**EVOLUTIONARY CHANGE IN TESTES TISSUE COMPOSITION AMONG EXPERIMENTAL POPULATIONS OF HOUSE MICE**

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EVOLUTIONARY CHANGE IN TESTES TISSUE COMPOSITION AMONG EXPERIMENTAL POPULATIONS OF HOUSE MICE

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Running head: Evolutionary change in the testes in mice
Theory assumes that postcopulatory sexual selection favours increased investment in testes size because greater numbers of sperm within the ejaculate increase the chance of success in sperm competition, and larger testes are able to produce more sperm.

However, changes in the organization of the testes tissue may also affect sperm production rates. Indeed, recent comparative analyses suggest that sperm competition selects for greater proportions of sperm-producing tissue within the testes. Here, we explicitly test this hypothesis using the powerful technique of experimental evolution.

We allowed house mice (*Mus domesticus*) to evolve via monogamy or polygamy in six replicate populations across 24 generations. We then used histology and image analysis to quantify the proportion of sperm-producing tissue (seminiferous tubules) within the testes of males. Our results show that males that had evolved with sperm competition had testes with a higher proportion of seminiferous tubules compared with males that had evolved under monogamy. Previously, it had been shown that males from the polygamous populations produced greater numbers of sperm in the absence of changes in testes size. We thus provide unequivocal evidence that sperm competition selects for an increase in the density of sperm-producing tissue, and consequently increased testes efficiency.

**KEY WORDS:** Sperm competition, postcopulatory sexual selection, sperm production, testosterone production, histology
Parker's game theoretic models of ejaculate evolution assume that sperm competition conforms to a raffle, with the relative number of sperm from each competitor being the primary determinant of paternity success (Parker and Pizzari 2010). In support of sperm competition theory, evolutionary and reproductive biologists focusing on in vivo sperm competition dynamics have shown that males that produce ejaculates with high numbers of sperm typically have a fertilization advantage over their rival/s (e.g. domestic species, Beatty 1957; Martin et al. 1974; wild species, Gage and Morrow 2003; Boschetto et al. 2011; Firman and Simmons 2011). Thus, when male fitness is contingent on the number of sperm within the ejaculate, it is likely that postcopulatory selection will target the organ responsible for sperm production, and evolutionary change will ensue (Parker 1970).

A positive correlation between testes size and the level of sperm competition, as determined by variation in mating system, the frequency of female remating, differences in population sex ratio, etc, has been observed. A multitude of studies have implicated intense selection via sperm competition as favoring the evolution of larger testes both within (Ribble and Millar 1992; Brown and Brown 2003; Long and Montgomerie 2005; Firman and Simmons 2008; Dzminiski et al. 2009) and between species (e.g. primates, Harcourt et al. 1981; butterflies, Gage 1994; bats, Hosken 1997; frogs, Byrne et al. 2002; birds, Pitcher et al. 2005; scorpions, Vrech et al. 2014). To date however, the most convincing evidence for the role of sperm competition in testes size evolution comes from investigations that have maintained evolving laboratory populations, and found reductions in testes mass with the removal of sperm competition (Hosken and Ward 2001; Pitnick et al. 2001; Simmons and Garcia-Gonzalez 2008). This significant body of literature has resulted in testes size (relative to body size) being routinely used as an indirect measure of sperm competition risk.
Unfortunately, the underlying critical assumption that testes size accurately reflects sperm production rate has largely been overlooked (discussed by Schärer et al. 2004). Certainly, variation in testis size alone may not necessarily reflect differences in sperm production rate, and evidence has emerged to support the idea that sperm competition selects to maximise testicular efficiency beyond changes in testes size (reviewed in Ramm and Schärer 2014).

The testis consists of spermatogenic tissue, which includes germ cells and somatic cells (called Sertoli cells in mammals), and non-spermatogenic tissue with somatic cells only (e.g. blood vessels and Leydig cells). In higher vertebrates the germ cells are located within the long and convoluted seminiferous tubules (Gier and Marion 1970; Roosen-Runge 1977; Wistuba et al. 2007). During the process of spermatogenesis the germ cells develop from spermatogonia into spermatocytes, then to spermatids, and finally spermatozoa (Roosen-Runge 1977; Wistuba et al. 2007). Spermatozoa are released from the nourishing Sertoli cells to enter the lumen of the seminiferous tubule before being passed into the efferent ducts and transported to the epididymis for maturation. The interstitial tissue that surrounds the seminiferous tubules supplies blood to the testis and produces endocrine signals via the Leydig cells (Wistuba et al. 2007).

It has long been known that seasonal variation in the amount of sperm-producing tissue versus non sperm-producing tissue occurs in many different taxa (e.g. Mayhew and Wright 1970; Goldberg and Parker 1975; Hochereau-de Reviers and Lincoln 1978; Fuentes et al. 1991). Since Harcourt et al. (1981) first indicated that sperm competition has the potential to select for increased sperm production rates via alternations in the spatial organization of the testes, a number of comparative studies have explored the association between the proportion of sperm-producing
tissue within the testes and postcopulatory sexual selection (Wistuba et al. 2003; Lüpold et al. 2009; Rowe and Pruett-Jones 2011; Montoto et al. 2012). Interspecies comparisons have provided general evolutionary patterns, but fail to demonstrate cause and effect. In their review on the evolutionary ecology of testicular function, Ramm and Schärer (2014) called for researchers to explore directly the consequences of sperm competition for testes machinery by using the powerful technique of experimental evolution. Here we provide the first account. We quantified the amount of sperm-producing and interstitial tissue from images of histological preparations of the testes of male house mice (Mus domesticus) from six replicate populations that had been evolving under a monogamous or polygamous mating regime for 24 generations. Our analyses revealed that the testes of males that had evolved with sperm competition had greater proportions of seminiferous tubules compared to males that had evolved via monogamy. Previously, it had been shown that males from the polygamous populations produced ejaculates with elevated sperm numbers in the absence of changes in testes size (Firman and Simmons 2010). Thus, our result here suggests that sperm competition acts directly on the spatial organisation of the testes, which is likely to select for enhanced efficiency and the production of more sperm.

Materials and Methods

EXPERIMENTAL ANIMALS

House mice are actively polygamous in nature, with both males and females copulating with multiple mates (Dean et al. 2006; Firman and Simmons 2008). However, laboratory strains of house mice are typically maintained under an enforced monogamous mating regime. The Uwa:MMD colony of wild-derived mice was established in 1997 at the Animal Resources Centre (ARC) (Murdoch, Western
Australia) with 90 breeding pairs sourced from six wild, Australian *Mus domesticus* populations. From 1997 to 2002 the colony was maintained as an outbred population under the Poiley outbreeding system (Poiley 1960), after which the outbreeding regime changed to a dedicated program based on the coefficient of inbreeding (Firman and Simmons 2010). Consequently, the Uwa:MMD colony was maintained under a strict monogamous mating regime for approximately 30 generations prior to the establishment of the selection lines. Male/female pairs (*n* = 60) reproduced to establish a base population from which our selection lines were derived, for which we referred to the ARC colony pedigree to ensure that close relatives did not breed.

At the University of Western Australia the animals were maintained in a constant temperature room under identical conditions (24°C; reversed 14:10 hour light-dark cycle), and food and water was provided *ad libitum*. We established four monogamous and four polygamous lines with 18 males and 18 females in each. Subsequently, 18 males and 18 females contributed to each generation. In the monogamous lines, almost all selection on adult fitness was eliminated by ensuring that every male and female pair contributed one son and one daughter to the next generation (Shabalina et al. 1997). Although greatly relaxed, selection may not have been completely eliminated because sometimes a pair did not mate or produce offspring. In the polygamous lines, adult females had equal fitness (two offspring), and adult males had equal mating success but not equal fertilisation success due to sperm competition and/or cryptic female choice. Four polygamous lines were established with 18 females and 18 males, but potentially <18 sires. In the polygamous lines, the same three males mated with the same three females. Thus, males in the polygamous lines competed for fertilizations, and the number of males who contributed to successive generations was determined by the relative paternity...
success of each male. This mating design ensured that the effective population sizes of the monogamous lines were potentially greater than the polygamous lines at each generation. Therefore, any observed fitness benefits associated with sperm competition would be conservative, as the polygamous lines would be expected to have higher inbreeding coefficients than the monogamous lines (documented at generation 12; Firman & Simmons 2011). As with the monogamous lines, one male and one female were selected at random from each polygamous line litter and used to produce the next generation. Consequently, while postcopulatory sexual selection on males was reinstated in the polygamous lines, natural selection and precopulatory sexual selection remained absent in both treatments.

Anatomical measurements and sperm quality assays initially performed after eight generations of experimental evolution revealed that males had diverged in sperm number without changes in testes size (Firman and Simmons 2010; Table S1 in the online supplementary material). Males from the polygamous lines produced more sperm, had a greater proportion of motile sperm, and sperm with greater swimming velocities than males from the monogamous lines, as would be expected following selection via sperm competition (Firman and Simmons 2010). Further, testes size among males from the different selection regimes had not changed following 16 (Firman et al. 2011) or 18 (Table S1; Table S2) generations of selection. Males from the 24th generation were used here to assess evolutionary change in the spatial organisation of the testes. Due to time and resource restrictions, we were limited to using a randomly selected sample of three monogamous (n = 10 males/line) and three polygamous (n = 10 males/line) selection lines.

Variation in the social environment can induce plasticity in sperm production in house mice (Ramm and Stockley 2009; Firman et al. 2013). Consequently, the
animals used in this experiment were reared under identical conditions. Thus, after being weaned from their mother at three weeks of age, each male was housed in an individual cage until they reached sexual maturity (12 weeks of age). To ensure that males acquired the appropriate olfactory cues during their sexual development they were placed within close proximity to cages housing females. All males were virgins at the time of sacrifice.

TESTES HISTOLOGY AND IMAGE ANALYSIS

Males were sacrificed via lethal injection and stored at -20°C. Prior to dissection, the bodies were defrosted and weighed. A single testis from each male was chosen at random and fixed in 10% buffered formal saline. Each testis was dehydrated through a series of graded ethanol baths, chloroform baths, and paraffin, and then embedded in paraffin wax. The specimens were sectioned through the midline, and centre sections of the testis were mounted on slides. The slides were stained with Gill’s haematoxylin and Eosin and viewed under a BX50 (Olympus) light microscope (×10 objective). Five different images per testis were captured using an Olympus DP72 camera attached to the microscope. Testis tissue covered the entire area in each image (Fig. 2).

Each image was visualized using the image analysis software ImageJ. We quantified the proportion of sperm-producing tissue within each image. Thus, we measured and recorded the area of each seminiferous tubule (including the tubule lumen) within each image (see Fig. S1 in the online supplementary material). We then calculated the proportion of sperm-producing tissue per image: sum[seminiferous tubule area]/total image area. The 300 images were distributed randomly among four different investigators, and each investigator was blind to the treatment from which
the images were sourced.

STATISTICAL ANALYSES

To confirm that investigator bias did not influence our measurements and results, a set of eight images were measured by the four investigators, which allowed us to perform a repeatability analysis on those measurements. A Levene’s Test was applied to test the equality of variances in the proportion of sperm-producing tissue among the replicate lines (SciStat Calc 2013). We used JMP statistical software to perform a nested analysis of variance (ANOVA) with replicate lines nested within selection treatments (random effect) to account for non-independence of replicate lines. Effect size analysis was calculated using the library “compute.es” in R. All means are presented ±1 SE.

Results

SPERM NUMBER AND TESTES SIZE MEASUREMENTS

The repeatability analysis revealed that there was no measurement bias among the investigators. There was significantly more variation between images than within images ($F_{7, 24} = 17.171, P < 0.001; R = 0.500$, calculated following Becker 1984).

We calculated the mean proportion of sperm-producing tissue for each male. A Levene’s Test revealed that the variances in the mean proportion of sperm-producing tissue were equal among the replicate lines ($n = 6, W = 0.778, P = 0.570$). Thus, we looked for an effect of selection history among males from the different experimental populations.

A nested ANOVA revealed significant variation in the proportion of total tubule area among males from the polygamous and monogamous selection lines (Table 1).
The mean difference in the proportion of sperm-producing tissue between treatments was 4.6%. Males from the polygamous lines had testes with greater proportions of sperm-producing tissue \((n = 3, 0.747 \pm 0.008)\) compared with males from the monogamous lines \((n = 3, 0.701 \pm 0.009)\) (Fig. 1; Fig. 2). We calculated a standardized mean difference effect size (Cohen’s \(d\)) and the associated 95% confidence intervals (CIs) to gauge the magnitude of the observed effect of selection history \((d = 4.66 [0.29, 9.03])\) on the proportion of total tubule area. The effect size is large, and the 95% CIs give us confidence in rejecting the hypothesis that selection history had no effect on tubule area.

**Discussion**

Testes size evolution via postcopulatory sexual selection is well evidenced (Gomendio et al. 1998; Simmons 2001; Birkhead et al. 2009). Both inter- and intra-species comparisons have shown that sperm competition typically favours an increase in the amount of spermatogenetic tissue, and thus larger testes (e.g. Gage 1994; Hosken 1997; Byrne et al. 2002; Firman and Simmons 2008; Dzminiski et al. 2009). However, in addition to gross testes size, the relative proportion of sperm-producing tissue within the testes could also be an important factor determining spermatogenic investment (Ramm and Schärer 2014). Certainly, the capacity of the mammalian testes to produce sperm varies considerably across taxa; among six rodent species the proportion of sperm-producing tissue within the testes has been shown to range from 33% to 90% (Russell et al. 1990). Within *Mus*, the level of sperm competition (as estimated by relative testes size) correlates with both the number of sperm produced (Montoto et al. 2011) and the proportion of seminiferous tubules within the testes (Montoto et al. 2012). Similar evolutionary associations between the intensity of
sperm competition and testes tissue organization have been observed in comparative
studies of two groups of birds, the New World blackbirds (Icteridae) and the
Australian passarines (Maluridae) (Lüpold et al. 2009; Rowe and Pruett-Jones 2011).
Here, we show that male house mice from experimental populations evolving with
sperm competition have testes with greater proportions of sperm-producing tissue
compared to the testes of males from populations maintained under enforced
monogamy.

An increase in the proportion of spermatogenic tissue in the testes of males from
the polygamous lines could be ascribed to selection acting on standing genetic
variation in testes tissue organisation. Alternatively, as we reinstated sperm
competition in a source population that had experienced a long history of monogamy,
in which deleterious mutations might have accumulated, our result could be explained
by sperm competition successfully purging mutations that influenced testes
phenotype. While theory predicts that sexual selection on males can substantially
reduce the accumulation of mildly deleterious mutations (McLearn and Manning
1985; Whitlock and Agrawal 2009), experimental evidence among different
invertebrate species is contradictory (e.g. Radwan 2004; Hollis et al. 2009; Arbuthnott
and Rundle 2012; Almbro and Simmons 2014). To date, there have been no direct
tests of whether mutation accumulation influences testes tissue composition, however
it is interesting to note that a recent study of the dung beetle Onthophagus taurus
found that sexual selection was ineffective in removing deleterious mutations that
reduced testes mass (Almbro and Simmons 2014).

Deleterious recessive mutations can be exposed under conditions of inbreeding
(Charlesworth and Charlesworth 1999). Consequently, if individuals from our
monogamous selection lines were ‘carrying’ high mutation loads they might be
expected to experience greater fitness consequences from reproducing with relatives compared with individuals from the polygamous lines. Within-line experimental matings performed after 16 generations of selection revealed that full-sibling parental pairs and unrelated parental pairs had equivalent fitness (Firman et al. 2011). Further, the resulting ‘inbred’ polygamous and ‘inbred’ monogamous male offspring had, on average, comparable testes sizes and sperm numbers (Firman et al. 2011). Thus, although we are unable to eliminate conclusively that sperm competition purged deleterious alleles that influenced testes phenotype and suppressed testes function, available data suggests that this was not the case. Regardless, we have provided compelling evidence that sperm competition selects for enhanced levels of sperm-producing tissue, either by selecting against males with high mutation loads, or by acting on standing genetic variation. Certainly, these two mechanisms are not mutually exclusive and are likely to co-occur in both experimental and natural populations.

A series of previous investigations performed using these lineages of house mice had revealed that males from populations evolving via sperm competition produced more sperm compared with males from populations evolving under monogamy, but that changes in sperm production were not associated with changes in testes size (Firman and Simmons 2010; Firman et al. 2011). Moreover, the increase in sperm production resulted in enhanced success in sperm competition (Firman and Simmons 2011). We do not have testes size data from males from the 24th generation, however testes size had not diverged after eight (Firman and Simmons 2010), 16 (Firman et al. 2011) or 18 generations of selection. Thus, it is likely the increase in sperm production among males from the polygamous lines is attributable to an increase in the proportion of sperm-producing tissue, which was documented here.
Sperm production is also influenced by the efficiency of the sperm-producing tissue, for example the rate at which individual sperm can be manufactured. In mammals, the duration of spermatogenesis is defined as the ‘seminiferous epithelium cycle length’ (SECL), describing one complete series of cell associations that occurs in the seminiferous epithelium (Clermont 1972). Across species, variation in SECL has been shown to correlate negatively with relative testes size, supporting the idea that sperm competition selects for a faster rate of spermatogenesis (Pierce and Breed 2001; Paraponov et al. 2008; Ramm and Stockley 2010). In addition to evolutionary responses, variation in the speed of spermatogenesis might also account for the adaptive plasticity in sperm production that has been observed in response to local sperm competition conditions (delBarco-Trillo and Ferkin 2004; Ramm and Stockley 2009; Firman et al. 2013). Our findings on house mice have important implications for the general assumption that larger testes confer greater rates of sperm production, and emphasise the need for explorations beyond a simple measure of testes size and into testes tissue organisation and function (Ramm and Schärer 2014).

As almost all male sex hormone production occurs in the testes interstitium (Stocco and McPhaul 2006), we contend that an increase in the proportion of seminiferous tubules and a reduction in the amount of interstitial tissue could have important implications for precopulatory sexual selection. Testosterone, which is produced by the Leydig cells, plays a critical role in the promotion of secondary sexual characters (Dufau 1996). For example, testosterone-dependent urine scent-marking behaviour in male house mice is indicative of dominance status and territory defense (Desjardins et al. 1973; Hurst 1990), and is assessed by females to gauge the quality of potential mates (Wolff 1985, Penn and Potts 1998). Variation in testosterone levels can lead to changes in the major urinary proteins that mediate the
release of pheromones (Harvey et al. 1989). Therefore, a reduction in testes interstitial
tissue, and consequently a reduction in the density of Leydig cells, could result in a
reduction in testosterone production and influence the quality or frequency of scent-
marks produced by males. A recent study has revealed that sexually receptive females
spent more time associating with males from the monogamous populations compared
to males from the polygamous populations, suggesting that these males have a
precopulatory advantage which could be attributable to an intrinsic quality associated
with the scents that they produce (Firman 2014). The divergence in testes tissue
composition among males evolving with and without sperm competition reported here
certainly warrants further research into evolutionary trade-offs between pre- and
postcopulatory sexually selected traits.

In summary, we have documented divergence in testes tissue composition
among house mice from populations that had evolved under a polygamous or
monogamous mating regime for 24 generations. Our statistical analysis revealed that
males that had evolved with sperm competition had testes with a significantly greater
proportion of seminiferous tubules compared with males that had evolved under
enforced monogamy. Our finding accounts for the previously reported divergence in
sperm number and sperm competitiveness that had been observed in the absence of
evolutionary changes in testes size (Firman and Simmons 2010; Firman and Simmons
2011). This study has important implications for the general, mostly untested
assumption that testes size is a strong predictor of sperm production, and emphasizes
the need for future research to explore the evolutionary implications of sperm
competition for testicular efficiency.
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Table 1. ANOVA comparing the proportion of sperm-producing tissue in the testes of males that have evolved under either a polygamous or monogamous selection regime.

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Figure 1. The mean proportion of seminiferous tubules in the testes of house mice from monogamous (3) and polygamous (3) selection lines (as measured from images of histological preparations).

Figure 2. Example images displaying the difference in the density of seminiferous tubules in the testes of house mice from monogamous (1-3) and polygamous (4-6) selection lines.
Figure 1.

Proportion of spermatogenic tissue (mean + se)

Selection line

Monogamous Polygamous

1 2 3 1 2 3
Figure 2.