehmcke J., Wistuba J. 2014. Sperm competition and the evolution of spermatogenesis. Molecular Human Reproduction 20:1169–1179.

Ramm S.A., Stockley P. 2010. Sperm competition and sperm length influence the rate of mammalian spermatogenesis. Biology Letters 6:219–221.

Russell L.D., Ren H.P., Sinha Hikim I., Schulze W., Sinha Hikim A.P. 1990. A comparative study in twelve mammalian species of volume densities, volumes, and numerical densities of selected testis components, emphasizing those related to the Sertoli cell. American Journal of Anatomy 188:21–30.

Sarasa M., Serrano E., Perez J.M., Soriguer R.C., Gonzalez G., Joachim J., Fandos P., Granados J.E. 2010. Effects of season, age and body condition on allocation to testes mass in Iberian ibex. Journal of Zoology 281:125–131.

Scantlebury M., Speakman J.R., Bennett N.C. 2006. The energy costs of sexual dimorphism in mole-rats are morphological not behavioural. Proceedings of the Royal Society of London, B:Biological Sciences 273:57–63.

Schneider C.A., Rasband W.S., Eliceiri K.W. 2012. NIH Image to ImageJ: 25 years of image analysis. Nature Methods 9:671–675.

Schulte-Hostedde A.I., Millar J.S., Hickling G.J. 2005a. Condition dependence of testis size in small mammals. Evolutionary Ecology Research 7:143–149.

Schulte-Hostedde A.I., Zinner B., Millar J.S., Hickling G.J. 2005b. Restitution of mass-size residuals: validating body condition indices. Ecology 86:155–163.

Sikes R.S., And the Animal Care and Use Committee of the American Society of Mammalogists. 2016. 2016 Guidelines of the American Society of Mammalogists for the use of wild mammals in research and education. Journal of Mammalogy 97:663–688.

Smith A., Bruton J. 1977. Colour atlas of histological staining techniques. Wolfe Medical Publications, London, United Kingdom.

Stocco D.M., McPhaul M.J. 2006. Physiology of testicular steroidogenesis. In: Neill J.D., Plant T.M., Pfaff D.W., Challis J.R.G., De Kretser D.M., Richards J.S., Wassarman P.M., editors. Knobil and Neill's physiology of reproduction. Academic Press, Burlington; p. 977–1016.

Thomas H.G., Bateman P.W., Le Comber S.C., Bennett N.C., Elwood R.W., Scantlebury M. 2009. Burrow architecture and digging activity in the Cape dune mole-rat. Journal of Zoology 279:227–284.

Thomas H.G., Bateman P.W., Scantlebury M., Bennett N.C. 2012. Season but not sex influences burrow length and complexity in the non-sexually dimorphic Cape mole-rat (Rodentia: Bathyergidae). Journal of Zoology 288:214–221.

Thorley J., Mendonça R., Vullioud P., Torrents-Ticó M., Zöttl M., Gaynor D., Clutton-Brock T. 2018. No task specialization among helpers in Damaraland mole-rats. Animal Behaviour 143:9–24.

Torrents-Ticó M., Bennett N.C., Jarvis J.U.M., Zöttl M. 2018. Sex differences in timing and context of dispersal in Damaraland mole-rats (*Fukomys damarensis*). Journal of Zoology 306:252–257.

van Sandwyck J.H.D.T., Bennett N.C. 2005. Do solitary, seismic signally Cape mole-rats (*Georychus capensis*) demonstrate spontaneous or induced ovulation? Journal of Zoology 267:75–80.

Visser J.H., Bennett N.C., Jansen Van Vuuren B. 2017. Distributional range, ecology and mating system of the Cape mole-rat, *Georychus capensis* (family Bathyergidae). Canadian Journal of Zoology 95:713–726.

Voight C., Medger K., Bennett N.C. 2021. The oestrous cycle of the Damaraland mole-rat revisited: evidence for induced ovulation. Journal of Zoology 314:85–95.

Voigt C., Leitner S., Bennett N.C. 2016. Breeding status affects the expression of androgen and progesterone receptor mRNA in the brain of male Damaraland mole-rats. Journal of Zoology 298:209–216.

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