

EVOLUTIONARY REDUCTION IN TESTES SIZE AND COMPETITIVE FERTILIZATION SUCCESS IN RESPONSE TO THE EXPERIMENTAL REMOVAL OF SEXUAL SELECTION IN DUNG BEETLES

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Sexual selection is thought to favor the evolution of secondary sexual traits in males that contribute to mating success. In species where females mate with more than one male, sexual selection also continues after copulation in the form of sperm competition and cryptic female choice. Theory suggests that sperm competition should favor traits such as testes size and sperm production that increase a male's competitive fertilization success. Studies of experimental evolution offer a powerful approach for assessing evolutionary responses to variation in sexual selection pressures. Here we removed sexual selection by enforcing monogamy on replicate lines of a naturally polygamous horned beetle, *Onthophagus taurus*, and monitoring male investment in their testes for 21 generations. Testes size decreased in monogamous lines relative to lines in which sexual selection was allowed to continue. Differences in testes size were dependent on selection history and not breeding regime. Males from polygamous lines also had a competitive fertilization advantage when in sperm competition with males from monogamous lines. Females from polygamous lines produced sons in better condition, and those from monogamous lines increased their sons condition by mating polygamously. Rather than being costly for females, multiple mating appears to provide females with direct and/or indirect benefits. Neither body size nor horn size diverged between our monogamous and polygamous lines. Our data show that sperm competition does drive the evolution of testes size in onthophagine beetles, and provide general support for sperm competition theory.

KEY WORDS: Laboratory evolution, *Onthophagus taurus*, polyandry, sperm competition.

Sexual selection arises because of competition between males for access to females, and is typically held responsible for the evolution of secondary sexual traits that serve as weapons and/or ornaments for the monopolization of breeding females (Darwin 1871; Andersson 1994). Sexual selection can also continue after copulation in the form of sperm competition (Parker 1970; Simmons 2001). Sperm competition theory predicts that when a male's fertilization success depends on the numbers of sperm present at the site of fertilization relative to other males, selec-

tion should favor increased male investment in sperm production (Parker 1998). It has also been suggested that the multiple mating behavior of females that generates sperm competition can facilitate cryptic female choice via the selective fertilization of eggs with sperm that confer fitness benefits for offspring. For example, if a male's investment in sperm production and competitive fertilization success depends on heritable variation in genetic quality, then females may confer these genetic benefits on their offspring by promoting sperm competition (Eberhard 1996; Yasui 1997;

Evans and Simmons 2008). However, male and female interests over fertilization may not always coincide. For example, male adaptations to sperm competition can incur costs for females in the form of physical harm and reduced lifetime reproductive success (Stockley et al. 1993), and the ensuing sexual conflict can generate sexual selection on male and female reproductive traits (Parker 1979; Arnqvist and Rowe 2005).

Consistent with sperm competition theory, there is now considerable evidence from comparative studies of animal mating systems to show that sperm competition is positively correlated with male investment in sperm production; species under stronger selection from sperm competition tend to have larger testes (e.g., Gage 1994; Harcourt et al. 1995; Hosken 1997; Byrne et al. 2002; Pitcher et al. 2005). Generally, there does appear to be sufficient standing genetic variation in testes size upon which selection could act, and responses to direct selection on testes size have been reported (reviewed in Simmons and Moore 2008). Variation in testes size among populations of the same species has also been shown to be associated with variation in the strength of selection arising from sperm competition (Brown and Brown 2003; Tan et al. 2004). However, there is evidence that male investment in sperm production can be developmentally plastic, depending on local variation in the strength of sperm competition (Gage 1995; Schärer and Ladurner 2003), so that among population covariation between the strength of selection due to sperm competition and testes size need not be indicative of evolutionary change. Few studies have actually documented evolutionary changes in testes size in response to variation in the strength of selection via sperm competition, and even fewer have documented evolutionary responses in competitive fertilization success.

Studies of experimental evolution offer a powerful approach for observing responses to selection in male and female reproductive traits. Perhaps not surprisingly given their short generation times, studies of experimental evolution have generally been restricted to muscid flies, and in particular to *Drosophila*. These studies have focused on systems in which there is sexual conflict, and have examined changes in male harm to females, and female resistance to male harm. Thus, the experimental removal of sexual selection by enforced monogamy in the naturally polygamous *D. melanogaster* has been shown to lead to evolutionary reductions in the extent to which males reduce female longevity, and to increases in female lifetime productivity (Holland and Rice 1999). Although male flies from monogamous lines had a reduction in testes size and sperm count, and their ejaculates became less effective in inhibiting female remating, competitive fertilization success did not show a consistent evolutionary response to the removal of sexual selection (Pitnick et al. 2000, 2001). Sexual selection intensity in evolving lines of *D. melanogaster* has also been manipulated by varying the adult sex ratio (Wigby and Chapman 2004, 2006; Linklater et al. 2007). In these exper-

iments females also evolved increased resistance to male harm, but there were no changes in testes size, the sizes of male accessory glands, or in female remating rates in response to a 3:1 male-biased sex ratio. More extreme manipulations of the sex ratio however, revealed that testis size was increased in populations with a 10:1 female-biased sex ratio, where the demands on male remating rates were high, and males were sperm limited (Reuter et al. 2008).

Decreased male harm and increased female fitness have also been reported from experimental reductions in the intensity of sexual selection within evolving populations of *D. subobscura* (Crudginton et al. 2005), and the dung fly *Sepsis cynipsea* (Martin and Hosken 2003a,b, 2004). However, only one study has found a significant evolutionary response in competitive fertilization success. In yellow dung flies, *Scathophaga stercoraria*, enforced monogamy was associated with a reduction in testes size and sperm competitiveness after just 10 generations of laboratory evolution (Hosken et al. 2001; Hosken and Ward 2001). In the only study not to have examined muscid flies, Tilszer et al. (2006) documented evolutionary reductions in the competitive fitness of male bulb mites, *Rhizoglyphus robini*, following 37 generations of enforced monogamy. In their male fitness assay, Tilszer et al. (2006) allowed a male from each selection line to compete for access to two females with two other control males, so that decreased fitness of males from monogamous lines could have been the product of reductions in their ability to secure matings and/or in their sperm competitiveness. Collectively, these studies of experimental evolution, although taxonomically restricted, show that sexual selection can be a persuasive force in the evolution of male reproductive physiology. However, the evidence for direct effects of sexual selection on testes evolution, and importantly male competitive fertilization success, is limited. Here, we extend the approach of experimental evolution to nonmodel systems by describing changes in testes size and competitive fertilization success of males from lines of a naturally polygamous dung beetle, *O. taurus*, evolving under enforced monogamy or polygamy.

Sexual selection is thought to have been important in the evolution of onthophagine dung beetles. The males of many species possess horns on the head and/or thorax that are used in contest competition over access to breeding tunnels excavated by females; males with larger horns are at a competitive advantage (Emlen 1997; Moczek and Emlen 2000) and enjoy greater fitness (Hunt and Simmons 2001). Comparative analyses across 14 species of *Onthophagus* support a role for sexual selection in the evolutionary radiation of onthophagine horns; male-biased operational sex ratios are evolutionarily associated with the presence of horns, although increased crowding and reduced ability for males to monopolize females is associated with hornlessness (Pomfret and Knell 2008).

Sperm competition is also an important aspect of onthophagine mating systems. The males of many onthophagines exhibit a suite of behavioral and morphological traits that characterize alternative mating tactics (Emlen 1997; Moczek and Emlen 2000; Emlen et al. 2005). Major males have enlarged horns and compete for access to females. Minor males have rudimentary horns or remain hornless, often resembling females, and sneak into breeding tunnels to copulate with females that are guarded by major males. By virtue of their alternative tactic, minor males are always subject to sperm competition, whereas major males are subject to varying risk and intensity of sperm competition dependent on the number of sneaks in the population (Simmons et al. 1999b; Hunt and Simmons 2002a). Comparative analyses across 16 species of *Onthophagus* have revealed positive associations between testes size and the proportion of males adopting the sneaking tactic (Simmons et al. 2007), equivalent to sperm competition risk in Parker's theoretical models (Parker 1990). Moreover, within species, sneaks invest more heavily in testes growth (Simmons et al. 2007), and a behavioral response to sperm competition risk in *O. taurus* is an increase in the frequency with which they mate with their female partners (Hunt and Simmons 2002a). These studies suggest that sperm competition favors the evolution of increased physiological investment in testes by male onthophagines. However, it has not yet been established whether testes size contributes to a male's competitive fertilization success, or if testes size can respond to selection via sperm competition.

Sperm competition is particularly strong in Australian populations of *O. taurus*, where up to 60% of the male population adopt the sneaking tactic (Simmons et al. 1999b). There is some evidence to suggest that both testes allometry, and the proportion of males adopting the sneak tactic have undergone evolutionary change since the species was introduced into Australia in the 1970s, driven by the high population densities in which this exotic population occurs (Moczek et al. 2002; Moczek 2003; Simmons et al. 2007). Quantitative genetic analysis of ejaculate traits suggest that there is certainly enough standing genetic variation within this population on which selection could act; the coefficient of additive genetic variation in testes weight is in the region of 16%, and the heritability is around 0.97 (Simmons and Kotiaho 2002). Moreover testes size appears to be condition dependent, being genetically correlated with heritable variation in male condition, measured as somatic weight after controlling for body size (Kotiaho et al. 2001; Simmons and Kotiaho 2002). We experimentally removed sexual selection by enforcing monogamy over 21 generations in three independent lines of *O. taurus*, and contrasted changes in body size and condition, testes size, and competitive fertilization success of males from these lines with males from three independent lines in which the normal polygamous mating system had been maintained. We predict that polygamy should select for males with relatively larger testes and higher competi-

tive fertilization success, whereas the reverse should be true for monogamy. Moreover, if females mate multiply to obtain fitness benefits for their offspring, we would expect the sons of females from polygamous lines to be of greater fitness than the sons of females evolving under enforced monogamy.

Methods

SOURCE POPULATION AND CULTURE

Approximately 1000 beetles were collected in December 2002, from fresh droppings at a dairy farm in Byford, Western Australia. They were maintained in the laboratory in mix sex cultures, and provided with unlimited access to fresh dung for 1 week. Females (300) were then separated and established in individual breeding chambers (PVC piping, 30 cm in length and 9 cm in diameter, three quarters filled with moist sand, and topped with 25 mL of fresh cow dung) and left to construct broods for 1 week. Breeding chambers were sieved, and batches of ~50 broods were buried in moist sand in 10-L plastic boxes. A single brood provides the resources for growth and development of a single offspring. Broods were incubated under constant temperature $28 \pm 2^\circ\text{C}$ and a 12 h:12 h light:dark cycle. After 3 weeks of incubation, brood boxes were checked every 2 days, for a period of 2 weeks, for emerging adults, which were housed in single sex cultures with constant access to fresh dung for 1 week of feeding and maturation. Mixed sex cultures of ~200 beetles were established in 30-L buckets, three quarters filled with moist sand and provided with 100 mL of fresh dung. After 1 week of mating, females were separated and established in individual breeding chambers and left to construct broods for 1 week. Breeding chambers were sieved, broods were incubated, and the sexes were separated on emergence as above. The total generation time was 8 weeks. This second generation of laboratory-reared beetles were used as the base stock for our selection lines.

MONOGAMOUS SELECTION LINES

We initiated three independent selection lines that were breeding monogamously (M) (without sexual selection). After maturation feeding, 180 females and 180 males were selected at random from the base stock, and 60 males and 60 females were allocated to each of the three replicate monogamous selection lines. From this point the lines were maintained in reproductive isolation from one another. Females were placed individually into a small plastic container (7 cm \times 7 cm \times 5 cm) three quarters filled with moist sand and topped with 1 teaspoon of fresh dung. A single randomly chosen male was introduced into each female's mating container and the pair was left for 1 week. Males were then discarded, and females were established in individual breeding chambers for 1 week. Breeding chambers were sampled at random, and the broods of 50 females that had produced ≥ 5 broods were

combined, and incubated as described above. Thus, the effective population size for each line was 100 (50 males and 50 females). On emergence, the offspring from each line were housed in single sexed cultures for 1 week of maturation feeding before beginning the next generation.

POLYGAMOUS SELECTION LINES

We also initiated three independent selection lines that were breeding polygamously (P) (with sexual selection). After maturation feeding, 180 females and 180 males were selected at random from the base stock, and 60 males and 60 females were allocated to each of the three replicate polygamous selection lines. Again, from this point the lines were maintained in reproductive isolation from one another. Ten males and 10 females were introduced into each of 6 30-L buckets, three quarters filled with moist sand and topped with 1 L of fresh dung. These populations were left for 1 week after which females were retrieved and males were discarded. Females were established in individual breeding chambers for 1 week, and the broods of 50 females that had produced ≥ 5 broods were incubated. In this case the effective population size was ~ 106 (50 females and ~ 56 males). The exact effective population size is unknown and would have depended on the mating success and paternity success of individuals within each population bucket (see below). On emergence, offspring were housed in single sexed cultures for 1 week of maturation feeding before beginning the next generation.

The extent of sexual selection operating within our polygamous populations can be estimated from a previous study that employed amplified fragment length polymorphism to analyze parentage within eight replicate populations of 10 male and 10 female beetles (Simmons et al. 2004). These populations were established, and allowed to breed under the same protocol as our polygamous lines. Based on the number of patriline represented within a female's brood, females within these populations mated polyandrously, with a mean \pm SE of 4.5 ± 0.2 , and a range of 1–8 males. Sperm utilization by multiple mated females conforms to a fair raffle, in which paternity is equally distributed, on average, across a female's mates (Simmons et al. 2004). Thus, there was strong sperm competition within our polygamous lines. Based on the number of females producing one or more offspring sired by each male, the effective mating frequency of males in these populations was 3.85 ± 0.02 , and ranged from 0 to 9. Across the eight populations in Simmons et al.'s (2004) study, the average standardized slope of male fitness (total number of offspring sired by each male) on number of mates (Bateman's gradient, Arnold and Duvall 1994) was 0.832 ± 0.019 , indicative of the opportunity for strong sexual selection within our polygamous populations.

Finally, we can use the data on parentage from these eight populations to estimate the average number of males that are likely

to have contributed to subsequent generations in our polygamous lines. From the eight populations in Simmons et al. (2004) we know how many males sired offspring produced by each of 80 females. From these real data we randomly extracted 50 females, and determined the number of males contributing to the offspring they produced. We resampled 10,000 times and calculated the average (95% CI) number of males contributing to offspring production when 10 females were housed with 10 males as 9.38 (8.88, 9.75). Thus, for the six populations that made up a polygamous selection line, we would expect 56.3 (53.3, 58.5) males to contribute to each generation of offspring.

MEASURES OF BODY SIZE, CONDITION, AND TESTES WEIGHT

We monitored changes in body size and testes weight across 21 generations of selection spanning 4.5 years of continuous breeding. At generations 6, 10, 11, 16, 18, and 20, samples of 10–20 males were taken at random following maturation feeding; thorax width to the nearest 0.01 mm was measured as an index of body size, and males were weighed to the nearest 0.01 mg. Males were then dissected, and their testes were removed and weighed, again to the nearest 0.01 mg. To avoid spurious correlations due to part-whole relationships (Christians 1999), we calculate soma weight as total body weight minus testes weight, and use this as our measure of body weight.

At generations 5, 14, and 17 we measured the length of the head horns and thorax width for 50 males from each selection line. For horn length we used measure 1 of Tomkins et al. (2006). Male *O. taurus* exhibit dimorphic male morphology; males larger than ~ 5 mm thorax width develop enlarged head horns whereas those smaller than ~ 5 mm thorax width have rudimentary horns (Simmons et al. 1999b). Horn length thus exhibits a bimodal distribution that cannot be normalized, and a nonlinear relationship with body size. Minor males are generally more numerous in *O. taurus* populations, so that when sampled at random, they will outnumber major males by $\sim 60\%$. To obtain an accurate estimate of the scaling relationship between thorax width and horn length, males were selected for measurement so that we had relatively equal numbers of individuals across the horn length distribution. For each population, we calculated the position of the switch point, the body size at which males switch to the major phenotype, using established protocols (Simmons et al. 1999b). We calculated 95% confidence limits and the mean from 500 bootstrapped values of the switch point, using custom written code (Knell 2008) for the software package R (R development core team 2008).

Previous studies have revealed considerable developmental plasticity in male allocation to sperm production in response to environmental cues of sperm competition risk (e.g., Gage 1995; Schärer and Ladurner 2003), so that differences in testes size between our polygamous and monogamously breeding lines could

arise from phenotypic plasticity in testes growth, rather than from genetic divergence. To examine genetic versus environmental effects on testes size we conducted a common garden experiment at generation 20. Thus, 30 unmated beetles from each line were sampled at random following maturation feeding, and established to breed using the opposite protocol to the selection line from which they were sourced; monogamous beetles were bred polygamously, and polygamous beetles were bred monogamously. Once generation 21 offspring had emerged and completed maturation feeding, we contrasted body size, condition, and testes weights of males bred under the appropriate selection protocols, with those from beetles that had been bred from monogamous or polygamous lines but with the reversed mating regime.

SPERM COMPETITION ASSAY

We conducted two replicate sperm competition assays, at generation 11 and 16. Females used in these assays were the unmated laboratory-reared offspring of females collected from the same location as the source population used to start the selection lines. Males were sampled at random from the lines following maturation feeding. We competed polygamous males against monogamous males, reversing their sequence position (first or second male to mate), and using all 18 possible line combinations. Individual males were used only once.

We used the irradiated male technique to assign offspring within families to each of a female's mates (Simmons 2001). Half of the subject males were anesthetized for 5 min in nitrogen, and then exposed to a 10 krad dose of gamma radiation from a Cobalt 60 source. This dose rendered males completely sterile. Thus, when a female was mated with both an irradiated and a nonirradiated male, those eggs that successfully hatched were scored as being fertilized by the nonirradiated male.

Matings were conducted in artificial tunnels constructed from clear rectangular plastic vials measuring $60 \times 36 \times 13$ mm. Vials were half filled with plaster to create a tunnel measuring $60 \times 17 \times 13$ mm. The plaster floors of tunnels were smeared with cow dung and dried. Before use, tunnels were lightly moistened with fresh water. An unmated female, randomly selected from stock culture, was placed into an artificial tunnel. Within 5 min, one experimental male was introduced and the pair observed until mating. Following their first copulation, females were housed individually in small plastic containers, and provided with a teaspoon of fresh dung. Twenty-four hours later, females were returned to the artificial tunnels and provided with their second mate. After the second copulation, females were established in individual breeding chambers and left to produce broods for 1 week. Broods were sieved and opened to determine paternity. At generation 11 we obtained a total of 50 mixed paternity families distributed equally across the 18 possible line combinations (2.8 ± 0.3 families per line combination). At generation 16 we obtained a further 63 mixed

paternity families, again distributed equally across the 18 possible line combinations (3.5 ± 0.4 families per line combination).

Results

EVOLUTIONARY RESPONSE IN BODY SIZE AND HORN ALLOMETRY

Male thorax width and body weight both varied across generations. However, there were no significant effects of our selection treatments on either measure (Supporting Table S1). The mean thorax width of male beetles in our populations declined from 4.73 ± 0.04 mm to 4.59 ± 0.03 mm, and body weight declined from 65.94 ± 1.69 mg to 60.22 ± 0.22 mg, from generation 6 to 20.

Measurement of horn morphology showed no changes due to selection history in absolute horn length (Supporting Table S2). Horn length was on average greater at generations 7 ($M = 1.38 \pm 0.07$ mm, $P = 1.26 \pm 0.07$ mm) and 19 ($M = 1.21 \pm 0.07$ mm, $P = 1.36 \pm 0.07$ mm) than at generation 16 ($M = 0.84 \pm 0.07$ mm, $P = 0.69 \pm 0.07$ mm). To examine relative horn length, we used the cubic spline fitting function in JMP to fit a smoothing spline ($\lambda = 0.01$) through the plot of horn length on thorax width for the entire dataset in Figure 1 ($N = 900$). The r^2 for the spline was 0.89, and residuals were saved as our measure of relative horn length. There was no significant effect of selection history or generation on relative horn length (Table S2). Neither were there any significant effects of selection history or generation on the switch point body size at which males adopt the major male phenotype (Fig. 1 and Supporting Tables S3 and S4).

EVOLUTIONARY RESPONSE IN CONDITION

We examined changes in the condition of male beetles in our lines by examining body weight controlling for thorax width (Kotiaho et al. 2001). Condition declined across the 20 generations (effect estimate ($\times 10 - 4$) = -5.8 ± 2.3), although the data suggested a marginally nonsignificant tendency for male condition to have remained higher in our polygamous lines (mean weight adjusted for body size: 73.76 ± 0.57 mg) compared with our monogamous lines (71.70 ± 0.58 mg) (Table 1). The effect of selection history on male condition was more evident in our experimental reversal of mating regimes at generation 20. Selection history and mating regime had significant effects on the condition of male offspring, and there was a significant interaction effect (Table 2). Monogamous mating was associated with reduced male offspring condition for monogamous lines, but not for polygamous lines (adjusted means for selection history[mating regime]: $M[M] = 55.34 \pm 0.74$ mg; $M[P] = 61.12 \pm 0.69$ mg; $P[M] = 62.59 \pm 0.76$ mg; $P[P] = 61.41 \pm 0.67$ mg).

EVOLUTIONARY RESPONSE IN TESTES WEIGHT

The mean testes weight for beetles in the source population was 2.91 ± 0.07 mg. Given the changes in body weight across

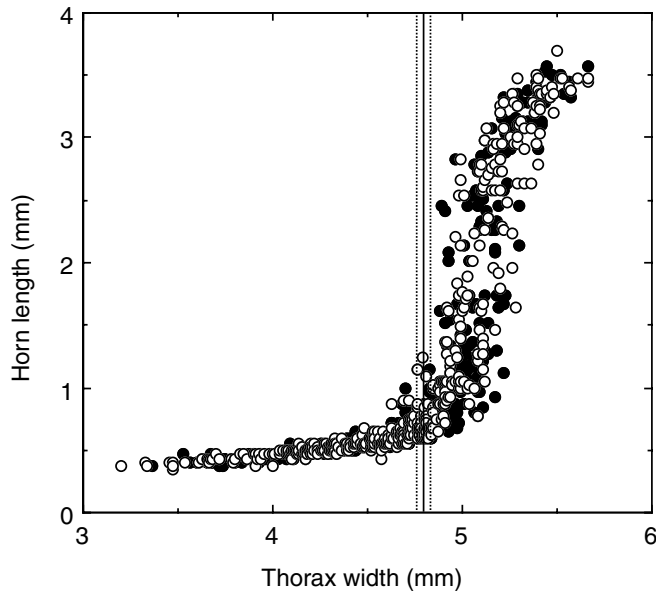


Figure 1. Combined horn allometry from males sampled at generations 5, 14, and 17 from three replicate lines breeding monogamously (open symbols) or polygamously (closed symbols). The grid lines provide the mean and 95% confidence intervals of the switch point body size between minor and major males (see text for more details).

generations in our lines, we controlled for body weight in our analyses of testes weight. We found significant effects of both selection history and generation on relative testes weight, and an interaction effect between selection history and generation (Table 3). Posthoc analyses within selection treatments revealed the source of the interaction effect; after controlling for body weight, there was a significant linear reduction in the average testes weights across generations of monogamous line males (partial effect of

Table 1. ANOVA of condition (log body weight after controlling for thorax width), across 20 generations of three replicate lines of *Onthophagus taurus* breeding either monogamously or polygamously.

Effect	SS	df	MS	F	P
Selection history	0.030	1	0.030	6.60	0.062
Line[selection history] ¹	0.018	4	0.005	5.06	0.001
Generation	0.005	1	0.005	6.27	0.013
Selection × Generation	0.001	1	0.001	0.15	0.716
Generation × Line [selection history] ²	0.019	4	0.005	6.74	<0.001
Thorax width	4.595	1	3.196	6377.85	<0.001
Error ³	0.448	622			

¹Error term for effect of selection history.
²Error term for effect of selection × generation.
³Error term for effects of the covariates generation and thorax width.

Table 2. ANOVA of condition (log body weight controlling for thorax width), for males at generation 21 of the polygamous and monogamous selection lines, that were bred under both polygamous and monogamous mating regimes.

Effect	SS	df	MS	F	P
Selection history	0.016	1	0.016	11.82	0.026
Line[selection history] ¹	0.005	4	0.001	3.23	0.143
Breeding regime	0.006	1	0.006	14.24	0.019
Selection × Breeding	0.012	1	0.012	28.52	0.006
Breeding × Line [selection history] ²	0.002	4	0.000	0.58	0.676
Thorax width	0.427	1	0.427	600.96	<0.001
Error ³	0.058	82			

¹Error term for the effect of selection history.
²Error term for the effect of breeding regime, and selection × breeding regime.
³Error term for replicate lines nested within selection history, breeding history × replicate lines nested within selection history, and the covariate thorax width.

generation $F_{1,15} = 11.03, P = 0.004$). The addition of a quadratic term for generation did not improve the model fit ($F_{1,14} = 1.26, P = 0.281$). In contrast, there was a significant nonlinear increase in testes weights across generations of polygamous line males (partial effect of generation $F_{1,14} = 5.83, P = 0.030$, quadratic term for generation $F_{1,14} = 5.03, P = 0.042$) (Fig. 2, patterns of variation in raw testes weights are depicted in Supporting Fig. S1).

Differences in testes weights between lines could in theory stem from environmental effects associated with the mating regimes. Experimental reversal of mating regimes within lines at generation 20 suggest that this was not the case. Selection history had a significant effect on testes weight for males at generation 21, but the mating regime experienced by their parents did

Table 3. ANOVA of log testes weight after controlling for log body weight, across 20 generations of three replicate lines of *Onthophagus taurus* breeding either monogamously or polygamously.

Effect	SS	df	MS	F	P
Selection history	0.519	1	0.519	24.36	0.008
Line[selection history] ¹	0.086	4	0.021	5.13	<0.001
Generation	0.070	1	0.070	17.11	<0.001
Selection × Generation	0.096	1	0.096	17.82	0.014
Generation × Line [selection history] ²	0.022	4	0.005	1.31	0.265
log body weight	0.904	1	0.904	219.80	<0.001
Error ³	2.559	622			

¹Error term for the effect of selection history.
²Error term for the effect of selection × generation.
³Error term for effect of the covariate generation.

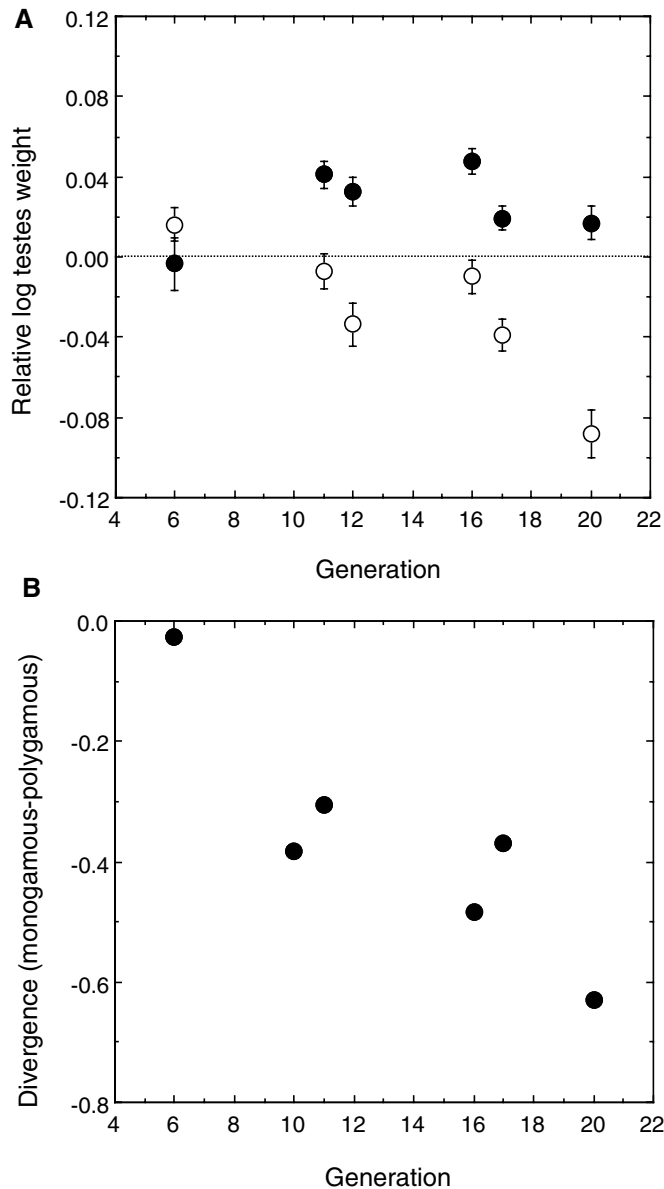


Figure 2. (A) Mean (\pm SE) log testes weights controlling for body weight across 20 generations of three replicate monogamous (open symbols) and polygamous (closed symbols) breeding lines of *Onthophagus taurus*; (B) Observed divergence (monogamous-polygamous) in log testes weights controlling for body weight between treatments.

not, and there was no significant interaction between selection history and mating regime (Tables 4 and 5). Males from monogamous lines had smaller testes than males from polygamous lines. These data suggest that the observed differences in testes size between selection treatments were genetically based, rather than environmental.

SPERM COMPETITION ASSAY

There were no significant selection history, generation, or interaction effects on the proportion of eggs that were fertilized when

Table 4. Mean (\pm SE) testes weights at generation 21 when animals were derived from monogamous and polygamous selection lines breeding under both monogamous and polygamous mating regimes.

Selection lines	Mating regime	
	Polygamous	Monogamous
Polygamous (1)	2.63 \pm 0.09	2.93 \pm 0.13
Polygamous (2)	2.62 \pm 0.10	3.11 \pm 0.15
Polygamous (3)	2.57 \pm 0.11	2.49 \pm 0.11
Mean (\pm SE)	2.60 \pm 0.06	2.81 \pm 0.09
Monogamous (1)	2.03 \pm 0.13	2.11 \pm 0.07
Monogamous (2)	2.15 \pm 0.20	1.87 \pm 0.10
Monogamous (3)	2.23 \pm 0.11	1.98 \pm 0.15
Mean(\pm SE)	2.13 \pm 0.09	2.00 \pm 0.07

field collected females were mated with nonirradiated males from the six selection lines (whole model $F_{11,24} = 0.413, P = 0.936$). Thus, noncompetitive fertilization success did not vary between our monogamous and polygamous treatments. The proportion of eggs fertilized when females mated with a nonirradiated male was less than 1 ($0.88 \pm 0.02, N = 36$). Because a nonirradiated male's paternity is scored based on hatched versus unhatched eggs, the absolute competitive fertilization success of nonirradiated males in our sperm competition trials represent conservative estimates. The proportion of offspring sired by nonirradiated males when females mated with both a nonirradiated and an irradiated male in the replicate sperm competition assays are shown in Figure 3. The overall mean proportion of offspring sired by nonirradiated males was $0.66 \pm 0.02 (n = 113)$. We analyzed the data using

Table 5. ANOVA of log testes weight controlling for log body weight, for males at generation 21 of the polygamous and monogamous selection lines, that were bred under both polygamous and monogamous mating regimes (data in Table 4).

Effect	SS	df	MS	F	P
Selection history	0.263	1	0.263	23.43	0.008
Line[selection history] ¹	0.046	4	0.011	3.02	0.154
Breeding regime	0.001	1	0.001	0.25	0.644
Selection \times Breeding	0.014	1	0.014	3.75	0.123
Breeding \times Line [selection history] ²	0.015	4	0.004	0.79	0.536
log body weight	0.104	1	0.104	21.49	<0.001
Error ³	0.397	82			

¹Error term for the effect of selection history.

²Error term for the effect of breeding regime, and selection \times breeding regime.

³Error term for replicate lines nested within selection history, breeding history \times replicate lines nested within selection history, and the covariate log body weight.

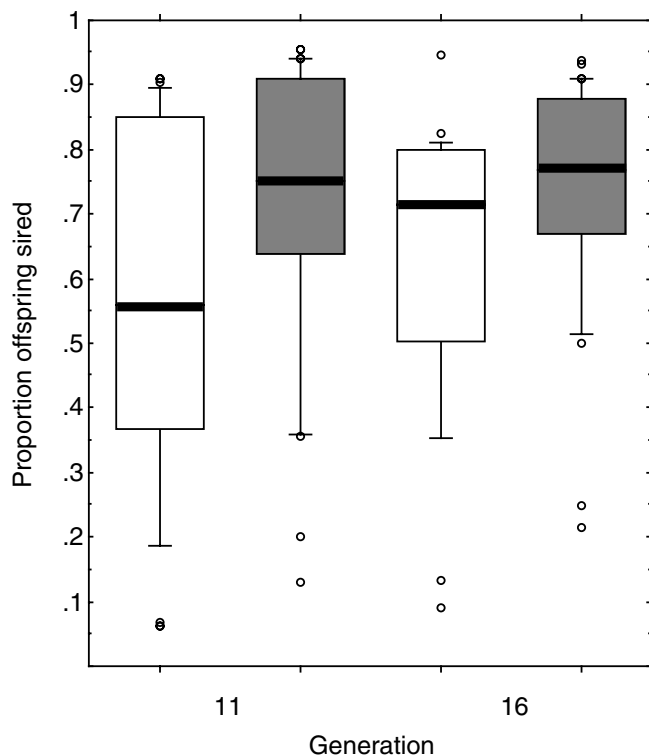


Figure 3. Box plot of the proportion of offspring sired by focal male *Onthophagus taurus* from monogamous lines when in competition with irradiated males from polygamous lines (open bars), or focal males from polygamous (shaded bars) lines when in competition with irradiated males from monogamous lines. Two replicate experiments were conducted, at generations 11 and 16.

a generalized linear model, with the number of larvae (and thus eggs fertilized by the nonirradiated male) as the dependent variable, the number of broods as the binomial denominator, and a logit link function. Because of overdispersion, we used F -tests, rather than chi-square, to test statistical significance (Crawley 1993). Again interaction effects were not significant and removed from the model (all P s > 0.525). There was a significant effect of the nonirradiated male's selection history on his fertilization success ($F_{1,109} = 7.03$, $P = 0.009$), and no effect of the generation at which the experiment was run ($F_{1,109} = 0.54$, $P = 0.463$). Males from polygamous lines had a fertilization advantage when competing against males from monogamous lines (Fig. 3). The effect of the nonirradiated male's sequence position was also significant ($F_{1,109} = 4.20$, $P = 0.043$). Although nonirradiated males always out-competed irradiated males as expected (Simmons 2001), they had a slightly greater advantage when mating second (first to mate: 0.62 ± 0.03 , second to mate: 0.69 ± 0.03).

Discussion

Experimental removal of sexual selection by enforced monogamy generated an evolutionary reduction in testes weight and compet-

itive fertilization success in replicated lines of a naturally polygamous onthophagine dung beetle. The decline in male fitness is unlikely to have been due to inbreeding depression in the monogamous lines. Our experimental design minimized differences in effective population sizes between monogamous and polygamous treatments, and although the reduced size and weight of beetles in all lines could be indicative of inbreeding depression (but see below), this effect did not differ between our selection treatments (see also Rice and Holland 2005). These data provide empirical evidence that selection via sperm competition can be responsible for the evolution of testes size and competitive fertilization success for male *Onthophagus* (Simmons et al. 2007). More generally they provide empirical support for theoretical models of ejaculate evolution that predict increased testes size in response to sperm competition (Parker 1998).

The response in testes size across our experimental treatments was asymmetrical. Response to selection is often weaker in the direction of increased fitness (Hill and Caballero 1992), and our data suggest that this was the case for our polygamous populations; although testes size increased initially, the strength of sexual selection was insufficient to generate continuous increases in testes size. Conversely, a continuous reduction in testes size was favored in our monogamous populations where sexual selection was removed. Evolutionary reversals to ancestral character states following the experimental removal of selection have been reported previously (Hill and Caballero 1992; Teotónio and Rose 2000; Beldade et al. 2002; Hall and Colegrave 2008), and are expected where traits under selection are costly to produce. In *Onthophagus*, there are resource allocation trade-offs between morphological traits that occur throughout the developing organism (Emlen and Allen 2004), including a trade-off between testes and morphological traits such as body and horn size (Simmons and Emlen 2006). Investment in testes size and sperm production has been shown to have physiological costs for male immune function in other insects (Hosken 2001; Simmons and Roberts 2005), and these costs are likely to favor the evolutionary reduction in testes size observed here, and to oppose the evolutionary increase in testes size in polygamous populations.

Our findings are congruent with a previous study in which sexual selection was removed from lines of yellow dung flies, *S. stercoraria*. Hosken and Ward (2001) enforced monogamy in lines of these flies, finding decreases in testes size and competitive fertilization success (Hosken et al. 2001) after just 10 generations of selection. In contrast, although removal of sexual selection from lines of *D. melanogaster* (Pitnick et al. 2001) decreased testes size, it had no impact on competitive fertilization success even after 81 generations of selection. Moreover, experimental manipulation of sexual selection for 67 generations had no impact on testes size in Linklater et al.'s (2007) study of *D. melanogaster*. We believe that differences in the mechanisms of sperm competition

between species may underlie these different responses to selection.

In *O. taurus*, the outcome of sperm competition conforms to a fair raffle in which the proportion of offspring sired by a given male depends on the relative number of males competing for fertilizations (Tomkins and Simmons 2000; Simmons et al. 2004; House and Simmons 2006). Variation about the average paternity expected from a fair raffle does occur in this species, and depends on aspects of male morphology such as genital morphology (House and Simmons 2003, 2005) and sperm length (García-González and Simmons 2007). Nevertheless, under the fair raffle a male's share in paternity will depend largely on the number of sperm he has in the fertilization set (Parker et al. 1990), so that selection from sperm competition is expected to act directly on sperm production, and thus testes size, as found in our study. In *S. stercoraria*, the sperm from copulating males displace rival sperm from the female's sperm stores during copulation (Parker and Simmons 1991; Simmons et al. 1999a), so that again the ability of males to transfer large numbers of sperm, and thus testes size, should be subject to selection under sperm competition. Like *S. stercoraria*, male *D. melanogaster* displaces rival sperm from the female's sperm stores to gain a fertilization advantage. However, in *D. melanogaster*, accessory gland proteins (acps) play a major role in the mobilization and displacement of rival sperm (recently reviewed in Ram and Wolfner 2007). As such, the amount of sperm transferred, and thus testes size, may not be the primary focus of selection under sperm competition. Rather, acp production is expected to respond to experimental manipulations of sperm competition intensity. Indeed, although the actual size of accessory glands did not respond to manipulations of operational sex ratio in *D. melanogaster*, their rate of depletion did, with males from male-biased lines becoming acp depleted more rapidly with successive copulations than males from female-biased lines (Linklater et al. 2007). These data suggest that when sperm competition is common and mating opportunities rare, males are selected to invest more acps per mating and suffer from accessory gland depletion. Moreover, an extreme female-biased sex ratio, which imposed high mating demands on male sperm production to maintain fertility but little or no sperm competition, was shown to generate evolutionary increases in testes size in *D. melanogaster*, whereas an extreme male-biased sex ratio, and thus intense sperm competition, was shown not to influence testes size (Reuter et al. 2008). Collectively, these data suggest, therefore, that selection from sperm competition acts on acp rather than sperm production in *Drosophila*, a conclusion congruent with the finding that experimentally enforced monogamy leads to a reduction in the harmful effects of male acps on females (Holland and Rice 1999).

Previous studies of experimental evolution have manipulated sexual selection to explore the evolutionary potential of sexual

conflict. Enforced monogamy generates sexual harmony because a male's fitness depends on the fitness of his mate. Therefore, any male traits that reduce female fitness should be lost, resulting in an increased fitness of females and their male partners. Consistent with sexual conflict theory, studies of experimental evolution have often found that reductions in traits that contribute to male fitness under sexual selection are associated with increased female fitness under enforced monogamy (Holland and Rice 1999; Martin and Hosken 2004; Crudgington et al. 2005). However, for the most part these studies have been conducted using species in which, a priori, mating is known to be costly for females so that sexual conflict seems likely. In *O. taurus* there is little evidence to suggest that multiple mating is costly for females. Females do not suffer a longevity cost of mating (Hunt et al. 2002). Rather, major males offer extensive paternal assistance during brood provisioning that ameliorates the cost of reproduction for females (Hunt et al. 2002). Even in the absence of paternal care, seminal fluid effects have been found to increase female life span and lifetime reproductive success (Kotiaho et al. 2003). These data suggest, therefore, that polyandry may provide females with direct fitness benefits. Moreover, there is also evidence to suggest that females might obtain indirect fitness benefits from polyandry. Both courtship rate and testes size are positively genetically correlated with male condition, whereas sperm length is negatively genetically correlated with condition (Kotiaho et al. 2001; Simmons and Kotiaho 2002). Females prefer males with high courtship rates in precopulatory sexual selection (Kotiaho et al. 2001), there is a female-mediated fertilization advantage for shorter sperm (García-González and Simmons 2007), and the results from our selection lines indicate that testes size contributes positively to competitive fertilization success. Thus, polyandrous females could ensure their offspring are sired by males in good condition who offer indirect genetic benefits to offspring (Keller and Reeve 1995; Yasui 1997; Evans and Simmons 2008). We found a significant interaction effect between selection history and mating regime on the condition of male offspring; females from monogamous lines produced sons of equivalent condition to those from polygamous lines when allowed to mate polygamously, but of lower condition when mating monogamously. Such an interaction effect might be expected if pre- and postcopulatory mechanisms of sexual selection had removed deleterious mutations that affect condition in our polygamous lines, but were unable to do so under enforced monogamy (Radwan 2004). Thus, the fitness benefits of polyandry were more pronounced in lines with a history of enforced monogamy. In general, the data seem more consistent with direct and/or indirect benefit models of sexual selection in *O. taurus*, than with sexual conflict.

We found no consistent response in body size or horn length to the removal of sexual selection. The lack of response in these traits is perhaps not surprising. Both traits are determined

predominantly by the amount of dung provided by the female in the brood mass; estimates of the levels of additive genetic variance in body size and horn length are consistently low, and influenced strongly by maternal brood size effects (Hunt and Simmons 2002b; Kotiaho et al. 2003). Nevertheless, we did find significant reductions in body size in all lines, irrespective of selection regime. Reduced body size could theoretically have arisen as a result of inbreeding depression. Alternatively, or in addition, we may have imposed phenotypic selection for decreased body size in our lines. A key aspect of the breeding system of *O. taurus* that we removed from both monogamous and polygamous lines was the provision of paternal care; females produced broods alone. Paternal care contributes significantly to brood size and realized adult offspring size in this system (Hunt and Simmons 2000). Furthermore, maternal provisioning is positively correlated with female size (Hunt and Simmons 2000, 2002b). These two phenotypic effects are likely to have had reinforcing effects in reducing the size of beetles in our lines; absence of paternal care is expected to reduce the size of female adult offspring who are in turn expected to produce smaller broods and grand-offspring. Phenotypic evolution in the absence of additive genetic variance is expected when environments contributing to phenotype are themselves heritable. Such effects can reinforce or inhibit evolutionary change across generations (Wolf et al. 1998; Rauter and Moore 2002), and have been identified as potentially influential in the evolution of body size in *O. taurus* (Hunt and Simmons 2002b).

Although body size and horn length show little additive genetic variance, previous studies of onthophagines have found genetic variance in the allometric relationship between body size and horn length; specifically, direct selection for relatively large or small horns generated increases or decreases in relative horn length, and also corresponding changes in the position of the body size switch point delineating minor and major phenotypes (Emlen 1996). Moreover, evolutionary divergence in horn allometry has been reported from exotic populations of *O. taurus*, and is thought to be the consequence of selection arising from variation in population density (Moczek et al. 2002; Moczek 2003). The removal of sexual selection from our laboratory populations had no impact on the position of the switch point between minor and major male phenotypes, or in relative horn length. Given that this population is known to have responded previously to selection on these traits (Moczek et al. 2002; Moczek 2003), our data suggest either that our treatments were insufficient to impose variation in pre-mating sexual selection, or that longer periods of selection are required to realize such a response. We suspect the latter may be the case, because populations of *O. taurus* established under the same breeding regime as our polygamous lines did show evidence of selection on male horn morphology (Hunt and Simmons 2001) that would have been absent in our monogamous treatment.

In conclusion, we found that the experimental removal of sexual selection from replicate lines of *O. taurus* led to a reduction in testes size and male competitive fertilization success. Our findings lend support to theoretical expectations that sperm competition should favor the evolution of increased testes size (Parker 1998; Parker and Ball 2005). Like Hosken and Ward's (2001) work on yellow dung flies, our findings also provide direct experimental evidence for the evolutionary change thought to underlie macroevolutionary patterns in testes size variation across animal taxa.

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Supporting Information

The following supporting information is available for this article:

Table S1. ANOVAs of thorax width and log body weight, across 20 generations of three replicate lines of *Onthophagus taurus* breeding either monogamously or polygamously.

Table S2. ANOVAs of absolute log horn length, and residual horn length from a smoothing spline fitted to the plot of horn length on thorax width, across generations 5, 14, and 17 of three replicate lines of *Onthophagus taurus* breeding either monogamously or polygamously.

Table S3. Switch point thorax widths and their 95% confidence intervals for populations of males sampled at generations 5, 14, and 17 of three replicate lines of *Onthophagus taurus* breeding either monogamously or polygamously.

Table S4. ANOVA of the switch point thorax width between minor and major male phenotypes, across generations 5, 14, and 17 of three replicate lines of *Onthophagus taurus* breeding either monogamously or polygamously.

Figure S1. Changes in raw testes weight across 20 generations and three replicate monogamous (open symbols) and polygamous (closed symbols) breeding lines of *Onthophagus taurus*.

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