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Benefits of polyandry: Molecular evidence from field-caught dung beetles

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Abstract

When females mate with multiple males, they set the stage for postcopulatory sexual selection via sperm competition and/or cryptic female choice. Surprisingly little is known about the rates of multiple mating by females in the wild, despite the importance of this information in understanding the potential for postcopulatory sexual selection to drive the evolution of reproductive behaviour, morphology and physiology. Dung beetles in the genus *Onthophagus* have become a laboratory model for studying pre- and postcopulatory sexual selection, yet we still lack information about the reproductive behaviour of female dung beetles in natural populations. Here, we develop microsatellite markers for *Onthophagus taurus* and use them to genotype the offspring of wild-caught females and to estimate natural rates of multiple mating and patterns of sperm utilization. We found that *O. taurus* females are highly polyandrous: 88% of females produced clutches sired by at least two males, and 5% produced clutches with as many as five sires. Several females (23%) produced clutches with significant paternity skew, indicating the potential for strong postcopulatory sexual selection in natural populations. There were also strong positive correlations between the number of offspring produced and both number of fathers and paternity skew, which suggests that females benefit from mating polyandrously by inciting postcopulatory mechanisms that bias paternity towards males that can sire more viable offspring. This study evaluates the fitness consequences of polyandry for an insect in the wild and provides strong evidence that female dung beetles benefit from multiple mating under natural conditions.

KEYWORDS
microsatellites, *Onthophagus*, paternity skew, polyandry, postcopulatory sexual selection

1 | INTRODUCTION

Polyandry, or multiple mating by females with different males, is a powerful driver in the evolution of male reproductive behaviour, morphology and physiology, and a widespread phenomenon across diverse animal taxa (Birkhead & Møller, 1998; Eberhard, 1996; Jennions & Petrie, 2000; Parker, 1970; Simmons, 2001, 2005; Zeh & Zeh, 2001). Despite the potential costs of polyandry, including increased risk of predation (Arnqvist, 1989; Rowe, 1994) and disease (Knell & Webberley, 2004), there is now strong evidence that females gain both direct and indirect benefits from mating polyandrously. For example, female insects often gain nuptial gifts and/or hormonal stimulants in the male's ejaculate that can enhance egg production and female fertility (Arnqvist & Nilsson, 2000), and polyandry can also improve female fitness by promoting postcopulatory mechanisms (e.g., sperm competition and cryptic female choice) that bias paternity towards more competitive, viable and/or genetically compatible sperm (Jennions & Petrie, 2000; Tregenza & Wedell, 2000; Zeh & Zeh, 1997). Although polyandry was once considered an evolutionary puzzle, the accumulating evidence on the benefits of mating with multiple males suggests that explaining the evolution and maintenance of the rare cases of monandry is the real

conundrum (Arnqvist & Nilsson, 2000; Hosken, Stockley, Tregenza, & Wedell, 2008).

Innovative laboratory experiments over the past four decades have clarified the specific costs and benefits of multiple mating to both males and females, and the many ways by which the two sexes compete for control of fertilizations (reviewed in Arnqvist & Nilsson, 2000; Jennions & Petrie, 2000; Simmons, 2005; Tregenza & Wedell, 2000). Controlled laboratory studies are essential for disentangling the various factors that can influence fertilization success, such as distinguishing between the benefits of true polyandry vs. repeated mating (Tregenza & Wedell, 1998). However, to understand the evolutionary significance of multiple mating, it is also essential to identify the actual rates of polyandry under natural conditions. The number of males with which a female typically mates determines the risk and intensity of sperm competition, which shapes the evolution of male investment in sperm production (Parker, Ball, Stockley, & Gage, 1996, 1997). The level of polyandry also influences the relative costs and benefits of multiple mating, which should contribute to the evolution of female mating patterns and optimal mating rates (Arnqvist & Nilsson, 2000). Knowledge of the levels of polyandry in natural populations is therefore essential for interpreting the biological relevance of laboratory findings. Unfortunately, there are still relatively few studies that examine the rates of multiple mating and patterns of sperm utilization for insects in the wild (e.g., Bretman & Tregenza, 2005; Bundgaard, Barker, Frydenberg, & Clark, 2004; Frentiu & Chenoweth, 2008; Good, Ross, & Markow, 2006; Haddrill, Shuker, Amos, Majerus, & Mayes, 2008; Harshman & Clark, 1998; Imhof, Harr, Brem, & Schlötterer, 1998; Oneal & Knowles, 2015; Simmons & Beveridge, 2010; Simmons, Beveridge, & Kennington, 2007a; Song, Drew, & Hughes, 2007) and only a few studies that measure the fitness consequences of polyandry for any animal in their natural habitat (Fisher, Double, Blomberg, Jennions, & Cockburn, 2006; Gerlach, McGlothlin, Parker, & Ketterson, 2012; Rodríguez-Muñoz, Bretman, Slate, Walling, & Tregenza, 2010).

Dung beetles in the genus *Onthophagus* have proved to be a valuable system for studying pre- and postcopulatory sexual selection processes in the laboratory (reviewed in Simmons, 2011). In particular, Onthophagine dung beetles have been important in testing theoretical predictions of how selective pressures from sperm competition and cryptic female choice have driven the evolution of male ejaculate expenditure (Simmons, Emlen, & Tomkins, 2007b; Simmons & García-González, 2008; Simmons, Tomkins, & Hunt, 1999), how female choice and polyandry allow females to gain indirect benefits through the production of more viable offspring (García-González & Simmons, 2011; Kotiaho, Simmons, & Tomkins, 2001; Simmons & Holley, 2011), and how postcopulatory processes affect the evolution of horns used in precopulatory male–male competition (Pomfret & Knell, 2008; Simmons & Emlen, 2006; Simmons & Fitzpatrick, 2016).

Female dung beetles dig tunnels beneath fresh dung pads where they mate and build brood balls in which to lay their eggs (Halffter & Edmonds, 1982). In many species, males are morphologically and behaviourally dimorphic: large males develop horns on their head and/or pronotum that they use in male–male contests over ownership of breeding tunnels, while small males remain hornless and dig intercepting side tunnels to sneak matings inside the tunnels guarded by larger males (Emlen, 1997; Moczek & Emlen, 2000). Females therefore encounter and mate with several different males, which creates the opportunity for sexual selection to persist after copulation in the form of sperm competition and/or cryptic female choice (Eberhard, 1996; Parker, 1970; Simmons, 2001). The strength of these postcopulatory sexual selection pressures is expected to differ according to the proportion of the male population that adopts the sneaking tactic, and indeed, previous studies have found that variation in the frequency of sneakers contributes to the macroevolutionary patterns in testis size variation among *Onthophagus* species (Simmons & García-González, 2008; Simmons et al., 2007b). However, all of this work has been conducted in the laboratory, and essentially nothing is known about the actual levels of polyandry and strength of postcopulatory sexual selection under natural conditions.

Here, we develop polymorphic microsatellite markers for the bull-horned dung beetle, *Onthophagus taurus*, and use the markers to genotype the offspring of wild-caught females. Our paternity data allow us to estimate the rates of female multiple mating and patterns of sperm utilization in a natural population, and determine the fitness consequences of variation in polyandry naturally occurring in the wild.

2 | METHODS

2.1 | Isolation and characterization of microsatellite loci

We used salt precipitation (Aljanabi & Martinez, 1997) to extract genomic DNA from the legs of *O. taurus* adults collected from a dairy farm in southwest Western Australia. DNA (36.9 μg) from one individual was sent to the Australian Genomic Research Facility for Illumina MiSeq sequencing. The sample produced 236,197 sequence reads, with an average fragment size of 215 bp. We used the program QDD version 3.1.2 (Meglécz et al., 2010) to screen the sequences for microsatellites with a minimum of eight di- to pentabase repeats and detected 15,413 microsatellite loci.

Thirty-nine loci were chosen for further development based on their primer design scores (no homopolymers allowed in flanking region or primer, no nanosatellites allowed in flanking region or primer and no compound repeats), repeat motif (dinucleotide or greater) and product size (80–300 bp). The loci were screened for amplification in 10 μl reactions containing 10 ng of DNA, 1× PCR buffer, 3 mM $MgCl_2$, 0.5 μg bovine serum albumin, 0.2 mM dNTPs, 0.5 μM of each primer and 0.5 U Platinum Taq DNA polymerase (Invitrogen). The following PCR conditions were used: 94°C for 3 min, followed by 30 cycles at 94°C for 20 s, 60°C for 30 s and 72°C for 45 s and then a final elongation step at 72°C for 10 min. PCR products were visualized on 3% agarose gels stained with GelRed (Biotium).

Thirty-four loci amplified a PCR product of the expected size and were subsequently tested for polymorphism in six individuals. Of these loci, 18 (53%) were polymorphic, and 16 (47%) were unclear. The forward primers for the 18 polymorphic loci were labelled with one of four fluorescent dyes: NED (Thermo Fisher), FAM, PET or VIC (AlphaDNA). We then screened the loci for variation in 50 wild-caught adult females. PCR products were analysed on an ABI 3730 xl DNA Analyzer using GENESCAN-500 LIZ internal size standard, and the profiles were scored using GENEMARKER software (SoftGenetics). Two loci failed to show strong and unambiguous peaks for all samples, so they were excluded from further analyses.

We used the online version of GENEPOP 4.0 (Raymond & Rousset, 1995) to test whether any loci deviated from Hardy–Weinberg equilibrium. We used CERVUS version 3.0 (Kalinowski, Taper, & Marshall, 2007; Marshall, Slate, Kruuk, & Pemberton, 1998) to calculate the observed and expected heterozygosities and the polymorphic information content of each locus and to estimate the frequency of null alleles (Table 1).

2.2 | Collection of samples

Adult beetles were collected from a dairy farm in southwest Western Australia in early and mid-October, when adults are most actively burying dung and producing brood masses in the field (Ridsdill-Smith, 1993). Approximately 60 females were established in individual breeding chambers (PVC piping, 25 cm in length and 6 cm in diameter) filled three-quarters with moist sand and topped with 100 g cow dung. Females were left undisturbed for 7 days to build brood balls. The chambers were sieved for brood balls and females, and the females were re-established in the breeding chambers with fresh dung for another 7 days before being sieved again for brood balls.

TABLE 1 Summary data for 16 microsatellite loci for *Onthophagus taurus*

Loci	Primer sequence (5′–3′)	Repeat motif	N_A	Allele size	PIC	H_{obs}	H_{exp}	F_{null}	p_{HWE}
59	F: TCCCGAGATCAACGAACACC R: TGATCTCCCGTCGTACCTCA	ATT	2	170–201	0.143	0.102	0.157	0.207	.042
61	F: TACGAGCGGTTGAAATTGGC R: TTCCATCGACCGACGTTGAA	AG	4	98–106	0.516	0.483	0.593	0.112	.058
65	F: AATGATCGACAGAATATGCACG R: GCGAAGAATCTGCAGATTGGT	AT	6	132–145	0.676	0.683	0.724	0.034	.398
70	F: GGCAAAGGTTGGTTCGATTCG R: TGTGGTTCAAATAGTTACGGACT	AG	5	79–87	0.660	0.717	0.716	0.000	.852
71	F: AGTTAGAGGATAAAGTGAGGCGT R: ACTGATGTTATAGGTTCCAACGGT	AT	2	257–287	0.077	0.083	0.081	0.000	.999
72	F: CCCAAGTCTCAAATCCAAACGT R: CATGGACGAGACATGCTGAA	TC	10	91–117	0.792	0.767	0.819	0.023	.674
74	F: AGATTGATATGGAGGATCTGGCA R: TGTCTGATGAAGGTAGGCTGT	AT	8	150–169	0.775	0.431	0.808	0.305	<.001*
76	F: CAGACTCACGGCTAAACACG R: TCGTGCGTTGATAACTCCACT	TA	10	165–213	0.667	0.635	0.694	0.035	.501
78	F: AAAGGACTTTCTACGCCGGG R: ACGTCAACTCATTAGAGCAGTGA	TA	9	96–166	0.455	0.390	0.479	0.097	.139
80	F: GTTGCCACCTCTGACTTGCT R: CGCAATCACGTCTGCATTCT	AT	4	97–103	0.509	0.424	0.598	0.163	.004
84	F: GCCGGATATCCATTCCACCA R: TGATTAAGCCGAGGTTGTCCA	AT	6	85–94	0.667	0.412	0.717	0.268	<.001*
86	F: TGTAGAGTCTTATGGTGCACAA R: GTTGATTTATATGTAGCGTCATGTCT	ATT	4	89–104	0.108	0.117	0.112	0.000	.999
88	F: CCAGCCGGAATGTTAGTTGG R: TTCAGCAAAGATCCGAACCA	TA	5	126–146	0.615	0.483	0.675	0.165	.003*
89	F: ACACTGCATAACTCGGGTTCT R: TAGGCTGAAGCTGTCGCAAA	TA	5	103–113	0.632	0.559	0.687	0.107	.231
90	F: GCAGACGTTTCGCCAAGATC R: CGACTTGGTTCAGCCCATCT	AT	4	249–263	0.553	0.132	0.610	0.638	<.001*
93	F: ACCACTTTGGCAATTTATATGCA R: GCTCATTCACCGAATCTCAACC	AT	6	141–170	0.690	0.438	0.740	0.262	<.001*

N_A, number of alleles; PIC, polymorphic information content; H_{obs}, observed heterozygosity; H_{exp}, expected heterozygosity; F_{null}, estimated frequency of null alleles; p_{HWE}, p-value for Hardy–Weinberg equilibrium tests. Loci that deviated from Hardy–Weinberg equilibrium after Bonferroni's correction ($p < .003$) are indicated with an asterisk.

TABLE 2 Summary of paternity results from clutches of 40 wild-caught females, ranked most to least fecund (as in Figure 1)

# Larvae	# Sires	Paternity skew ± 95% CI	H_0 skew	*p*-value
29	3	0.443 ± 0.017*	0.310	.002
25	4	0.271 ± 0.004	0.219	.175
25	3	0.576 ± 0.024*	0.306	<.001
22	4	0.259 ± 0.004	0.214	.309
22	4	0.302 ± 0.005	0.214	.071
21	4	0.243 ± 0.002	0.213	.468
20	3	0.574 ± 0.000*	0.298	<.001
20	4	0.261 ± 0.004	0.211	.280
19	3	0.452 ± 0.011*	0.296	.008
19	2	0.509 ± 0.000	0.472	.169
18	2	0.791 ± 0.000*	0.471	<.001
17	4	0.248 ± 0.005	0.203	.446
17	2	0.539 ± 0.042	0.469	.148
17	5	0.176 ± 0.002	0.150	.757
17	3	0.430 ± 0.006*	0.292	.033
16	3	0.340 ± 0.017	0.289	.349
15	2	0.524 ± 0.000	0.464	.119
14	3	0.342 ± 0.010	0.282	.296
14	5	0.149 ± 0.002	0.139	.972
14	3	0.492 ± 0.013*	0.282	.015
14	3	0.449 ± 0.013*	0.282	.034
12	4	0.199 ± 0.002	0.182	.789
11	4	0.193 ± 0.003	0.175	.910
11	3	0.313 ± 0.007	0.267	.420
11	2	0.564 ± 0.000	0.450	.069
11	1	NA		
10	3	0.269 ± 0.002	0.259	.785
10	3	0.267 ± 0.000	0.259	.785
10	2	0.509 ± 0.052	0.444	.351
10	1	NA		
8	2	0.528 ± 0.056	0.429	.289
8	2	0.518 ± 0.086	0.429	.289
8	3	0.247 ± 0.002	0.238	.999
7	3	0.243 ± 0.005	0.222	.711
6	2	0.422 ± 0.080	0.400	.680
6	2	0.413 ± 0.017	0.400	.680
5	2	0.451 ± 0.022	0.375	.338
5	1	NA		
2	1	NA		
2	1	NA		

H_0 skew is the paternity skew under a null expectation in which all sires gain equal paternity share, corrected for small sample size (see Methods for details).

p-values represent the probability that the observed estimate of paternity skew is equal to or greater than the null expectation, given sampling error in the number of larvae and sires.

Clutches with significant paternity skew are denoted with an asterisk ($p < .05$).

95% confidence intervals were calculated from the 50 most likely minimum-father solutions generated by GERUD (or fewer where the program detected fewer possible sire combinations).

Clutches that are sired by a single father have no potential for paternity skew.

The females were retrieved from the breeding chambers, and their body size (pronotum width) was measured to the nearest 0.01 mm with digital callipers. The females were then placed in Eppendorf tubes and frozen at −20°C. Brood balls were opened carefully, and any developing larvae were placed in Eppendorf tubes and frozen at −20°C. 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 2 days for a developing larva. Brood balls that contained dead or unfertilized eggs were discarded.

Several females failed to produce any viable offspring, presumably because they were unmated. Additionally, several females could not be found in the breeding chambers, presumably because they died and had started to decompose in the dung. Our final sample included 548 larvae from 40 females (Table 2).

2.3 | Genotyping and paternity analysis

We used the EDNA HiSpEx tissue kit (Fisher Biotec) to extract genomic DNA from the hind legs of each female and the head capsule of each larva. We determined the genetic profile of all individuals at six microsatellite loci: 61, 65, 70, 72, 76 and 89 (see Table 1). These loci were selected based on their ease of peak assignment and high polymorphic information content. PCR conditions were the same as those described above.

We used GENEMARKER to score the genetic profiles of each mother and all of her larvae at the six microsatellite loci. All profiles were individually verified, and only samples showing strong and unambiguous peaks were included in the final analysis. We estimated the extent of multiple paternity using GERUD version 2.0, a sibship analysis program that estimates the minimum number of fathers that are needed to account for the genotypes in a progeny array (Jones, 2005). The program also reconstructs the genotypes of candidate fathers and estimates the number of offspring sired by each father. When multiple sire genotype solutions are possible for a given progeny array, GERUD ranks the solutions in order of their relative probabilities based on patterns of Mendelian segregation (Jones, 2005). The expected exclusion probability using all six loci and known maternal genotypes was 98.2%, giving an error rate of less than 2%.

We evaluated the power of the six microsatellite loci to detect multiple paternity using GERUDSIM version 2.0 (Jones, 2005). Specifically, we simulated progeny arrays based on observed allele frequencies and patterns of paternity skew and then tested how often the GERUD 2.0 algorithm recovered the correct number of sires. We ran

simulations (1,000 iterations each) for two-, three- and four-sire clutches based on an average clutch size (14 larvae) and average patterns of paternal contributions (10:4 in two-sire clutches, 8:4:2 in three-sire clutches and 5:4:3:2 in four-sire clutches).

In addition to estimating the minimum number of fathers in each clutch, we calculated the degree of paternity skew, defined as the sum of the squared proportions of offspring sired per father (Simmons et al., 2007a; Starr, 1984). Paternity skew equals 1/(number of fathers) when paternity is distributed equally among all fathers in a clutch, and increases with the disparity in paternity share among candidate fathers. We calculated the average paternity skew for each clutch based on the 50 most likely minimum-father solutions generated by GERUD (or fewer where the program detected fewer possible sire combinations). However, this estimate of skew may be affected by the number of offspring in a clutch, making it likely that the offspring of particular fathers (i.e., those with low paternity contributions) are unrepresented in the sample collected for genetic analyses (Boomsma & Ratnieks, 1996). We therefore corrected our estimate of paternity skew for sampling error using a general expression developed by Pamilo (1993):

$$\text{Corrected paternity skew} = (N\Sigma p_i^2 - 1)/(N - 1)$$

where p_i is the proportion of offspring sired by the ith father, and N is the number of offspring sampled. Our results are qualitatively the same if we calculated average paternity skew for each clutch based on the 10 most likely minimum-father solutions rather than the 50 most likely minimum-father solutions generated by GERUD because the two estimates of paternity skew were tightly correlated (Pearson's correlation: $r_{33} = .997$).

We tested whether our estimates of paternity skew were statistically significant by comparing the observed skew estimates against null distributions in which males have equal siring probability, accounting for sampling error in the number of offspring and sires. Specifically, we generated 10,000 hypothetical clutches for a given female by randomly assigning each of the observed number of offspring to one of the potential sires (R code is available from the Dryad Digital Repository; McCullough, Buzatto, & Simmons 2017). We then calculated the paternity skew (corrected for sample size) for each simulated clutch and considered the observed estimate to be statistically significant if it was equal to or greater than 95% of the skews from the simulated data set.

To examine the combined effects of paternity skew and minimum number of fathers on the number of larvae produced in a clutch, we first calculated a standardized index of paternity skew. This standardization was necessary because the values of corrected paternity skew are dependent on the number of candidate sires and therefore are not directly comparable across families with different numbers of sires. Standardized paternity skew was calculated as:

$$(\text{Corrected paternity skew} - \text{Minimum skew})/(\text{Maximum skew} - \text{Minimum skew})$$

where minimum skew and maximum skew represent the minimum and maximum possible values of skew for each family, given the observed number of fathers and clutch size, and corrected for sample size (see Dryad file for more details; McCullough et al., 2017). We then examined the combined effects of standardized paternity skew and minimum number of fathers on clutch size by fitting a generalized linear model (GLM) with a log link function and Poisson errors. The full model included standardized paternity skew, minimum number of fathers and their interaction as explanatory variables. The interaction term was not statistically significant ($\chi^2 = 3.63$, $p = .16$) and therefore was removed from the final model. Clutches with one or five minimum fathers were excluded from these analyses because single-father clutches have no potential for paternity skew, and there were only two clutches with five minimum fathers.

3 | RESULTS

3.1 | Microsatellite variability

The number of alleles per locus ranged from two to ten (mean ± *SD*: 5.6 ± 2.5). The observed and expected heterozygosities ranged from 0.083 to 0.767 and 0.081 to 0.819, respectively (Table 1). Based on our sample of 50 females collected in the field to assay microsatellite variability, five loci deviated significantly from Hardy–Weinberg expectations, possibly due to the presence of null alleles (Table 1).

3.2 | Minimum number of fathers

Thirty-five of the 40 wild-caught females (88%) produced clutches that were sired by multiple males (Table 2; Figure 1). There was no correlation between female body size and either number of offspring produced ($r_{38} = -.05$, $p = .77$) or minimum number of fathers ($r_{38} = .21$, $p = .20$). We therefore did not include female body size as a covariate in subsequent analyses. Clutch sizes ranged from 2 to 29 larvae (mean ± *SD*: 13.7 ± 6.5).

The minimum number of fathers ranged from one to five, with a mean ± *SD* of 2.8 ± 1.1. We note that three of the five clutches that were sired by a single male were the smallest clutches in our sample, with just 5, 2 and 2 larvae, respectively.

Using the population allele frequencies of our six microsatellite loci and average patterns of paternity skew for simulations in GERUDSIM, we found that our analyses in GERUD had a very high probability (99.4%) of determining the correct number of sires for two-sire clutches, a high probability (84.2%) of determining the correct number of sires for three-sire clutches and a moderate probability (49.7%) of determining the correct number of sires for four-sire clutches. These results suggest that we were able to correctly detect double and triple matings, but the actual incidence of multiple paternity (especially for higher-order matings) is probably greater than we detected.

There was a significant positive correlation between minimum number of fathers and number of offspring produced in a clutch (Figure 2; $r_{38} = .56$, $p < .001$). Because the minimum number of fathers is likely to be underestimated in small clutches, we reran the analysis

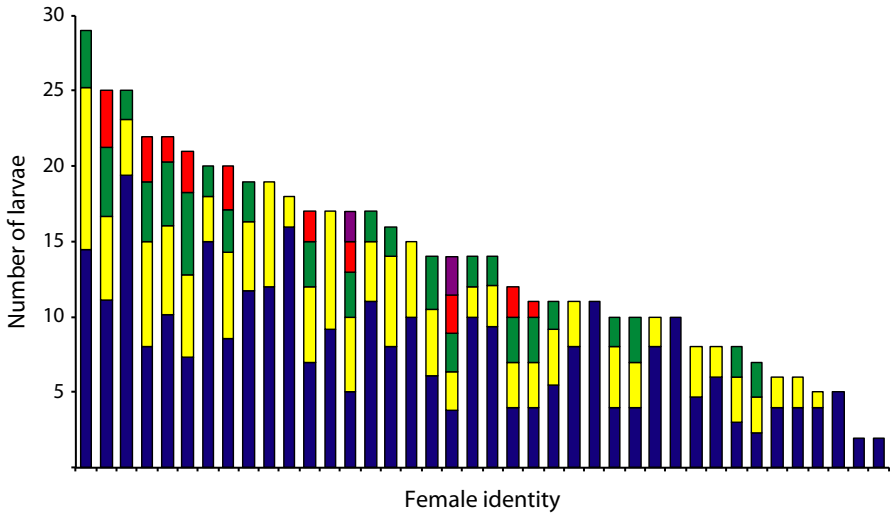

FIGURE 1 Patterns of multiple paternity in the clutches of 40 wild-caught females. Each bar represents the clutch of an individual female, and different colours within each bar indicate the number of larvae sired by different fathers based on the most likely minimum-father solution generated by GERUD [Colour figure can be viewed at wileyonlinelibrary.com]

with the four smallest clutches excluded and found that the relationship remained significant (r_{34} = .41, p = .014).

3.3 | Paternity skew

Of the 35 clutches that were sired by multiple males, eight (23%) had significant paternity skew (p < .05), and an additional two trended towards significance (p < .10; Table 2). There was a significant effect of both standardized paternity skew (χ^2 = 16.03, p < .001) and number of sires (χ^2 = 18.62, p < .001) on the number of offspring produced in a clutch (Figure 3). There was no effect of number of sires on standardized paternity skew ($F_{2,\ 30}$ = 2.15, p = .13).

Our method of brood collection made it possible to categorize offspring into two general cohorts (early = offspring from first brood sieving; late = offspring from second brood sieving) and test for heterogeneity in paternity skew across the brood production period. For each clutch, we compared the number of offspring sired by the most successful male between the two brood production periods. There was no evidence of heterogeneity in sperm utilization over time (Wilcoxon rank-sum test: W = 743, p = .12).

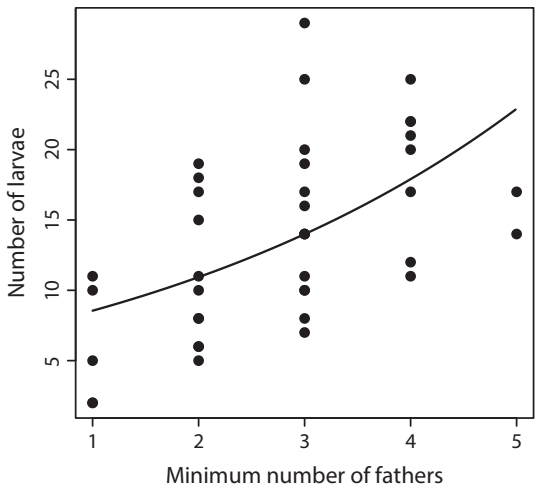

FIGURE 2 Relationship between the minimum number of fathers and number of larvae produced in a clutch. The fitted line is from a generalized linear model with a log link function and quasi-Poisson errors to account for overdispersed data: log(number of larvae) = 1.90 + 0.25 × minimum number of fathers

4 | DISCUSSION

Dung beetles in the genus *Onthophagus* have become a model system for testing predictions of pre- and postmating sexual selection theory (e.g., Kotiaho, Simmons, Hunt, & Tomkins, 2003; Kotiaho

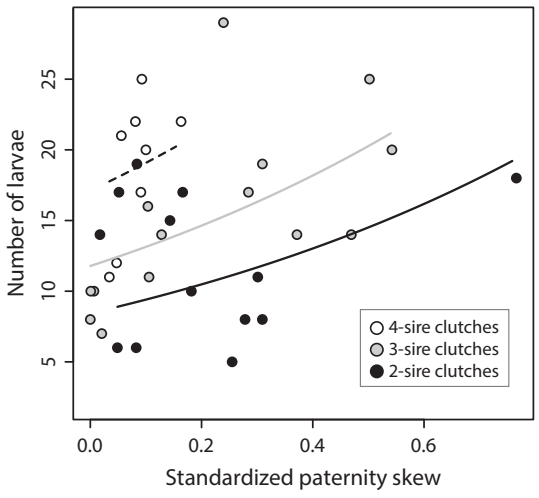

FIGURE 3 Relationship between standardized paternity skew and number of larvae produced in a clutch. Open points represent clutches with four minimum sires; grey points represent clutches with three minimum sires; closed circles represent clutches with two minimum sires. The fitted lines are from a generalized linear model with a log link function and Poisson errors. Four-sire clutches (dashed line): log (number of larvae) = 2.84 + 1.08 × standardized paternity skew; three-sire clutches (grey line): log(number of larvae) = 2.47 + 1.08 × standardized paternity skew; two-sire clutches (black line): log(number of larvae) = 2.13 + 1.08 × standardized paternity skew

et al., 2001; Simmons & Emlen, 2006; Simmons & García-González, 2008; Simmons, House, Hunt, & García-González, 2009; Simmons & Kotiaho, 2007; Simmons et al., 2007b). However, these studies have been conducted exclusively under controlled laboratory conditions, and even basic information about the reproductive behaviour of female dung beetles in natural populations remains largely unknown. In this study, we developed microsatellite markers for the species *Onthophagus taurus* and then used the markers to genotype the offspring of wild-caught females. Our results provide the first estimates of the rates of multiple paternity and patterns of sperm utilization for dung beetles in the wild and support the findings of previous laboratory studies that females gain indirect benefits from polyandry (Simmons, 2011; Simmons & Holley, 2011).

We found that female *O. taurus* in the wild are highly polyandrous. The majority of females (88%) produced offspring that were sired by at least two males, and two females (5%) produced clutches with as many as five sires. The average number of sires in a clutch was 2.8. We note that our results represent minimum estimates of the true rates of multiple paternity for at least two reasons. First, extensive allele sharing in the population would reduce our power to discriminate among potential fathers. Second, females may have already produced broods prior to collection and exhausted supplies of sperm from previous mates. The latter seems highly unlikely, however, given that *O. taurus* females produce fertile broods for their entire lifespan even when they are exposed to a single male for a single day at the beginning of their reproductive lives (Hunt, Simmons, & Kotiaho, 2002). Despite these potential limitations, our results indicate that females frequently mate with and store sperm from multiple males.

While our paternity analyses estimate the number of males that sired offspring in each clutch, they do not accurately estimate the number of males with which each female has mated. First, females may not store sperm from all matings, and they may only use a subset of stored sperm to fertilize their eggs. As a result, the number of fathers in a female's clutch is likely to underestimate the total number of mates (Bretman & Tregenza, 2005; Eberhard, 1996; Simmons, 2001; Simmons & Beveridge, 2010). Controlled experiments that compare the number of mates with the number of fathers will provide insights into how much control females have in biasing paternity during copulation and/or after insemination (Eberhard, 1996; Jennions & Petrie, 2000; Simmons, 2001, 2005). Second, levels of polyandry may be underestimated if offspring from genetically incompatible males fail to hatch and thus are not genotyped. However, previous work on *O. taurus* that used amplified fragment length polymorphism fingerprinting to assign paternity to larvae from laboratory populations found no evidence that a male's genetic dissimilarity with a female influenced his fertilization success (Simmons, Beveridge, & Krauss, 2004). These findings suggest that our estimates of polyandry are unlikely to be biased due to genetic incompatibilities. Third, sperm stratification within the female reproductive tract can affect patterns of temporal sperm utilization and sperm precedence in insects, potentially leading to underestimates in the levels of multiple mating (Harvey & Parker, 2000; Simmons, 2001). However, previous studies have shown that sperm utilization conforms to a model of random sperm mixing in *O. taurus* (Hunt & Simmons, 2002; Simmons et al., 2004; Tomkins & Simmons, 2000), and our own data showed no evidence of heterogeneity in sperm utilization over time.

In addition to finding high levels of polyandry, we found a strong positive correlation between the number of fathers and overall clutch size. Thus, mating with multiple males appears to increase female reproductive success. The same effect was found in laboratory studies, with clutch size depending on mating frequency and the quality of mates, implicating both direct and indirect benefits of polyandry (Simmons & Holley, 2011). We also found high levels of paternity skew within clutches, and a positive relationship between paternity skew and clutch size that was independent of the number of fathers. These results suggest that females benefit from mating with multiple males because they can thereby incite sperm competition and/or exercise cryptic female choice that skews paternity towards males capable of siring offspring of higher viability. To our knowledge, this is the first study to show a positive relationship between paternity skew in the wild and reproductive success for an insect (but see Fisher et al., 2006, for an example in a mammal) and provides strong evidence that female dung beetles benefit from polyandry under natural conditions.

We do not yet know whether the observed patterns of paternity bias are the result of sperm competition or cryptic female choice. Ascribing the variation in paternity skew to either sperm competition or sperm selection by females is likely to be an exceptionally difficult task, not only because the two processes are obviously intimately related (Jennions & Petrie, 2000; Simmons, Stockley, Jackson, & Parker, 1996), but also because both processes create the opportunity for females to gain genetic benefits in this species (Simmons, 2011). Specifically, previous studies in *O. taurus* have found that males with larger testes have a competitive fertilization advantage (Simmons & García-González, 2008) and that females preferentially use shorter sperm to fertilize their eggs (García-González & Simmons, 2007). Both large testes and short sperm are genetically correlated with body condition (Simmons & Kotiaho, 2002), so sperm competition and cryptic female choice should act synergistically as they both select for males of high genetic quality (Simmons, 2011). Indeed, experimental evolution studies find that females from polygamous lines produce sons of higher condition than females from monogamous lines, and females from monogamous lines can improve their sons' condition by mating polyandrously (Simmons & García-González, 2008). The evidence to date therefore supports a scenario of sexual selection for good genes: by mating with multiple males and inciting sperm competition, females can ensure that fertilization will be biased towards competitive males of high genetic quality.

Although our results are consistent with the hypothesis that females are polyandrous to gain genetic benefits, we cannot rule out the possibility that the positive correlation between reproductive success and number of fathers is also driven by direct benefits or maternal effects. For example, hormonal stimulants and accessory gland products that are transferred in the male's ejaculate often stimulate investment in egg production, and females that mate

multiply should acquire more of these substances (Arnqvist & Nilsson, 2000; Simmons, 2001). As a result, direct benefits from gonadotropic substances may contribute to the higher reproductive success of polyandrous females. Previous laboratory research has shown that even in the absence of paternal assistance in brood provisioning, *O. taurus* females have higher lifetime reproductive success and longer lifespan when mated with large males than with small males, which suggests that males do indeed provide nutrients in their seminal fluids that females use in reproduction (Kotiaho et al., 2003).

Females may also vary the amount of resources they allocate to their offspring depending on the phenotype of their mates (Mousseau & Fox, 1998; Sheldon, 2000). Specifically, if females invest more heavily in offspring production when they have greater scope for being choosy (Cunningham & Russell, 2000; Simmons, 1987; Wedell, 1996), then differential maternal allocation may confound the relationship between mating patterns and female reproductive success (Sheldon, 2000; Simmons, 2005). Maternal effects are known to be an important source of variation in offspring size and development in *O. taurus*: large females provide more resources for their offspring than small females (Hunt & Simmons, 2000), and female provisioning also increases after mating with large, long-horned males (Kotiaho et al., 2003). However, a previous study that controlled for maternal effects through a maternal split clutch design found significant covariation between the condition of fathers and sons (Simmons, 2011), and previous work has shown that females that mate multiply with high quality males produce offspring of higher viability than females that mate the same number of times to low quality males (Simmons & Holley, 2011). These results indicate that there is a clear genetic effect of male quality on offspring viability, such that females can gain indirect benefits through mate choice and polyandry (García-González & Simmons, 2011; Simmons, 2011; Simmons & Holley, 2011).

Finally, despite finding evidence for significant paternity skew, we do not have phenotypic data for the preferred fathers. Using the polymorphic microsatellite markers developed here, studies will now be able to evaluate the degree to which *O. taurus* females bias the paternity of their offspring towards males with particular traits. Our data provide clear evidence that female dung beetles engage in high levels of polyandry in the wild and thus have ample opportunity to promote postcopulatory mechanisms that bias paternity. Future molecular and experimental work in this model system promises to shed important insights into how these postcopulatory processes interact with precopulatory mate choice and male–male competition, offering a more holistic picture of the nature and strength of sexual selection.

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AUTHOR CONTRIBUTIONS

All authors conceived and designed the study; E.L.M. and B.A.B. collected the samples; E.L.M. performed the molecular work; E.L.M. and B.A.B. analysed the data; E.L.M. wrote the initial manuscript; all authors revised the manuscript and approved the final version.

DATA ACCESSIBILITY

The data used in these analyses and code for our paternity skew simulations are available from the Dryad Digital Repository: doi: 10.5061/dryad.2t53m.

REFERENCES

Aljanabi, S. M., & Martinez, I. (1997). Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucleic Acids Research*, *25*, 4692–4693.

Arnqvist, G. (1989). Multiple mating in a water strider: Mutual benefits or intersexual conflict? *Animal Behaviour*, *38*, 749–756.

Arnqvist, G., & Nilsson, T. (2000). The evolution of polyandry: Multiple mating and female fitness in insects. *Animal Behaviour*, *60*, 145–164.

Birkhead, T. R., & Møller, A. P. (Eds.) (1998). *Sperm competition and sexual selection*. London: Academic Press.

Boomsma, J. J., & Ratnieks, F. L. W. (1996). Paternity in eusocial hymenoptera. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *351*, 947.

Bretman, A., & Tregenza, T. (2005). Measuring polyandry in wild populations: A case study using promiscuous crickets. *Molecular Ecology*, *14*, 2169–2179.

Bundgaard, J., Barker, J. S. F., Frydenberg, J., & Clark, A. G. (2004). Remating and sperm displacement in a natural population of *Drosophila buzzatii* inferred from mother–offspring analysis of microsatellite loci. *Journal of Evolutionary Biology*, *17*, 376–381.

Cunningham, E. J. A., & Russell, A. F. (2000). Egg investment is influenced by male attractiveness in the mallard. *Nature*, *404*, 74–77.

Eberhard, W. G. (1996). *Female control: Sexual selection by cryptic female choice*. Princeton, New Jersey: Princeton University Press.

Emlen, D. J. (1997). Alternative reproductive tactics and male-dimorphism in the horned beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). *Behavioral Ecology and Sociobiology*, *41*, 335–341.

Fisher, D. O., Double, M. C., Blomberg, S. P., Jennions, M. D., & Cockburn, A. (2006). Post-mating sexual selection increases lifetime fitness of polyandrous females in the wild. *Nature*, *444*, 89–92.

Frentiu, F. D., & Chenoweth, S. F. (2008). Polyandry and paternity skew in natural and experimental populations of *Drosophila serrata*. *Molecular Ecology*, *17*, 1589–1596.

García-González, F., & Simmons, L. W. (2007). Shorter sperm confer higher competitive fertilization success. *Evolution*, *61*, 816–824.

García-González, F., & Simmons, L. W. (2011). Good genes and sexual selection in dung beetles (*Onthophagus taurus*): Genetic variance in egg-to-adult and adult viability. *PLoS ONE*, *6*, e16233.

Gerlach, N. M., McGlothlin, J., Parker, P., & Ketterson, E. (2012). Reinterpreting Bateman gradients: Multiple mating and selection in both sexes of a songbird species. *Behavioral Ecology*, *23*, 1078–1088.

Good, J. M., Ross, C. L., & Markow, T. A. (2006). Multiple paternity in wild-caught *Drosophila mojavensis*. *Molecular Ecology*, *15*, 2253–2260.

Haddrill, P. R., Shuker, D. M., Amos, W., Majerus, M. E. N., & Mayes, S. (2008). Female multiple mating in wild and laboratory populations of the two-spot ladybird, *Adalia bipunctata*. *Molecular Ecology*, *17*, 3189–3197.

Halffter, G., & Edmonds, W. D. (1982). *The nesting behavior of dung beetles (Scarabaeinae). An ecological and evolutive approach*. New Mexico: Instituto de Ecologia.

Harshman, L. G., & Clark, A. G. (1998). Inference of sperm competition from broods of field-caught *Drosophila*. *Evolution*, *52*, 1334–1341.

Harvey, I. F., & Parker, G. A. (2000). "Sloppy" sperm mixing and intraspecific variation in sperm precedence (P_2) patterns. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *267*, 2537–2542.

Hosken, D. J., Stockley, P., Tregenza, T., & Wedell, N. (2008). Monogamy and the battle of the sexes. *Annual Review of Entomology*, *54*, 361–378.

Hunt, J., & Simmons, L. W. (2000). Maternal and paternal effects on offspring phenotype in the dung beetle *Onthophagus taurus*. *Evolution*, *54*, 936–941.

Hunt, J., & Simmons, L. W. (2002). Confidence of paternity and paternal care: Covariation revealed through the experimental manipulation of the mating system in the beetle *Onthophagus taurus*. *Journal of Evolutionary Biology*, *15*, 784–795.

Hunt, J., Simmons, L. W., & Kotiaho, J. S. (2002). A cost of maternal care in the dung beetle *Onthophagus taurus*? *Journal of Evolutionary Biology*, *15*, 57–64.

Imhof, M., Harr, B., Brem, G., & Schlötterer, C. (1998). Multiple mating in wild *Drosophila melanogaster* revisited by microsatellite analysis. *Molecular Ecology*, *7*, 915–917.

Jennions, M. D., & Petrie, M. (2000). Why do females mate multiply? A review of the genetic benefits. *Biological Reviews*, *75*, 21–64.

Jones, A. G. (2005). gerud 2.0: A computer program for the reconstruction of parental genotypes from half-sib progeny arrays with known or unknown parents. *Molecular Ecology Notes*, *5*, 708–711.

Kalinowski, S. T., Taper, M. L., & Marshall, T. C. (2007). Revising how the computer program cervus accommodates genotyping error increases success in paternity assignment. *Molecular Ecology*, *16*, 1099–1106.

Knell, R. J., & Webberley, K. M. (2004). Sexually transmitted diseases of insects: Distribution, evolution, ecology and host behaviour. *Biological Reviews*, *79*, 557–581.

Kotiaho, J. S., Simmons, L. W., Hunt, J., & Tomkins, J. L. (2003). Males influence maternal effects that promote sexual selection: A quantitative genetic experiment with dung beetles *Onthophagus taurus*. *American Naturalist*, *161*, 852–859.

Kotiaho, J. S., Simmons, L. W., & Tomkins, J. L. (2001). Towards a resolution of the lek paradox. *Nature*, *410*, 684–686.

Marshall, T. C., Slate, J., Kruuk, L. E. B., & Pemberton, J. M. (1998). Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, *7*, 639–655.

McCullough, E. L., Buzatto, B. A., & Simmons, L. W. (2017). Data from: Benefits of polyandry: Molecular evidence from field-caught dung beetles. *Dryad Digital Repository*. doi: 10.5061/dryad.2t53m.

Meglécz, E., Costedoat, C., Dubut, V., Gilles, A., Malausa, T., Pech, N., & Martin, J. F. (2010). QDD: A user-friendly program to select microsatellite markers and design primers from large sequencing projects. *Bioinformatics*, *26*, 403–404.

Moczek, A. P., & Emlen, D. J. (2000). Male horn dimorphism in the scarab beetle, *Onthophagus taurus*: Do alternative reproductive tactics favour alternative phenotypes? *Animal Behaviour*, *59*, 459–466.

Mousseau, T. A., & Fox, C. W. (1998). The adaptive significance of maternal effects. *Trends in Ecology & Evolution*, *13*, 403–407.

Oneal, E., & Knowles, L. L. (2015). Paternity analyses in wild-caught and laboratory-reared Caribbean cricket females reveal the influence of mating environment on post-copulatory sexual selection. *Journal of Evolutionary Biology*, *28*, 2300–2307.

Pamilo, P. (1993). Polyandry and allele frequency differences between the sexes in the ant Formica aquilonia. *Heredity*, *70*, 472–480.

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

Parker, G. A., Ball, M. A., Stockley, P., & Gage, M. J. G. (1996). Sperm competition games: Individual assessment of sperm competition intensity by group spawners. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *263*, 1291–1297.

Parker, G. A., Ball, M. A., Stockley, P., & Gage, M. J. G. (1997). Sperm competition games: A prospective analysis of risk assessment. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *264*, 1793–1802.

Pomfret, J. C., & Knell, R. J. (2008). Crowding, sex ratio and horn evolution in a South African beetle community. *Proceedings of the Royal Society B: Biological Sciences*, *275*, 315–321.

Raymond, M., & Rousset, F. (1995). GENEPOP (Version 1.2): Population genetics software for exact tests and ecumenicism. *Journal of Heredity*, *86*, 248–249.

Ridsdill-Smith, T. J. (1993). Effects of avermectin residues in cattle dung on dung beetle (Coleoptera: Scarabaeidae) reproduction and survival. *Veterinary Parasitology*, *48*, 127–137.

Rodríguez-Muñoz, R., Bretman, A., Slate, J., Walling, C. A., & Tregenza, T. (2010). Natural and sexual selection in a wild insect population. *Science*, *328*, 1269.

Rowe, L. (1994). The costs of mating and mate choice in water striders. *Animal Behaviour*, *48*, 1049–1056.

Sheldon, B. C. (2000). Differential allocation: Tests, mechanisms and implications. *Trends in Ecology & Evolution*, *15*, 397–402.

Simmons, L. W. (1987). Female choice contributes to offspring fitness in the field cricket, *Gryllus bimaculatus* (De Geer). *Behavioral Ecology and Sociobiology*, *21*, 313–321.

Simmons, L. W. (2001). *Sperm competition and its evolutionary consequences in the insects*. Princeton, NJ: Princeton University Press.

Simmons, L. W. (2005). The evolution of polyandry: Sperm competition, sperm selection, and offspring viability. *Annual Review of Ecology, Evolution, and Systematics*, *36*, 125–146.

Simmons, L. W. (2011). Sexual selection after mating: The evolutionary consequences of sperm competition and cryptic female choice in onthophagines. In L. W. Simmons & T. J. Ridsdill-Simith (Eds), *Ecology and evolution of dung beetles* (pp. 66–86). West Sussex, UK: Wiley-Blackwell.

Simmons, L. W., & Beveridge, M. (2010). The strength of postcopulatory sexual selection within natural populations of field crickets. *Behavioral Ecology*, *21*, 1179–1185.

Simmons, L. W., Beveridge, M., & Kennington, W. J. (2007a). Polyandry in the wild: Temporal changes in female mating frequency and sperm competition intensity in natural populations of the tettigoniid *Requena verticalis*. *Molecular Ecology*, *16*, 4613–4623.

Simmons, L. W., Beveridge, M., & Krauss, S. (2004). Genetic analysis of parentage within experimental populations of a male dimorphic beetle, *Onthophagus taurus*, using amplified fragment length polymorphism. *Behavioral Ecology and Sociobiology*, *57*, 164–173.

Simmons, L. W., & Emlen, D. J. (2006). Evolutionary trade-off between weapons and testes. *Proceedings of the National Academy of Sciences*, *103*, 16346–16351.

Simmons, L. W., Emlen, D. J., & Tomkins, J. L. (2007b). Sperm competition games between sneaks and guards: A comparative analysis using dimorphic male beetles. *Evolution*, *61*, 2684–2692.

Simmons, L. W., & Fitzpatrick, J. L. (2016). Sperm competition and the coevolution of pre- and postcopulatory traits: Weapons evolve faster than testes among onthophagine dung beetles. *Evolution*, *70*, 998–1008.

Simmons, L. W., & García-González, F. (2008). Evolutionary reduction in testes size and competitive fertilization success in response to the experimental removal of sexual selection in dung beetles. *Evolution*, *62*, 2580–2591.

Simmons, L. W., & Holley, R. (2011). Offspring viability benefits but no apparent costs of mating with high quality males. *Biology Letters*, *7*, 419–421.

Simmons, L. W., House, C. M., Hunt, J., & García-González, F. (2009). Evolutionary response to sexual selection in male genital morphology. *Current Biology*, *19*, 1442–1446.

Simmons, L. W., & Kotiaho, J. S. (2002). Evolution of ejaculates: Patterns of phenotypic and genotypic variation and condition dependence in sperm competition traits. *Evolution*, *56*, 1622–1631.

Simmons, L. W., & Kotiaho, J. S. (2007). Quantitative genetic correlation between trait and preference supports a sexually selected sperm process. *Proceedings of the National Academy of Sciences*, *104*, 16604–16608.

Simmons, W. L., Stockley, P., Jackson, L. R., & Parker, A. G. (1996). Sperm competition or sperm selection: No evidence for female influence over paternity in yellow dung flies *Scatophaga stercoraria*. *Behavioral Ecology and Sociobiology*, *38*, 199–206.

Simmons, L. W., Tomkins, J. L., & Hunt, J. (1999). Sperm competition games played by dimorphic male beetles. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *266*, 145–150.

Song, S. D., Drew, R. A. I., & Hughes, J. M. (2007). Multiple paternity in a natural population of a wild tobacco fly, *Bactrocera cacuminata* (Diptera: Tephritidae), assessed by microsatellite DNA markers. *Molecular Ecology*, *16*, 2353–2361.

Starr, C. K. (1984). Sperm competition, kinship, and sociality in the aculeate Hymenoptera. In R. L. Smith (Ed.), *Sperm competition and the evolution of animal mating systems* (pp. 427–464). London, UK: Academic Press.

Tomkins, J. L., & Simmons, L. W. (2000). Sperm competition games played by dimorphic male beetles: Fertilization gains with equal mating access. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *267*, 1547–1553.

Tregenza, T., & Wedell, N. (1998). Benefits of multiple mates in the cricket *Gryllus bimaculatus*. *Evolution*, *52*, 1726–1730.

Tregenza, T., & Wedell, N. (2000). Genetic compatibility, mate choice and patterns of parentage: Invited review. *Molecular Ecology*, *9*, 1013–1027.

Wedell, N. (1996). Mate quality affects reproductive effort in a paternally investing species. *The American Naturalist*, *148*, 1075–1088.

Zeh, J. A., & Zeh, D. W. (1997). The evolution of polyandry II: Post–copulatory defenses against genetic incompatibility. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *264*, 69.

Zeh, J. A., & Zeh, D. W. (2001). Reproductive mode and the genetic benefits of polyandry. *Animal Behaviour*, *61*, 1051–1063.

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