

Population density mediates the interaction between pre- and postmating sexual selection

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When females mate with more than one male, sexual selection acts both before and after mating. The interaction between pre- and postmating episodes of selection is expected to be context dependent, but few studies have investigated how total sexual selection changes under different ecological conditions. We examined how population density mediates the interaction between pre- and postmating sexual selection by establishing replicate populations of the horned dung beetle *Onthophagus taurus* at low, medium, and high densities, and then using microsatellite-based parentage analyses to measure male fitness. We found that mating success and fertilization success were positively correlated at all three densities, but the strength of the correlation decreased with increasing density. We also found a shift from negative to positive linear selection on testes mass as density increased, and opposing selection on weapons and testes at high densities. These patterns suggest that the importance of postmating processes increases with increasing population density, which reduces the selective advantage of weapons for premating contest competition, and increases the selective advantage of large ejaculates for postmating sperm competition. We expect that density-dependent selection on testes mass has contributed to the phenotypic variation observed between natural populations of *O. taurus* that differ in density.

KEY WORDS: Dung beetle, multivariate selection, *Onthophagus*, sexual selection, sperm competition, variance decomposition.

Sexual selection is a powerful evolutionary force that is responsible for driving many of the differences in behavior, morphology, and physiology that are found among sexes and species (Darwin 1871; Andersson 1994). When Darwin first proposed his theory of sexual selection, he assumed that females were monogamous, and that sexual selection occurred exclusively before mating via male–male competition and/or female choice (Darwin 1871). Over the past few decades, however, it has become evident that females often mate with multiple partners, so that sexual selection can continue after mating, inside the female’s reproductive tract via sperm competition and/or cryptic female choice (Eberhard 1996; Birkhead and Møller 1998; Simmons 2001). A male’s overall reproductive success therefore depends on the outcomes of competitive interactions both before and after mating, and studies that only investigate pre- or postmating selective episodes are likely to

give poor estimates of the net effect of sexual selection in driving trait evolution (Andersson and Simmons 2006; Eberhard 2009; Hunt et al. 2009; Kvarnemo and Simmons 2013). Although there are now several studies across a broad range of taxa that quantify how pre- and postmating episodes interact (reviewed in Evans and García-González 2016), the body of literature is still too small to determine whether the two episodes of selection typically act concordantly or antagonistically, or to generalize about the relative importance of each episode in determining the overall strength, direction, and form of selection on pre- and postmating sexual traits.

The net effect of pre- and postmating sexual selection pressures is also expected to be sensitive to changes in a suite of environmental, ecological, and social conditions (Evans and García-González 2016). For example, seasonal variation in

resource availability can influence the strength and direction of mate choice (Gwynne and Simmons 1990; Cornwallis and Uller 2010; Gillespie et al. 2014; Miller and Svensson 2014); variation in population density and demographic structure can have strong effects on sexual selective pressures and mating system dynamics (Kokko and Rankin 2006; Kasumovic et al. 2008; Kvarnemo and Simmons 2013; McDonald et al. 2013); and differences in the degree of female monopolizability can influence the importance of male–male contest competition and thus investment in pre- and postmating sexual traits (Parker et al. 2013; Lüpold et al. 2014; Buzatto et al. 2015; Lüpold et al. 2017; Simmons et al. 2017). Because of the variability in how these factors can shape the strength, direction, and form of pre- and postmating episodes of selection and their covariance, a complete understanding of total sexual selection in any particular system will require investigation of pre- and postmating selection across different environmental and ecological contexts (Evans and García-González 2016).

The horned dung beetle *Onthophagus taurus* is an ideal species for investigating the interactions between pre- and postmating sexual selection because sexual selection is known to act strongly both before and after mating. Like many other *Onthophagus* species, *O. taurus* males are morphologically and behaviorally dimorphic. Males above a critical body size threshold develop a pair of long, curved head horns that they use in fights with rival males over ownership of breeding tunnels containing females, while males below the body size threshold have no horns, or only rudimentary horns, and rely on sneaking behaviors to gain matings inside the tunnels guarded by larger males (Emlen 1997; Moczek and Emlen 1999, 2000). Because matings occur exclusively within the underground tunnels, a male's ability to win fights and defend ownership of a breeding tunnel is critical to his ability to gain access to mates, and male–male competition favors large males with long horns (Emlen 1997; Moczek and Emlen 2000; Hunt and Simmons 2001; McCullough and Simmons 2016). Female choice is also important in determining a male's mating success because females cannot be forced to mate. In particular, a male that gains access to a breeding tunnel must court the resident female by vigorously tapping on her back with his forelegs and antennae to persuade her to open her genitalia before mating can occur (Werner and Simmons 2008), and females preferentially mate with males that deliver high courtship rates (Kotiaho et al. 2001; Kotiaho 2002; McCullough and Simmons 2016). Finally, turnovers in tunnel ownership following male contests are common, and small sneaker males frequently dig intercepting side tunnels to enter and mate inside the tunnels of larger males (Emlen 1997; Moczek and Emlen 2000), so females can encounter, mate with, and store sperm from many different males (Simmons et al. 2004; McCullough et al. 2017). Sexual selection thus continues after mating in the form of sperm competition and cryptic female choice, favoring males with large testes (Simmons

et al. 1999, 2007; Simmons and García-González 2008) and short sperm (Simmons and Kotiaho 2002, 2007; García-González and Simmons 2007). These studies indicate that all four mechanisms of sexual selection (pre mating male–male competition, female mate choice, sperm competition, and cryptic female choice) are important in this species, yet it is still unknown how pre- and postmating episodes interact to determine the net strength and pattern of selection on male traits.

Onthophagus taurus is also ideal for exploring the potential impact of ecology on the interplay between pre- and postmating sexual selection because the species exhibits a wide geographic range and diverse ecological conditions that appear to have influenced male investment in sexual traits (Moczek and Nijhout 2003). Although *O. taurus* originally exhibited a Mediterranean distribution (Balthasar 1963), it was accidentally introduced to the eastern United States in the 1960s (Fincher and Woodruff 1975), and deliberately introduced to Western Australia in the 1970s as part of a biological control program to remove cow dung and combat pest bush-fly populations (Tyndale-Biscoe 1990). The exotic populations have become established and expanded their range across both continents (Silva et al. 2016), and Western Australian populations, in particular, have grown explosively, and can reach exceptionally high densities in the field—sometimes exceeding 5000 adults per dung pad (Hunt et al. 1999). Intriguingly, the two populations have also diverged significantly in the threshold body size that separates major and minor male morphs. Males in the high-density Australian populations remain hornless over a much larger range of body sizes than males in the United States, such that Australian populations are comprised of a much larger proportion of the hornless sneaker morph (Moczek 2003; Moczek and Nijhout 2003). Additionally, the males of Australian populations invest more in testes growth than do males in the United States (Simmons et al. 2007). These patterns are consistent with the hypothesis that differences in population density can drive rapid and substantial changes in male investment toward traits used in pre-mating contest competition and postmating sperm competition. Under low-density conditions, large- and medium-sized males are expected to be successful in deterring rivals from breeding tunnels, so most males should benefit from fighting and developing horns. By contrast, under high-density conditions, males are expected to be unable to monopolize females through direct combat due to the higher number of intruders, so sperm competition will be more important, and most males should benefit from remaining hornless and investing heavily in ejaculates (Hunt and Simmons 2002; Moczek 2003). However, whether population density influences the relative strengths of selection acting on pre- and postmating traits is unknown.

This study aims to expand our understanding of how pre- and postmating sexual selection interact in affecting total sexual selection, using *O. taurus* dung beetles as a model system.

Specifically, we established replicate populations at low, medium, and high densities, and used microsatellite-based parentage analyses to reconstruct the mating success and fertilization success of each male within our experimental populations. We quantified the relative contributions of these fitness components in explaining the overall variance in male reproductive success, and also directly estimated the strength and direction of selection on both pre- and postmating phenotypic traits. Our data allow us to explore how total sexual selection is mediated by variation in population density.

Methods

EXPERIMENTAL POPULATIONS

We established replicate populations ($n = 3$) of freely interacting males and females at each of three different population densities: low (10 males and 10 females), medium (20 males and 20 females), and high (40 males and 40 females). The females used in the experiments were virgin, first-generation offspring from beetles that were collected from a dairy farm in southwest Western Australia and bred in the laboratory following published protocols (Simmons and Kotiaho 2002). Newly emerged females were kept in single-sex populations and fed fresh cow dung ad libitum for two weeks prior to experiments to ensure that they were sexually mature and unmated. The males used in the experiments were collected in the field from the same dairy farm and housed in single-sex populations with fresh cow dung for 10 days prior to experiments.

To ensure that male reproductive success was examined across the full range of male phenotypes, we assigned individuals to each of the nine experimental populations by selecting males that approximated a normal distribution of body sizes across seven body size classes (based on pronotum width), simulating the natural range and distribution of sizes found in field populations (Hunt and Simmons 2001). Assigned males and randomly selected females were placed in 15-liter plastic buckets (32 cm in height and 28 cm in diameter) that were half-filled with moist sand and topped with 1 L of cow dung, simulating a single dung pad. The populations were allowed to interact freely for seven days before being sieved for adults and brood balls. We chose seven days as an appropriate duration for the experiment because this is approximately how quickly the high-density populations (80 individuals) entirely consumed 1 L of dung (Ridsdill-Smith et al. 1982).

At the end of the experiment, females were placed in individual breeding chambers (PVC piping, 25 cm in length and 6 cm in diameter, filled with moist sand and 100 mL cow dung) and left undisturbed for 10 days to build brood balls. The females were then sieved from the breeding chambers and frozen at -20°C

in ethanol. Brood balls were collected and opened carefully, and any developing larvae were frozen at -20°C in ethanol. If a brood ball contained a viable egg, it was buried in moist sand in a small plastic container to allow the egg to hatch and checked again after two days for a developing larva. Brood balls that contained dead or unfertilized eggs were discarded.

Males were collected, weighed to the nearest 0.1 mg on a digital balance, and frozen at -20°C . They were then dissected, and their testes removed and weighed. The hind legs were removed and stored in ethanol at -20°C for subsequent DNA extraction. We also took a digital photograph of each male's head for horn measurements. Horn length was measured in ImageJ as the length of the horn curve from the lowest point on the top of the head to the center of the horn tip (Measurement 4 in Tomkins et al. 2006).

PATERNITY ANALYSIS

We used the EDNA HiSpEx tissue kit (Fisher Biotech) to extract genomic DNA from the hind legs of all mothers and putative fathers, and the head capsules of all larvae. We determined the genetic profile of all individuals and assigned paternity to males in each of the nine experimental populations using six microsatellite loci recently developed for *Onthophagus taurus* (McCullough et al. 2017; Table S1). The loci were amplified in 10 μL reactions containing 1 \times PCR buffer, 3 mM MgCl_2 , 0.5 μg bovine serum albumin, 0.2 mM dNTPs, 0.5 μM of each primer, 0.5 U Platinum Taq DNA polymerase (Invitrogen), and 1 μL DNA template. The forward primers were labeled with one of four fluorescent dyes: NED (Thermo Fisher), FAM, PET, or VIC (AlphaDNA). The cycling protocol included an initial denaturation step at 94°C for 3 min, 30 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s, and then a final elongation step at 72°C for 10 min. PCR products were visualized on an ABI 3730 \times 1 DNA Analyzer using GeneScan-500 LIZ as an internal size standard, and the profiles were scored using GENEMARKER software (SoftGenetics). All profiles were verified individually, and only samples showing strong and unambiguous peaks were included in the final analysis. Paternity was assigned to offspring at the 80% confidence level (Pemberton et al. 1999) using the paternity inference software CERVUS 3.0 (Marshall et al. 1998; Kalinowski et al. 2007).

BATEMAN GRADIENTS AND THE OPPORTUNITY FOR SEXUAL SELECTION

We evaluated the relationship between the number of mates and number of offspring (the Bateman gradient) for males and females by fitting generalized mixed models with a log link function, Poisson errors, and population replicate as a random effect. We only included individuals that produced at least one offspring in the analysis of the Bateman gradients to obtain the most comparable estimates between males and females (Gerlach et al. 2012).

We first ran the models for the sexes separately to examine the effect of density on the Bateman gradients, and then ran models with the sexes combined to examine the effects of density, sex, and their interaction on the Bateman gradients. We evaluated the significance of the fixed effects by using log-likelihood ratio tests to compare the goodness of fit of models with and without the variable of interest.

We also compared the sex difference in the opportunity for sexual selection (I_s) among the three different density treatments by calculating the opportunity for sexual selection for males and females in each density, and subtracting the estimates for females from that of males (Shuster 2009). The opportunity for sexual selection is a standardized index of the intensity of selection, and was calculated as the ratio between the variance in number of offspring and the square of the average number of offspring (Shuster 2009). Males that failed to mate were included in our calculation of I_s because excluding these individuals would clearly underestimate the true levels of variance in male reproductive success.

PARTITIONING VARIANCE IN MALE REPRODUCTIVE SUCCESS

Male reproductive success was measured as the number of offspring sired by each male divided by the total number of offspring produced in that male's population. Our estimates of reproductive success are thus comparable among experimental populations, which differed in the total number of offspring produced. Male reproductive success is comprised of three main fitness components: the number of females with which a male successfully mates, the average fecundity of these females, and the average paternity share of eggs produced by these females. We estimated mating success from the paternity data by calculating the number of females with which each male produced at least one offspring (i.e., number of effective mates). Because some females failed to produce broods and thus were not available as potential mates, we adjusted our estimate of mating success by dividing the number of effective mates of a given male by the total number of females that produced at least one offspring in that male's population (Devigili et al. 2015). We measured female fecundity as the average number of brood balls produced by females with which the focal male successfully mated. We estimated fertilization success as the mean paternity share of a given male across all of the females with which he successfully mated. Because paternity share is dependent on the number of sperm competitors within a clutch and therefore not directly comparable across clutches with different numbers of sires, we standardized our estimate of fertilization success using the formula developed by Devigili et al. (2015):

$$F_{std} = F_{obs} (n - 1) / (F_{obs} (n - 2) + 1)$$

where F_{obs} is the observed proportion of offspring sired by a given male in a female's clutch, and n is the number of males competing for fertilization within that clutch.

We divided each fitness component by the population mean to calculate relative reproductive success (T), relative mating success (M), relative female fecundity (B), and relative fertilization success (F). We then partitioned the variance in reproductive success into the three fitness components and their associated covariances following the decomposition model of Webster et al. (1995):

$$\begin{aligned} Var(T) = & Var(M) + Var(B) + Var(F) + 2Cov(M, F) \\ & + 2Cov(M, B) + 2Cov(B, F) + D \end{aligned}$$

where Var and Cov are variances and covariances, respectively, for the fitness components T , M , B , and F , and D is an error term that is calculated by subtracting the summed variances and covariance from the computed value of $Var(T)$. We estimated the contribution of each of these fitness components toward the total opportunity for sexual selection by dividing each term in the decomposition model by the variance in reproductive success (Webster et al. 1995). We only included males that produced at least one offspring in these analyses because there was no opportunity for selection on fertilization success in males that did not mate with any females. We note that by excluding all males with zero reproductive success, we almost certainly excluded some individuals that mated but failed to sire offspring, and therefore may have overestimated the true level of variance in mating success by falsely attributing variation in fertilization success to variance in mating success (Collet et al. 2014). We calculated 95% confidence intervals for each of the variance component estimates from a bootstrap distribution based on 10,000 samples with replacement using PopTools (Hood 2011). We evaluated the relationship between relative mating success and relative fertilization success across the different densities by fitting a generalized mixed model with a log link function, Poisson errors, and population replicate as a random effect.

ESTIMATING THE SPURIOUS CORRELATION BETWEEN MATING SUCCESS AND FERTILIZATION SUCCESS

Because we used paternity data to estimate both mating success (M) and fertilization success (F), our estimates of pre- and postmating sexual selection are not independent. Specifically, we may have underestimated the mating success of males that were the least successful sperm competitors simply because of the decreased probability of detecting offspring from these males based on relatively small clutch sizes (e.g., a male with $F = 0.10$ is likely to sire no offspring if there are fewer than 10 offspring in a female's clutch). Following the methods of Devigili et al. (2015), we addressed this problem by first generating a simulated paternity dataset in which the expected values of M and F for each male

varied independently of each other (hereafter M_{exp} and F_{exp}), and then calculating the values of M and F of each male using the simulated paternity dataset (hereafter M_{sim} and F_{sim}). Using a Monte Carlo procedure, we repeated this simulation 10,000 times to obtain a distribution of the correlation coefficients between M_{sim} and F_{sim} , and thereby estimate the extent to which our method of using paternity data with limited sample sizes generated a spurious correlation between estimates of M and F .

To generate the simulated paternity dataset, we first assigned a F_{exp} value to each male by sampling from an empirical distribution of P_N values (mean \pm SD [range] = 0.72 ± 0.21 [0.04, 0.95], $n = 142$) based on a previous study that examined sperm competition patterns in *O. taurus* using the irradiated male technique (L. W. Simmons and F. García-González, unpubl. data; available from the Dryad Digital Repository). Second, we assigned a mating probability to each male–female pair by sampling from a binomial distribution with a mean equal to the observed mating probability in each population. Specifically, the mating probability was calculated by dividing the average female mating rate (i.e., number of sires) in each population by the number of potential sires. Each male could potentially mate with several females, and we assumed (conservatively) that a male's F_{exp} was consistent across all the females with whom he mated. Third, we assigned a clutch size to each female by sampling from the distribution of observed clutch sizes in each population, including those with zero offspring (high-density: 4.73 ± 4.82 [0, 20]; medium-density: 4.12 ± 4.19 [0, 18]; low-density: 8.47 ± 8.04 [0, 26]). Finally, we assigned the paternity of each offspring in a given female's clutch by sampling from the list of potential sires with a probability that was equal to each male's F_{exp} (corrected to account for differences in the number of sperm competitors in each clutch). We then calculated the M_{sim} and F_{sim} of each male from this simulated paternity dataset to evaluate the strength of the correlation between M and F that is expected to arise from initially uncorrelated estimates when M is derived from paternity data. We repeated the simulations for each of the three different population densities, using the population-specific sample sizes (high-density: 40 males and 40 females; medium-density: 20 males and 20 females; low-density: 10 males and 10 females).

Preliminary simulations confirmed that some matings remain undetected when mating success is estimated from paternity data. When we ran the simulations based on the observed mating probability in each population, we found that the number of sires estimated from the simulated paternity datasets were slightly lower than the actual observed number of sires (simulated vs observed for high-density = 2.35 vs 3.21; medium-density = 1.83 vs 2.64; low-density: 3.15 vs 3.77). We therefore increased the mating probability values used in the simulations until the number of sires estimated from the simulated paternity datasets equaled the observed number of sires. We then compared the av-

erage simulated correlation coefficients between M_{sim} and F_{sim} with the observed correlation coefficients between M and F . The simulations were generated using PopTools (Hood 2011) and are available from the Dryad Digital Repository.

MULTIVARIATE SELECTION ANALYSIS

For each of the nine populations, we standardized male phenotypic traits to have a mean of zero and standard deviation of one, and converted fitness to relative fitness by dividing it by the mean fitness of the population (Lande and Arnold 1983). We then combined data from the three replicates within each density treatment, and used standard selection analyses to characterize the form and intensity of multivariate selection on pre- and postcopulatory sexual traits for the low-, medium-, and high-density populations (Lande and Arnold 1983). We fitted a linear regression to estimate the vector of standardized linear selection gradients (β) on testes mass and horn length, and then used a quadratic regression model that included all linear, quadratic, and cross-product terms to estimate the matrix of standardized nonlinear selection gradients (γ). We doubled the quadratic regression coefficients to obtain appropriate estimates of stabilizing and disruptive selection gradients (Stinchcombe et al. 2008). Our results are qualitatively the same if we fit the linear and quadratic regression models with relative testes mass, relative horn length, and soma mass (i.e., body mass minus testes mass) as predictor variables, or if we conducted our analyses on log transformed trait values. For ease of interpretation, we only present results based on the analyses using standardized trait values for absolute testes mass and absolute horn length.

To compare the linear selection gradients (β) among the three density treatments, we calculated the 84% confidence intervals around each estimate in order to visually assess the statistical significance of these differences based on interval overlap (Goldstein and Healy 1995). Typically, 95% confidence intervals are calculated to evaluate whether an estimate differs from a fixed expectation (e.g., 0 or 1) at a significance level of 0.05. However, to use the nonoverlap criterion to compare estimates between multiple samples, the width of the confidence interval needs to be adjusted to ± 1.39 times the standard deviation, or 84% (Goldstein and Healy 1995). We calculated the 84% confidence intervals from the linear regression estimates using the *confint* function in R.

Results

Of the 210 females used in the experiment, 161 produced viable offspring, and 1069 of the 1298 offspring (82.4%) could be assigned paternity with 80% confidence. Our results are qualitatively the same if we assigned paternity to the most-likely candidate father (1294 offspring assigned to a single sire, 99.7%), or

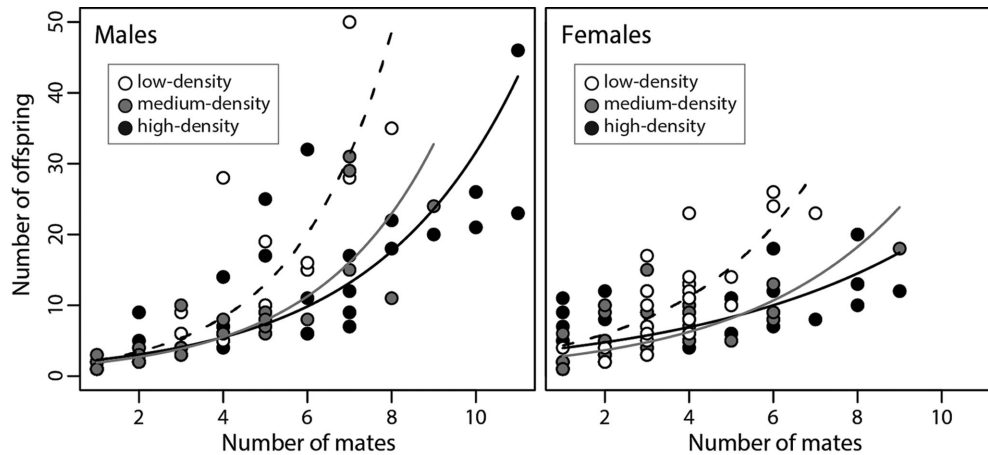


Figure 1. Bateman gradients showing the relationship between number of mates and number of offspring for males (left) and females (right) at each of the three different population densities. The fitted lines are from a generalized mixed model with a log link function, Poisson errors, and population replicate as a random effect. Open points (dashed line) represent individuals from low-density populations; gray points (gray line) represent individuals from medium-density populations; closed points (black line) represent individuals from high-density populations.

if we assigned paternity with 90% confidence (783 offspring assigned, 60.3%). On average, females produced 6.4 ± 4.1 (mean \pm SD) offspring in the high-density populations, 4.9 ± 4.1 offspring in the medium-density populations, and 11.5 ± 7.2 offspring in the low-density populations. Females in the medium- and high-density populations produced fewer brood balls than females in the low-density populations due to stronger competition for dung during the seven-day experiment in the buckets; on average, females produced 0.1 brood balls in the experimental buckets in the high-density populations, 2.3 brood balls in the medium-density populations, and 6.2 brood balls in the low-density populations.

We detected high rates of multiple mating by females, with 78.9% of productive females ($n = 127$) laying clutches that were sired by at least two males. The number of sires in a clutch ranged from one to nine (high-density: 3.21 ± 1.88 ; medium-density: 2.64 ± 1.71 ; low-density: 3.77 ± 1.41). There was a strong positive relationship between the number of sires and number of offspring ($\chi^2 = 204.94$; $P < 0.001$), and the slope of the female Bateman gradient increased with decreasing population density (Fig. 1; $\chi^2 = 10.81$; $P < 0.01$). We also detected high rates of multiple mating by males, with 50.5% of males ($n = 106$) siring clutches with at least two females. The number of effective mates ranged from zero to 11 (high-density: 2.38 ± 2.66 ; medium-density: 2.20 ± 2.36 ; low-density: 2.77 ± 2.40). As with the female data, there was a strong positive relationship between the number of effective mates and number of offspring ($\chi^2 = 940.90$; $P < 0.001$), and the slope of the male Bateman gradient increased with decreasing population density (Fig. 1; $\chi^2 = 31.78$; $P < 0.001$). When the sexes were considered together, there was a significant interaction between sex and number of mates, indicating that the reproductive benefit of gaining extra mates was greater

for males than females at each of the different densities ($\chi^2 = 37.64$; $P < 0.001$). However, the three-way interaction between number of mates, sex, and density was not significant ($\chi^2 = 0.34$; $P = 0.85$), indicating that there was no effect of density on the difference in the Bateman gradient between males and females.

In contrast to the patterns for Bateman gradients, which increased with decreasing density, the sex difference in the opportunity for sexual selection ($I_s = I_{male} - I_{female}$) increased with increasing density: from 1.70 in the low-density populations, to 1.80 in the medium-density populations, to 2.26 in the high-density populations. We note that these estimates for I_s represent an upper limit on the intensity of sexual selection in each population, rather than the actual, realized strength of selection (Jones 2009; Klug et al. 2010), and that our results should be considered with caution given the small sample sizes and high variance in the low- and medium-density populations.

The variance partitioning analysis indicated that most of the variance in total male reproductive success was attributable to variance in mating success. Specifically, in the high-density populations (for which our estimates are the most robust due to large sample size), 37% of the total variance was explained by mating success, 6% was explained by female fecundity, and 4% was explained by fertilization success (Table 1). As noted by Webster et al. (1995), the sum of the variance and covariance components may not equal 100% because higher order terms and skewness in the data also contribute to the total variance in reproductive success.

There was a positive correlation between mating success and fertilization success at all three densities (Fig. 2; $\chi^2 = 38.15$; $P < 0.001$), and the strength of this relationship increased with decreasing density ($\chi^2 = 25.26$; $P < 0.001$). The simulations

Table 1. Contributions of mating success (*M*), female fecundity (*B*), fertilization success (*F*), and their covariances to variance in total male reproductive success (*T*).

	Population density	Reproductive success (<i>T</i>)	Mating success (<i>M</i>)	Female fecundity (<i>B</i>)	Fertilization success (<i>F</i>)	Covariance (<i>M</i> × <i>F</i>)	Covariance (<i>M</i> × <i>B</i>)	Covariance (<i>B</i> × <i>F</i>)	Error (<i>D</i>)
Variance	High	1.58	0.59	0.09	0.06	0.15	-0.04	-0.06	0.79
% Explained			37.3	5.6	3.8	9.6	-2.6	-3.5	49.9
95% CI			[27.8, 57.9]	[3.2, 11.5]	[2.3, 7.1]	[5.8, 14.8]	[-8.8, 2.3]	[-8.0, -1.1]	[24.3, 61.4]
Variance	Medium	1.49	0.51	0.17	0.06	0.15	-0.11	-0.08	0.78
% Explained			34.3	11.1	4.3	10.3	-7.1	-5.1	52.3
95% CI			[19.3, 98.3]	[4.6, 49.4]	[2.6, 13.8]	[5.5, 22.2]	[-37.9, 5.9]	[-25.1, 0.3]	[-32.7, 70.8]
Variance	Low	1.36	0.34	0.09	0.16	0.39	-0.12	-0.11	0.61
% Explained			25.1	6.6	12.1	28.8	-9.0	-8.3	44.7
95% CI			[12.6, 72.1]	[2.6, 20.2]	[5.1, 36.4]	[-18.7, 19.0]	[-13.7, 13.4]	[-9.8, 9.9]	[-29.7, 80.2]

95% Confidence intervals were calculated from bootstrapping (10,000 iterations with replacement).

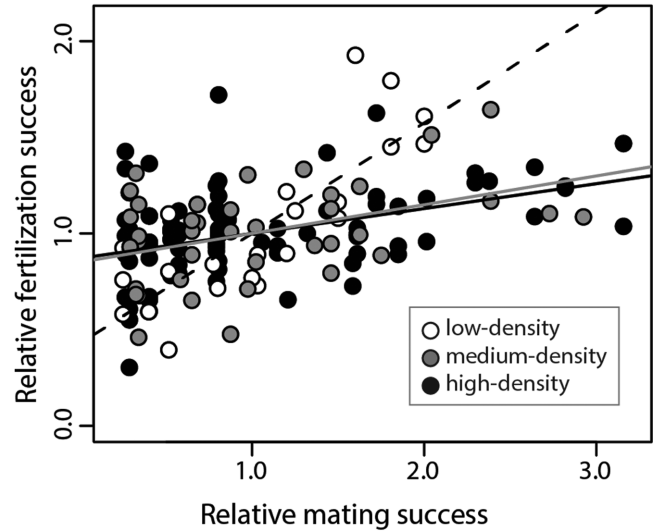


Figure 2. Relationship between relative mating success and relative fertilization success for males of different population densities. The fitted lines are from a linear mixed model with population replicate as a random effect. Open points (dashed line) represent males from low-density populations; gray points (gray line) represent males from medium-density populations; closed points (black line) represent males from high-density populations.

indicated that the correlation between mating success and fertilization success is expected to increase with decreasing density due to chance alone given that mating success and fertilization success were both estimated from paternity data, but the difference in the observed correlation coefficients between the low- and high-density populations (0.43) was eight times greater than the difference expected from the simulated paternity dataset (0.05).

In the high-density populations, we detected significant positive linear selection on testes mass, and significant negative linear selection on horn length (Fig. 3; Table 2). The linear multiple regression model including testes mass and horn length explained 12% of the variation in male fitness in the high-density populations. There was no evidence for nonlinear or correlational selection on testes mass or horn length (Table 2).

In the low- and medium-density populations, there was no significant linear or nonlinear selection on testes mass or horn length (Table 2). However, the strength of selection on testes mass increased with increasing population density, and differed significantly ($P < 0.05$) between the low- and high-density populations, based on nonoverlapping 84% confidence intervals (Fig. 4).

Discussion

This study offers new insights into how population density moderates the interaction between pre- and post-mating sexual selection in onthophagine dung beetles. Our results reveal that both pre- and

Table 2. Vector of standardized linear selection gradients (β) and matrix of standardized quadratic and correlational selection gradients (γ) on testes mass and horn length for high-, medium-, and low-density populations.

Density	Trait	β coefficients	γ coefficients	
			Testes mass	Horn length
High	Testes mass	0.525 (<0.001)	0.297 (0.127)	-0.144 (0.355)
	Horn length	-0.343 (0.02)		0.111 (0.773)
Medium	Testes mass	0.198 (0.376)	-0.709 (0.101)	0.317 (0.274)
	Horn length	-0.193 (0.387)		0.592 (0.483)
Low	Testes mass	-0.405 (0.134)	0.145 (0.743)	0.263 (0.393)
	Horn length	-0.361 (0.180)		0.399 (0.640)

Significant selection gradients are denoted in bold.

P-values for selection gradient estimates are reported in parenthesis.

Quadratic selection gradients are twice the coefficients returned from the multiple regression models (Stinchcombe et al. 2008).

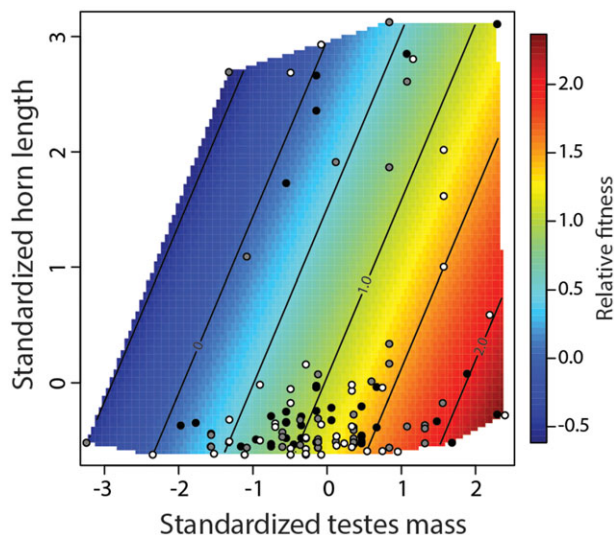


Figure 3. Visualization of significant linear selection on testes mass and horn length for males in the high-density populations. Heat colors indicate relative fitness. Plotted points represent the observed values for individual males from each of the three replicates (black, gray, and white points, respectively).

post-mating sexual selection and their covariance are important determinants of a male's overall reproductive success, but the relative importance of these fitness components depends on population density. We also found a shift from negative to positive linear selection on testes mass as population density increased, indicating that increasing population density strengthens post-mating sexual selection acting on male ejaculate expenditure. By combining variance-partitioning analyses with direct measures of selection on phenotypic traits, this study provides a more holistic picture of the strength and direction of overall sexual selection in these male dimorphic dung beetles, and how patterns of sexual selection can change among populations depending on their ecological conditions.

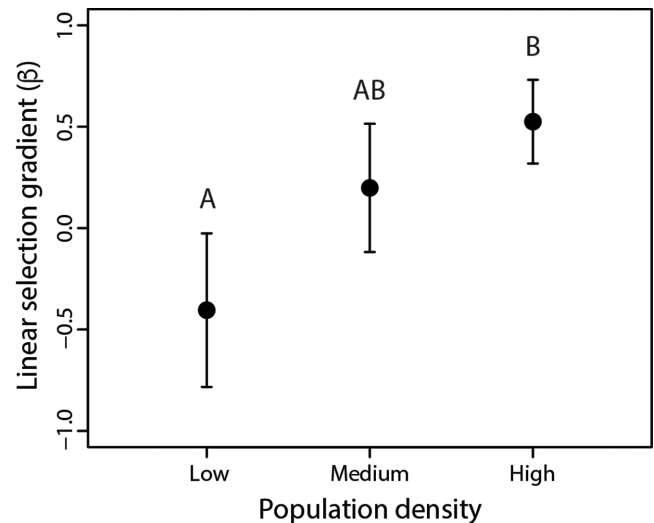


Figure 4. Variation in the strength of linear selection on testes mass within the low-, medium-, and high-density populations. Error bars represent 84% confidence intervals. Letters above the bars denote selection gradient estimates that are significantly different from each other ($P < 0.05$, nonoverlap criterion, see text).

We found that total sexual selection in male dung beetles was mostly attributable to pre-mating episodes of selection: 37% of the total variance in male reproductive success was explained by variance in mating success in the high density populations, and, although the confidence intervals are substantially larger for the medium- and low-density populations due to the smaller sample sizes, more than a quarter of the total variance arose from variance in mating success in the medium- and low-density populations. By contrast, our data suggest that fertilization success explained only a small proportion of the total variance in male fitness – only 4% in the high-density populations. These results were surprising given that numerous studies have found that sexual selection in *O. taurus* acts strongly after mating (e.g., García-González

and Simmons 2007; Simmons and Kotiaho 2007; Simmons and García-González 2008; Simmons et al. 2009). However, the variance partitioning analysis also found that the covariance between mating success and fertilization success explained a significant proportion of the total variance in male reproductive success (~10% in the high-density populations), which indicates that both pre-mating and post-mating selection make important contributions to total sexual selection, and that the two episodes act synergistically.

Because *O. taurus* mate exclusively underground, we were unable to directly observe matings in this study. Instead, we inferred mating success from genetic parentage data, but in doing so, we may have overestimated the relative contribution of mating success in explaining the total variance in reproductive success by overlooking matings that failed to result in successful fertilization. For example, Collet et al. (2014) found that by excluding no-progeny matings (which comprised 29% of all matings in their experiment on red jungle fowl), the proportion of total variance in male reproductive success explained by variance in mating success increased from 43% to 57%. We expect that the exclusion of no-progeny matings had a smaller effect in biasing our estimates of the variance in mating success because studies on sperm competition patterns between *O. taurus* males found that over 80% of matings result in successful fertilization (L. W. Simmons and F. García-González, unpublished data; available from the Dryad Digital Repository).

There was a strong positive correlation between mating success and fertilization success at all three population densities, indicating that males that were successful at gaining matings were also successful at gaining fertilizations. Intriguingly, our data show that the strength of this correlation decreased with increasing density, suggesting that mating success is a relatively poor predictor of fertilization success at high population densities. These patterns are similar to those of a previous study on red jungle fowl, *Gallus gallus*, that found that polyandry decreased the importance of mating success and increased the importance of fertilization success in explaining the total variance in male reproductive success (Collet et al. 2012). We also found that the slope of the male Bateman gradient decreased with increasing density, which is consistent with theoretical models that predict that high levels of polyandry will reduce the fitness payoff for males of obtaining more mates due to the loss of paternity under intense sperm competition (Parker and Pizzari 2010; Parker and Birkhead 2013). Although there was no association between population density and level of polyandry amongst our experimental populations, we note that our measures of polyandry are almost certainly underestimates given that they are inferred from genetic paternity data. Previous studies suggest that increased population density is indeed associated with stronger sperm competition in *O. taurus* and dung beetles more generally. For example, Hunt

and Simmons (2002) found that for *O. taurus*, a male's paternity share decreased as the number of sneaker males in the population increased, and Pomfret and Knell (2008) found that high population density (more crowding) was a strong predictor of the absence of horns among species in a community of South African dung beetles. The latter finding is consistent with the hypothesis that the evolutionary loss of horns in more crowded species is due to high levels of sperm competition and a trade-off between the size of horns and testes (Knell 2009). Collectively, these observations provide strong evidence that as population density increases, post-mating episodes of selection become more important, and shifts the mating system dynamics from direct male-male contests to scrambles (Parker et al. 2013).

We also found that the strength of linear selection on testes mass increased with increasing population density, which supports theoretical predictions that increased risk of sperm competition should select for increased male expenditure on ejaculates (Parker and Pizzari 2010). Density-dependent selection on testes mass may explain the patterns in phenotypic variation found between natural populations in Western Australia and the eastern United States that differ dramatically in population density (Moczek et al. 2002; Moczek 2003). In particular, the fact that there was strong positive selection on testes mass in high-density populations (typical in Western Australia) but negative selection on testes mass in low-density populations (typical in the eastern United States) suggests that males that engage in sperm competition are more successful at high densities, and may contribute to the phenotypic divergence in testes mass (Simmons et al. 2007) and the threshold for horn development (Moczek 2003) that is found between populations in Western Australia and the eastern United States.

Such patterns of density-dependent selection on weapons and testes have also been found in other taxa. For example, in Soay sheep, *Ovis aries*, horn size is a strong predictor of a male's siring success, but as the number of estrous females increases, testes size becomes an additional predictor of siring success because large-horned males are no longer able to monopolize access to females through direct male-male competition (Preston et al. 2003). Similarly, in the chorusing frog, *Crinia georgiana*, large arms (weapons) confer a selective advantage in gaining matings at low density, but selection on arm girth shifts from positive to negative as male density and the risk of sperm competition increases (Buzatto et al. 2015). Male density is also positively correlated with testes size and negatively correlated with arm girth across both populations and species (Dziminski et al. 2010; Lüpold et al. 2017), which supports the hypothesis that population density and the associated changes in female monopolizability mediate the relationship between male expenditure on weapons for winning mates and ejaculates for winning fertilizations (Parker et al. 2013; Lüpold et al. 2014, 2017; Simmons et al. 2017).

Sperm competition theory assumes that males have a limited pool of resources with which to invest in reproduction, and therefore cannot simultaneously maximize the development of both pre- and post-mating sexual traits (Parker et al. 2013). There is empirical support both within and among dung beetle species that males face resource allocation tradeoffs between weapons and testes (Simmons and Emlen 2006; Simmons et al. 2007), and our current data suggest that there is also opposing selection on weapons and testes in high-density populations that should reinforce this allocation tradeoff. Surprisingly, we found no evidence for opposing selection on horns and testes in the low-density populations, and no evidence that males benefit from developing horns and fighting under those conditions, when the risk of sperm competition was presumably low (Hunt and Simmons 2002; Pomfret and Knell 2008). One possibility for these findings is that our low-density treatment was not low enough for males to be able to deter rival males from breeding tunnels and effectively monopolize females. Indeed, the fact that at least one low-density male mated with up to eight females indicates that males are not investing in just one female under low-density conditions. The population densities imposed in this experiment are representative of those observed in the field (Moczek 2003), and the differences between the low- and high-density treatments were sufficient to switch the direction of selection on testes mass from significantly positive under high-density conditions to negative under low-density conditions (Fig. 4). However, it is possible that even lower population densities are required before horns become selectively advantageous, and future studies should investigate the selection regime on horns and testes across an even wider range of population densities.

Because *O. taurus* males are morphologically and behaviorally dimorphic, we predicted that there would be divergent selection on male phenotypes, and thus distinct peaks on the adaptive landscape—with one morph specialized for fighting and gaining high mating success, and a second morph specialized for sneaking and gaining high fertilization success. Contrary to these predictions, we found no evidence for non-linear selection in any of our experimental populations, and thus no evidence for complex or rugged fitness landscapes. So what maintains the coexistence of both major and minor morphs?

We suspect that the coexistence of the two morphs may not be evolutionarily stable and that the major morph may ultimately disappear from high-density populations. Comparative studies between exotic and native *O. taurus* populations in the early 2000s found that the position of the threshold body size separating major and minor morphs had evolved in less than 40 years since the introduction to new habitats, and that the threshold of the high-density Western Australian populations and low-density United States populations had evolved in opposite directions from their source Mediterranean populations (Moczek 2003; Moczek and

Nijhout 2003). To our knowledge, no follow-up studies have been conducted over the subsequent 20 years, yet there is no reason to expect that the threshold positions would not continue to evolve in their respective directions if the differences in intraspecific competition between high- and low-density populations were sustained. Very high population densities over sustained periods of time may lead to the evolutionary loss of horns if females become economically indefensible and non-aggressive “scramble” males are the most successful at gaining matings and fertilizations (Wiens 2001; Pomfret and Knell 2008; Knell 2009). As a result, it is possible that the maintenance of both male morphs is unstable in the exotic Western Australian populations if the exceptionally high-density conditions drives the evolution of an increasingly higher threshold (and thus increasingly fewer majors), potentially leading to the complete loss of horns.

Conclusions

Our study adds to a small but growing number of studies that aim to resolve the overall strength and pattern of sexual selection by quantifying the relative importance of pre- and postmating episodes of selection (e.g., Collet et al. 2012; Pischedda and Rice 2012; Rose et al. 2013; Péliissié et al. 2014; Devigili et al. 2015; Turnell and Shaw 2015; Marie-Orleach et al. 2016), and measuring the selective pressures on both pre- and postmating phenotypic traits (e.g., Danielsson 2001; Bangham et al. 2002; Preston et al. 2003; Thomas and Simmons 2009; South and Lewis 2012; Devigili et al. 2015; Buzatto et al. 2017). We found that both pre- and postmating episodes were important in the horned dung beetle *O. taurus*, which highlights the need to investigate how selection acts both before and after mating to gain an accurate picture of total sexual selection. Our results also indicate that the strength and direction of selection on particular phenotypic traits (e.g., testes mass) was dependent on population density, indicating that the interplay between pre- and postmating sexual selection is complex and context dependent. Finally, we found that the patterns observed among our experimental populations were largely consistent with the phenotypic variation observed among natural populations of *O. taurus* that experience different ecological conditions. We predict that variation in the relative importance of pre- and postmating sexual selection will be critical in understanding intra- and interspecific patterns in the degree of male dimorphism (Simmons et al. 2007) and investment in male weaponry (Pomfret and Knell 2008).

AUTHOR CONTRIBUTIONS

All authors conceived and designed the study; E.L.M. and B.A.B. collected the samples; E.L.M. conducted the paternity analyses; E.L.M. and B.A.B. analyzed the data; E.L.M. wrote the initial manuscript; all authors revised the manuscript and approved the final version.

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DATA ARCHIVING

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Summary data for the six microsatellite loci used to estimate paternity of experimental males.