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Multiple paternity and sperm competition in the sibling species *Drosophila buzzatii* and *Drosophila koepferae*

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Abstract

Sperm competition (SC) is a major component of sexual selection that enhances intra- and intersexual conflicts and may trigger rapid adaptive evolution of sexual characters. The actual role of SC on rapid evolution, however, is poorly understood. Besides, the relative contribution of distinctive features of the mating system to among species variation in the strength of SC remains unclear. Here, we assessed the strength of SC and mating system factors that may account for it in the closely related species *Drosophila buzzatii* and *Drosophila koepferae*. Our analyses reveal higher incidence of multiple paternity and SC risk in *D. buzzatii* wild-inseminated females. The estimated number of fathers per brood was 3.57 in *D. buzzatii* and 1.95 in *D. koepferae*. In turn, the expected proportion of females inseminated by more than one male was 0.89 in *D. buzzatii* and 0.58 in *D. koepferae*. Laboratory experiments show that this pattern may be accounted for by the faster rate of stored sperm usage observed in *D. koepferae* and by the greater female remating rate exhibited by *D. buzzatii*. We also found that the male reproductive cost of SC is also higher in *D. buzzatii*. After a female mated with a second male, first-mating male fertility was reduced by 71.4% in *D. buzzatii* and only 33.3% in *D. koepferae*. Therefore, we may conclude that postmating sexual selection via SC is a stronger evolutionary force in *D. buzzatii* than in its sibling.

Keywords: mating system, polyandry, sexual selection, sperm competition, sperm precedence, sperm usage

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Introduction

Sperm competition (SC) is a form of sexual selection that is widely recognized as a major and pervasive force in evolution (Parker 1998; Simmons 2001). It generates behavioural, physiological and morphological adaptations in males that facilitate displacement of sperm stored by females from previous matings and prevent their own sperm from being displaced (Parker 1970, 1998; Simmons 2001). These adaptations lead to conflicts between rival males and, potentially, between members of reproductive couples. Based on these conflicts, postmating sexual selection via SC has been proposed as responsible of the rapid evolution of male sexual traits such as genital morphology and seminal fluid proteins (Birkhead & Pizzari 2002; Swanson & Vacquier 2002; Hosken & Stockley 2004; Haerty *et al.* 2007). One prediction of this hypothesis is that interspecific differences in the strength of SC should account for evolutionary rate variation of rapidly evolving reproductive traits or genes (Wong 2011). Testing this prediction, which may clarify the actual scope of postmating sexual selection on rapid evolution, requires the quantification of the intensity of SC among related species in which divergent reproductive traits or genes have been identified (Wong 2011).

For any organism with internal fertilization, SC implies the co-occurrence of sperm from multiple males inside a female (Parker 1970). Thus, the strength of SC can be influenced by several mating system variables that affect the risk of SC by determining the chances

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that sperm from different males co-occur inside a female (Simmons 2001). Among *Drosophila* species, for instance, SC risk (i.e. the probability that the male's sperm will compete against the sperm from other males for a given set of ova) is expected to increase with female remating rate (FRR) and to decrease with sperm usage rate (SUR) (Parker 1970; Hurtado & Hasson 2013). FRR and SUR, however, may not be independent because *Drosophila* females often remate to reload sperm supply (Markow 1996, 2002; Singh *et al.* 2002). In such cases, species with higher SUR would have greater FRR (Markow *et al.* 2012), nevertheless, the intensity of SC would still depend on which FRR or SUR is the strongest determinant of SC. Thus, without accurate estimations of the intensity of SC, it would be difficult to infer the significance of SUR and FRR as determinants of SC risk. Up to now, estimations of the intensity of SC were fairly indirect (Simmons & Beveridge 2010) and the relative contribution of different mating system features to interspecific variation in the strength of SC remains unclear.

A prerequisite for SC is that females remate before depleting sperm supply. One way of exploring this prerequisite is to determine whether the time after mating required by inseminated females to exhaust sperm supply is longer than the time elapsed until remating. However, laboratory estimations of these variables may not reflect what actually occurs in nature as ecological conditions may affect SUR and FRR by determining oviposition and mating opportunities, respectively (Markow & O'Grady 2008). Another way of testing the co-occurrence of sperm from multiple fathers, which is not dependent on laboratory conditions, is by the recovery of genetic variation in the progeny of wild-caught females using highly polymorphic markers. This approach has been applied in a number of studies revealing that the incidence of polyandry in wild-inseminated females is highly variable among *Drosophila* species (Harshman & Clark 1998; Imhof *et al.* 1998; Jones & Clark 2003; Bundgaard *et al.* 2004; Schlotterer *et al.* 2005; Good *et al.* 2006; Frentiu & Chenoweth 2008). Although polyandry is necessary for SC, it does not guarantee a competition unless the number of eggs fertilized by the sperm of a father is reduced by the presence of another father's ejaculate (Simmons 2001). Thus, to estimate the strength of SC as an evolutionary selective pressure, it is necessary to assess multiple paternity as well as its reproductive consequences on stored sperm.

The South American *Drosophila buzzatti* and *Drosophila koepferae* are cactophilic sibling species that belong to the *buzzatii* complex of the *repleta* group (Fontdevila *et al.* 1988). Studies involving members of the *repleta* group have shown that females have high FRRs (Markow 1996; Bundgaard & Barker 2000; Good *et al.* 2006) and extremely rapid evolutionary rates of some reproductive tract proteins (Wagstaff & Begun 2005; Kelleher *et al.* 2011). In addition, it has been established that male genital morphology exhibits rapid adaptive evolution in *Drosophila mojavensis* (Richmond *et al.* 2012) and *Drosophila buzzatii* (Soto *et al.* 2013). Recently, Hurtado & Hasson (2013) found that female latency to remating is 14 times shorter in *D. buzzatii* than in *D. koepferae*, which suggests that female remating frequency and SC opportunities are quite higher in *D. buzzatii*. The fast evolutionary rate of sexual traits and distinctive features between *D. buzzatii* and *D. koepferae* mating systems make these siblings an attractive model for the study of the connection between mating system characteristics, SC and rapid evolution.

Here, we aim to assess both the strength of SC and mating system factors that account for it in *D. buzzatii* and *D. koepferae*. With these purposes, we examine the risk of SC by determining the incidence of multiple paternity in wild-caught females using genetic variation at five polymorphic microsatellite loci. In addition, we explore how FRR and the pattern of sperm usage affect the risk of SC. Finally, to evaluate the male reproductive cost of polyandry, we assess the reduction in male reproductive output when the female remates with a second male.

Materials and methods

Multiple paternity

To explore the risk of sperm competition in *Drosophila buzzatii* and *Drosophila koepferae*, we estimated the number of sires per brood and the proportion of females inseminated by more than one father. We accomplished this aim by paternity analyses by means of the genetic analysis of the progeny of wild-caught inseminated females using highly polymorphic markers.

Samples collection. A total of 204 females were collected by bait trapping in northwestern Argentina (La Rioja province) in February 2012. All collected flies were immediately aspirated into individual vials, transferred to new vials every 48 h until egg laying stopped and preserved in absolute ethanol at −20 °C. Sixty eight females did not produce any offspring after 2 weeks and were discarded. When adult offspring of the remaining females started to emerge from the vials, newly eclosed flies were re-covered every 24 h and preserved in ethanol at −20 °C. Species identification, accomplished by the inspection of the genitalia of one progeny adult male (as females of both species are morphologically indistinguishable), revealed that 72 collected females were *D. buzzatii* and 64 *D. koepferae*.

Molecular genotyping. We randomly chose, from the preserved females that had produced any progeny, 2. *buzzatii* and 14 *D. koepferae* mothers, and 17–23 (mean number = 21) offspring of each respective brood (a total of 748 flies) for genotyping.

DNA was extracted from individual ethanol-preserved flies following the Puregene (Gentra Systems) DNA Purification Kit Protocol. Multilocus genotypes were generated using five dinucleotide repeat microsatellite loci. Four loci, named B65, K72, K75 and K76, could be amplified in both species, while K60 could be only amplified in *D. koepferae*. All five markers were developed from genomic libraries according to Hamilton *et al.* (1999) with minor modifications. Deviations from Hardy–Weinberg expectations or linkage disequilibrium were not found for these microsatellite loci, which are neither linked to each other (Lipko & Hasson unpublished). Forward primers were tagged at their 5′ ends with four different fluorescent dyes and amplified in a single multiplexed polymerase chain reaction (PCR) using Platinum Multiplex PCR Master Mix (Applied Biosystems). Primer sequences of the five microsatellite loci employed in the study are presented in Table 1.

Polymerase chain reaction products were genotyped in the Genomics Unit of INTA Biotechnology Institute (Castelar, Argentina) using an ABI 3130XL Genetic Analyser (Applied Biosystems). We used Peak Scanner™ software v1.0 (Applied Biosystems) to score alleles. Multilocus genotypes can be found as supporting information in the online version of this study (Table S1, Supporting Information).

Paternity analyses. For each species, we fitted two parameters of a model that describes the number of mates per female (α) and the proportion of offspring sired by the last-mating male (β) (Jones & Clark 2003). The model assumes that the probability density for the number of mates per female is a truncated Poisson (eliminating the possibility of zero mates) with parameter α. Then, unlike the regular Poisson distribution, the mean number of mates is not α but α/(1-exp(-α)), and the probability of finding more than one father per brood is 1-α/(exp(α)-1). The model also assumes that each male following the first mate displaces a fraction β of the already-present sperm, so last-mating male sires a proportion β of the progeny. Parameter estimation was performed with the SCARE software (available at http://www.massey.ac.nz/~mbjones/research/content_local/scare.html) which applies a Markov chain Monte Carlo (MCMC) to generate 10 000 samples from the joint posterior of the two parameters. From these simulated samples, we computed mean values of α and β (and the corresponding 90% credible intervals), the expected proportion of females inseminated by multiple males and the expected number of sires per brood.

Table 1 Microsatellite primer sequences for polymerase chain reaction (PCR) amplification

Locus	Dye	Primer Sequences (5′–3′)
B65	6-FAM	F: GAATTGTGGCCAAGTTTCGTAGAATC
		R: CTGCCACTAGTGAAGTATCAACAATG
K60	6-FAM	F: CAACCATTGCCATTTCATCTTACTGC
		R: AAGACAATCCAGCTTTCTATATGGCG
K72	NED	F: CAAATGACCAGAGGGAAGCGGG
		R: CGCCGAGGCACAGGAGCTGTTG
K75	PET	F: TGCCCTGAATACCAGGAGCATAAT
		R: ATAGGCAAACAGAGCGGCAAATAAC
K76	VIC	F: TTAACAGACAAGTCGATGCCGCTTC
		R: CGATCTCAAATGCAAGCACTACCTG

Mating system effects on sperm competition risk

For females that store sperm in a single tubular seminal receptacle, like *D. buzzatii* and *D. koepferae* (data not shown), the chances that sperm of different mates meet inside the reproductive tract of a female depend on female remating frequency and on the rate of stored sperm usage. Hurtado & Hasson (2013) have already studied female remating frequency in the same strains we use in the present study. Here, we complemented these results and explored the stored sperm usage by daily monitoring egg laying after mating.

Fly stocks. Four *D. buzzatii* isofemale lines (DB1, DB2, DB3 and DB4) and four *D. koepferae* isofemale lines (DK1, DK2, DK3 and DK4) were established in the laboratory by rearing the progeny of wild-inseminated females collected in northwestern Argentina in February 2010 (see details in Hurtado & Hasson 2013).

Patterns of sperm usage. We aimed to assess how fast an inseminated female exhausts (uses or wastes) sperm that is functional for SC, that is, sperm that can reproductively alter or be altered by sperm from other males. We addressed this issue assuming that the number of stored sperm functional for SC equals the number of stored sperm functional for egg fertilization. Thereby, we described sperm usage by counting the number of eggs that are fertilized per day in single-inseminated females, until total depletion of sperm supply. From these numbers, we then calculated the times elapsed until females deplete different proportions of the effective sperm supply.

Sperm usage was assessed in 6–20 (mean number = 14) females per strain. To obtain these females, 12–24 single virgin females (5-day-old) per strain were

given the opportunity to mate by placing with two virgin males (5-day-old) in a 3 cm^3 vial containing *Drosophila* culture medium at 8 am until copulation. When copulation occurred, the female was gently aspirated and released in an individual vial attached to an oviposition chamber with fresh egg-collecting medium (egg chamber from hereafter). Few females (4–25%) did not mate within an hour and were discarded. Egg chambers were daily replaced for 8 days as preliminary assays showed no egg laying beyond the 8th day after insemination. *Drosophila* females often lay infertile eggs (Hanson & Ferris 1929); thus, the number of first instar larvae hatched from each egg chamber was determined as an indicator of the number of fertile eggs laid per day. The number of larvae observed in an egg chamber may be considered as a fair predictor of the number of fertile eggs laid per day as egg mortality is very low in *D. buzzatii* and *D. koepferae* (Fanara & Hasson 2001). Because sperm release for fertilization occurs immediately before egg deposition (Campos-Ortega & Hartenstein 1985), the number of first instar larvae per chamber is also a surrogate of the daily release of stored sperm for effective fertilization. Females that did not produce any progeny were assumed to be involved in sterile matings and were excluded from the analysis. Daily-fertilized eggs numbers per female can be found as supporting information in the online version of this study (Table S2, Supporting Information).

Statistical analysis. To evaluate whether *D. buzzatii* and *D. koepferae* differ in the rate at which females use sperm supply for fertilization, we tested the null hypothesis that single-inseminated females of both species spend the same time to exhaust a given proportion of their effective sperm supply. Thus, we established, for each strain, the number of days elapsed as mating until the number of fertile eggs laid by the entire group of females exceeded 30%, 60% and 90% of their total fecundity. These values were chosen because time elapsed as insemination until egg fertilization seemed to covary linearly with these particular proportions. We applied a repeated-measure ANOVA in R (*ezANOVA* function of *ez* package (Lawrence 2012)) with species (*D. buzzatii* and *D. koepferae*) as a between-strains categorical fixed factor and the proportion of female fecundity (30%, 60% and 90%) as a within-strains covariate, which was supposed to linearly affect the dependent variable.

Male reproductive cost of sperm competition

We investigated the consequences of SC on the reproductive capacity of males by assessing the reduction in male fertility when females remate with a second male. Therefore, we compared brood size between once-mated females (i.e. in the absence of SC) and twice-mated females (i.e. after SC).

Male reproductive output in once-mated females. We assessed male reproductive capacity in once-mated females using the data obtained in the analysis of sperm usage rate in which we estimated, for each strain, the number of fertile eggs laid per day by a single-mated female during 8 days. Females that did not produce any progeny were assumed to be involved in sterile matings and were excluded from the analysis.

Twice-mated female fecundity. wFive-day-old virgin females were released along with two 5-day-old virgin males in 3-cm^3 vials containing *Drosophila* culture medium at 8 am. When copulation occurred, both males were replaced with two virgin males. When a second copulation was observed, the remated female was gently aspirated and released in an individual vial attached to an egg chamber that was daily replaced for 8 days. A high proportion of females (59–89%) did not mate and remate within 90 min and were discarded. This was not surprising because a significant reduction in female sexual receptivity is observed in these flies after mating (Hurtado & Hasson 2013). Nine to 12 twice-mated females were obtained per strain. The number of first instar larvae counted in the egg chambers offered to each female along an 8 days period was employed as an indicator of fecundity. Females that did not produce any progeny were assumed to be involved in sterile matings and were excluded from the analysis. Number of fertile eggs laid by each twice-mated female can be found as supporting information in the online version of this study (Table S3, Supporting Information).

Sperm precedence. We assessed sperm precedence by measuring P1 and P2 in twice-mated females obtained as described in the previous section. Here, however, we used first- and second-mating males coming from different strains as each strain was homozygous for a distinctive second-chromosome arrangement (inversion) that was used as a cytogenetic marker. In these experiments, two pairs of strains were used for each species (DB1-DB2 and DB3-DB4; DK1-DK2 and DK3-DK4). For each pair of strains, sperm displacement was tested in both male-mating orders and with females from both strains, that is, four different combinations of males and female genotypes (chromosome arrangements). For each combination, P2 was determined in 4–11 (mean number = 6) females by cytological genotyping 7–20 (mean number = 10) progeny larvae per female. Cytological characterization was accomplished via the inspection of

salivary gland chromosomes. Polytene chromosomes slides were prepared according to Fontdevila *et al.* (1981) and observed in a light microscope at 400× magnification.

Statistical analyses. To test whether female fecundity differs between *D. buzzatii* and *D. koepferae* or whether it increases with the number of mates, a REsidual Maximum Likelihood (REML) approach was applied for fitting a mixed effects model in R (*lmer* function of *lme4* package (Bates *et al.* 2011)). The model included species (*D. buzzatii* and *D. koepferae*) as a categorical fixed factor, the number of mates (1 and 2) as a categorical fixed factor, the interaction between species and the number of mates, and strains (1, 2, 3 and 4) as a random factor nested in species. *P*-values were computed by means of MCMC simulations applying the *pvals.fnc* function of *languageR* package (Baayen 2011).

To test whether these species exhibit different patterns of sperm precedence, we analysed P2 by applying a logistic regression model in R (*lmer* function (link= 'logit') of the *lme4* package) with species as a categorical fixed factor and pairs of strains (1–2 and 3–4) as a random factor nested in species. For this analysis, we assumed that P2 did not vary between females of the same pair of strains as nonsignificant effects of the male or female strain on P2/P1 were found within each pair of strains (results not shown).

Finally, we calculated per strain the reduction in male reproductive output due to SC as $F_{om}-P1 \times F_{tm}$. Where F_{om} and F_{tm} represent for mean fecundity of once and twice-mated females, respectively, and P1 denotes the expected proportion of offspring sired by the first-mating male assuming that P2/P1 ratio did not vary between strains of the same species. We divided this variable by the mean fecundity of once-mated females to obtain a relative estimation of the cost of SC that is independent of fecundity. Thus, to test whether the male reproductive capacity is equally reduced in the strains of *D. buzzatii* and *D. koepferae*, an ANOVA of the relative cost of SC was applied in R (*lm* function of *stats* package (R Development Core Team 2012)) with species as a categorical fixed factor.

Results

Incidence of multiple paternity

Estimates of observed and expected heterozygosities for each locus, based on the genotypes of the 20 *Drosophila buzzatii* and 14 *Drosophila koepferae* wild-inseminated females, are given in Table 2. Expected heterozygosity of the four loci amplified in both species was greater in *D. buzzatii*. Nevertheless, the discriminatory power of the microsatellite loci did not appreciably differ between *D. buzzatii* (1.00) and *D. koepferae* (1.00) because we assessed an additional locus in *D. koepferae* that did not amplified in *D. buzzatii*. None of the loci showed significantly reduced heterozygosity in these samples compared with Hardy–Weinberg expectations.

Table 2 Variability of microsatellites among wild-inseminated mothers. Observed (Ho) and expected heterozygosities (He), and *P*-value for Hardy–Weinberg expectations [HWE.test function of *genetics* package (Warnes *et al.* 2012)] are shown for each locus

	Drosophila buzzatii			*Drosophila koepferae*		
Locus	Ho	He	*P*-value	Ho	He	*P*-value
B65	0.75	0.85	1.00	0.07	0.13	0.07
K60				0.48	0.53	0.65
K72	0.65	0.74	1.00	0.86	0.72	1.00
K75	1.00	0.90	1.00	0.57	0.53	1.00
K76	0.95	0.90	1.00	0.64	0.60	1.00

Multilocus genotypes inspection of each wild-caught mother and her offspring showed different mating patterns in *D. buzzatii* and *D. koepferae*. Ninety-five per cent (19/20) of the *D. buzzatii* broods could not be fathered by less than two different sires (presented more than two distinct nonmaternal alleles) and 40% (8/20) could not be fathered by less than three different sires (presented more than four nonmaternal alleles). In *D. koepferae*, only 36% (5/14) of the broods could not be fathered by less than two different sires, and 0% could not be father by less than three different sires.

Paternity analysis with SCARE revealed that among wild-caught females, the incidence of multiple paternity was significantly greater in *D. buzzatii* than in its sibling. Mean α was 3.46 with a 90% credible interval of 2.70–4.31 in *D. buzzatii*. Thus, according to the implemented model, the expected proportion of *D. buzzatii* females inseminated by more than one male was 0.89, and the mean number of fathers was 3.57. In *D. koepferae*, mean α was 1.53 with a 90% credible interval of 0.89–2.29. Therefore, the expected proportion of females inseminated by multiple males in this species was 0.58, and the mean number of fathers was 1.95. Histograms based on 10 000 samples of α for both species are shown in Fig. 1A,B.

Estimations of β indicated that sperm precedence in wild-caught females was very similar in both species. The expected proportion of offspring sired by the last-mating male was 0.57 with a 90% credible interval of 0.52–0.61 in *D. buzzatii*, and 0.57 with a 90% credible interval of 0.51–0.64 in *D. koepferae* (Fig. 1C,D).

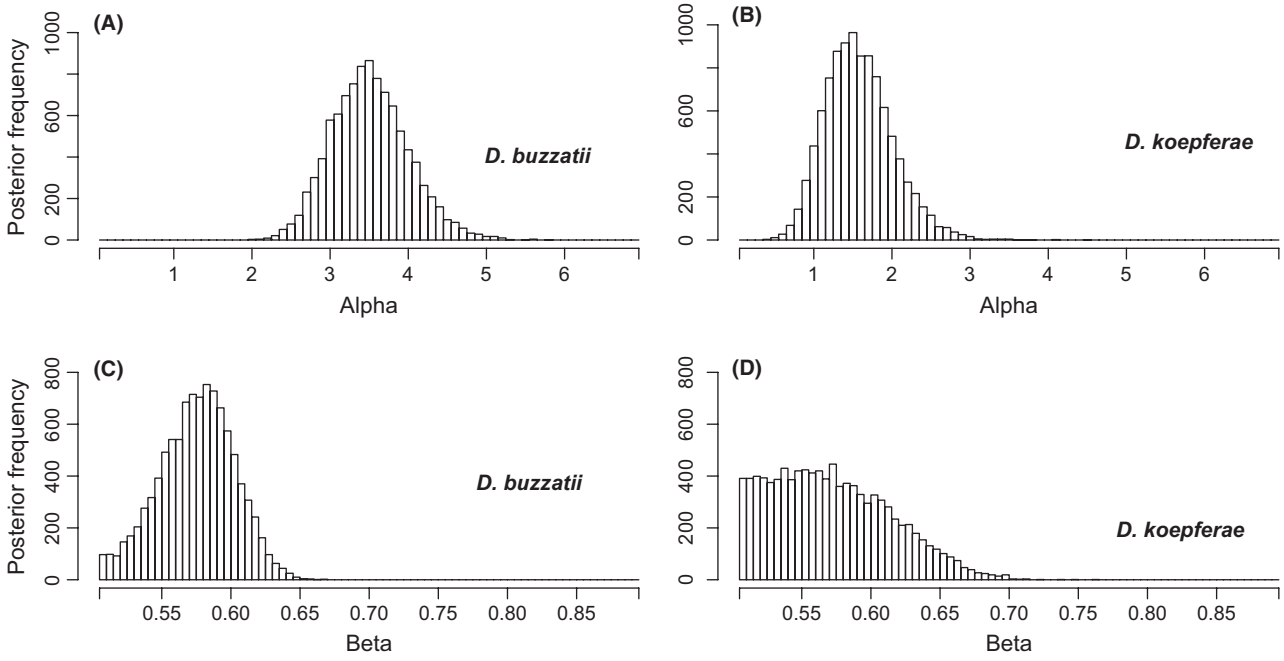

Fig. 1 Histograms of the posterior distribution of α (A, B) and β (C, D) for *Drosophila buzzatii* (left panel) and *Drosophila koepferae* (right panel). Charts are based on 10 000 samples obtained from the joint posterior produced by the Markov chain Monte Carlo.

Female rate of sperm usage

The mean time required by inseminated females to exhaust a given proportion (30%, 60% and 90%) of their effective sperm supply was longer for *D. buzzatii* than for *D. koepferae* ($F_{(6,1)}$ = 20.30, *P*-value = 0.004). Figure 2 shows the temporal pattern of sperm usage for egg fertilization in the four strains of each species.

Male reproductive cost of sperm competition

Analysis of the mean brood size revealed that the number of mates affected female fecundity in both species. Twice-mated females produced on average 37.13 more offspring than once-mated females (*P*-value <0.001), and the mean brood size of *D. buzzatii* females exceeded, though not significantly, *D. koepferae* mean brood size by an average of 31.36 offspring (*P*-value = 0.16). We did not detect interaction between the species factor and the number of mates (estimate = 2.16, *P*-value = 0.86). In addition, the interaction term failed to increase the model goodness of fit ($Chi^2_{(1)}$ = 0.048, *P*-value = 0.83), and therefore, it was excluded from the model for computing main effects. Figure 3 shows mean brood size of once and twice-mated females for each strain of both species.

The mean proportion of the offspring sired by the second male (P2) in *D. buzzatii* and *D. koepferae* is shown in Table 3. Although P2 varied slightly across

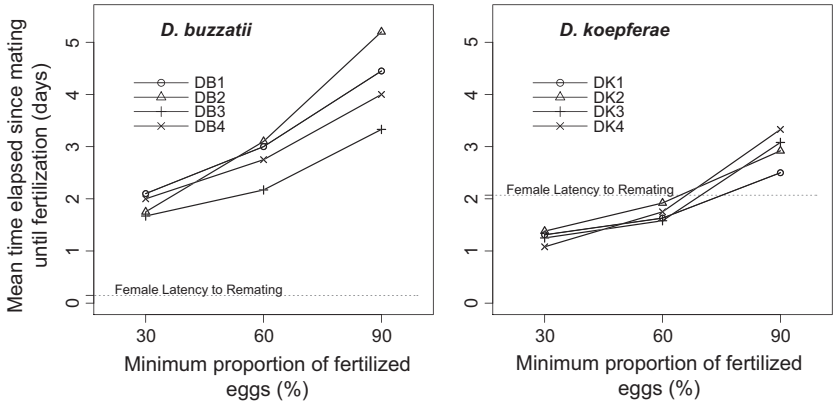

Fig. 2 Temporal pattern of sperm usage in *Drosophila buzzatii* (left) and in *Drosophila koepferae*. Each point represents the time females of each strain take to fertilize at least 30%, 60% and 90% of the total number of eggs. Time values were computed from the reproductive output of a group of 6–20 inseminated females per strain. Female latency to remating (extracted from Hurtado & Hasson 2013) is shown in discontinuous line.

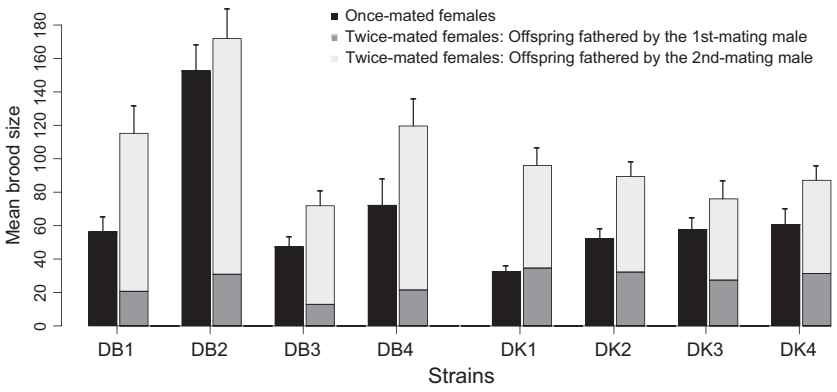

Fig. 3 Reproductive output of once and twice-mated females. Mean brood sizes with standard error, computed from the reproductive output of 6–20 inseminated females in each case, are displayed per strain. Left bars represent once-mated females and right ones twice-mated females. Lower and upper portions of the right bars distinguish the offspring expected to be sired by the first and second-mating males, respectively, assuming that P2/P1 ratio do not differ among strains of the same species.

Table 3 Mean proportion of offspring sired by the last-mating male (P2). Results [including mean P2 and the observed number of offspring (N)] are sorted by the female strain (FS), the strain of the first-mating male (FMS) and the strain of the second-mating male (SMS)

Drosophila buzzatii					*Drosophila koepferae*				
FS	FMS	SMS	N	P2	FS	FMS	SMS	N	P2
DB1	DB1	DB2	53	0.85	DK1	DK1	DK2	120	0.57
DB2	DB1	DB2	54	0.80	DK2	DK1	DK2	65	0.83
DB1	DB2	DB1	59	0.86	DK1	DK2	DK1	135	0.49
DB2	DB2	DB1	57	0.84	DK2	DK2	DK1	80	0.84
DB3	DB3	DB4	70	0.89	DK3	DK3	DK4	43	0.65
DB4	DB3	DB4	47	0.66	DK4	DK3	DK4	28	0.71
DB3	DB4	DB3	59	0.78	DK3	DK4	DK3	42	0.67
DB4	DB4	DB3	48	0.81	DK4	DK4	DK3	26	0.54
Total			447	0.82	Total			539	0.64

strains within species (results not shown), our results showed that sperm precedence differed between species. In effect, logistic regression analysis showed that P2/P1 ratio was on average 2.5 times higher in *D. buzzatii* (estimate = 0.91, *P*-value <0.001).

The expected number of twice-mated females offspring produced by the first-mating males (P1 × F_{tm}) was lower than the number of offspring produced by once-mated females for all strains except DK1 (Fig. 3), indicating that male reproductive output was reduced due to female remating. However, the relative cost of sperm competition strongly depended on the species (*P*-value = 0.033). On average, when the female mated with a second male, first-mating male fertility was reduced by 71.4% in *D. buzzatii* and only 33.3% in *D. koepferae*.

Discussion

Our study shows that both the risk of sperm competition and male reproductive cost of sperm competition are much greater in *Drosophila buzzatii* than in *Drosophila koepferae*. Thus, it may be argued that postmating sexual selection via sperm competition (SC) is a stronger evolutionary force in *D. buzzatii* than in its sibling. We also show that female remating rate (FRR) and the pattern of sperm usage may account for the interspecific differences in the strength of SC.

Paternity analysis performed with SCARE revealed that mean α was significantly greater in *D. buzzatii* (3.46) than in *D. koepferae* (1.53). We therefore may infer that the incidence of polyandry in wild-inseminated females was significantly higher in *D. buzzatii* than in *D. koepferae*. Thus, the chances that sperm from different males co-occur inside a female (i.e. SC risk) are greater in *D. buzzatii*. However, it does not necessarily mean that SC is stronger in *D. buzzatii* because multiple paternity does not necessarily imply reproductive costs for any of the fathers. To evaluate this issue, we compared the relative cost of SC between species. Our results showed that SC causes a higher reduction in first-mating male reproductive output in *D. buzzatii*. Thus, our findings imply that selection imposed by SC is stronger in *D. buzzatii* than in *D. koepferae*.

To evaluate the behaviour of the implemented model in the paternity analysis, we also run SCARE on the data of the sperm precedence assays in which we experimentally fixed the number of fathers per brood at 2. All mating flies used in these assays were homozygous for one of four alleles of a cytogenetic marker. Therefore, these samples' genotypes do not fit Hardy–Weinberg equilibrium frequencies, which are assumed by the model to assign mates' genotypes (Jones & Clark 2003). Nevertheless, we expected that the estimated number of fathers per brood would not markedly depart from 2 (α = 1.6). Also, we expected β to be similar to the actual proportion of progeny sired by the second male (P2), which was 0.82 in *D. buzzatii* and 0.64 in *D. koepferae*. In *D. buzzatii*, mean α was 2.02 with a 90% credible interval of 1.04–3.35, and mean β was 0.85 with a 90% credible interval of 0.77–0.92. Hence, parameters

estimate clearly met expectations in *D. buzzatii*. In *D. koepferae*, mean α was 3.16 with a 90% credible interval of 1.50–5.49, and mean β was 0.88 with a 90% credible interval of 0.81–0.93. In this species, α did not significantly depart from expectations, even though it was strikingly high with a very wide credible interval. Moreover, β estimate was markedly greater than P2. The model implemented in the SCARE analysis makes the assumption that the last-mating male is always the male with the largest proportion of progeny. This assumption is met by the vast majority of the *D. buzzatii* laboratory-based broods. In *D. koepferae*, however, many of the broods were mostly sired by the first-mating male (Table S4, Supporting Information). This fact may explain why β was inflated relative to P2 and the flat posterior distribution of α obtained for *D. koepferae* (Fig. S1, Supporting Information). However, we believe that this fact did not represent a problem in our analysis of multiple paternity in wild-caught females because, for both species, mean β (0.57) was very close to 0.5 (the minimum possible value of this parameter), and α posterior distribution was quite sharp in both species.

The model we used to analyse the incidence of multiple paternity in wild-inseminated females had been previously implemented in other studies. In some of them, α was interpreted as the mean number of sires per brood (Bundgaard *et al.* 2004; Frentiu & Chenoweth 2008). This interpretation would be accurate if the probability density for the number of sires was not truncated; however, as females cannot be inseminated by zero males, the model assumes that the probability density for the number of mates per female is a truncated Poisson (Jones & Clark 2003). Then, the mean number of mates should be equated by $\alpha/(1-\exp(-\alpha))$ instead of α. For this reason, we recalculated the mean number of fathers per brood from previously estimated α values to compare the incidence of multiple paternity among *Drosophila* species (Table 4). Our estimate of the number of fathers per brood in *D. buzzatii* (3.57) is among the highest compared with estimations obtained by means of similar approaches in other species of *Drosophila* (Table 4). In contrast, mean number of fathers per brood in *D. koepferae* (1.95) is among the lowest values for the genus. Thus, despite *D. buzzatii* and *D. koepferae* are closely related, they exhibit ample differences in their mating systems, making these siblings an attractive model for the study of the mechanisms and evolutionary consequences of SC. Multiple paternity in *D. buzzatii* was previously assessed by Bundgaard *et al.* (2004) in a population that was introduced to Australia in the 1930s (Barker 1982). Bundgaard *et al.* (2004) reported a α = 2.1–2.2 (2.4–2.5 fathers per brood), which seems quite lower than our estimation in the Argentinian native population studied in this study (Table 4). Although, at the present time, it might be speculative to argue about the reasons of this difference between a native and a non-native population, historical and ecological factors may be invoked. For instance, availability of different host plants, which are more diverse in native populations (Barker & Starmer 1982; Hasson *et al.* 1992), may affect SC risk by determining oviposition and mating opportunities.

The results of laboratory experiments showed that female *D. buzzatii* exhibits a slower sperm usage rate (SUR) and, recently, Hurtado & Hasson (2013) found that latency to remating is shorter in *D. buzzatii* (4 h) than in *D. koepferae* (50 h) suggesting that FRR is higher in *D. buzzatii*. Therefore, interspecific variation in SUR or FRR points in the same direction predicting that the chances for multiple paternity are much higher in *D. buzzatii* than in its sibling. Then, we cannot tell which one, SUR or FRR, is the main determinant of SC risk based on our paternity analyses, which confirmed the prediction. If we take into account the mean

Table 4 Estimated number of sires per brood in field *Drosophila* populations. All estimations were similarly inferred by the recovery of genetic variation in the progeny of wild-caught females using polymorphic microsatellite loci

Species	Collection site	Fathers per brood	Number of broods	References
Drosophila serrata	Brisbane, Australia	8.7 (α = 8.7)	19	Frentiu & Chenoweth (2008)
Drosophila melanogaster	Vienna, Austria	4–6	4	Imhof *et al.* (1998)
Drosophila buzzatii	La Rioja, Argentina	3.6 (α = 3.46)	20	Present study
Drosophila mojavensis	Arizona, USA	3.1	20	Good *et al.* (2006)
D. melanogaster	California, USA	2.7 (α = 2.4)	19	Harshman & Clark (1998), Jones & Clark (2003)
D. buzzatii	Queensland, Australia	2.4–2.5 (α = 2.1–2.2)	18	Bundgaard *et al.* (2004)
Drosophila koepferae	La Rioja, Argentina	2.0 (α = 1.5)	14	Present study
Drosophila simulans	Kisoro, Uganda	1.9 (α = 1.4)	11	Schlötterer *et al.* (2005)
D. simulans	Schabs, Italy	1.8 (α = 1.3)	10	Schlötterer *et al.* (2005)

proportion of eggs that were fertilized at the time mated females recover sexual receptivity, we can calculate the effective sperm load that still remains stored in the female reproductive tract (and is more likely involved in SC). This effective sperm load varied from 73 to 100% among *D. buzzatii* strains and from 10 to 21% in *D. koepferae* (Fig. 2). Thus, both SUR and FRR may account for the detected interspecific variation in the incidence of multiple paternity. However, interspecific differences may also be the outcome of others features of the mating system that we did not evaluate. For instance, sperm number transferred per copulation (ejaculate size) is expected to shape, together with SUR and FRR, the chances for SC.

We assessed sperm precedence in the field by estimating parameter β, which gives a measure of the proportion of offspring sired by the last-mating male, and in the laboratory using P2, the proportion of the brood sired by the second-mating male in twice-mated females. P2 was greater than β in both species suggesting that SC outcome is condition dependent. Besides, P2 was higher in *D. buzzatii* (0.82) than in *D. koepferae* (0.64), while mean β did not differ between species (0.57). Such pattern suggests some qualitative disagreement of postmating sperm behaviour between natural and experimental conditions. However, P2 and β are different variables that probably have different behaviour when, for instance, there is nonrandom sperm usage across time. If females first use the sperm from the last-mating male, it would not be surprising that β was lower than P2 as wild females may have partially exhausted the sperm supplied by the last-mating male at the time they were caught. Patterns of sperm precedence may also depend on the number of mates per inseminated female, which was always two in the laboratory, but was uncontrolled in nature. Sperm precedence was not only sensitive to mating conditions, but also depended on the species. Even though the β estimate did not differ between species, its posterior distribution was much flatter in *D. koepferae* (Fig. 1). This might be attributed to greater variation in sperm precedence in *D. koepferae*. On the other hand, P2 was significantly higher in *D. buzzatii* than in *D. koepferae* strains. One possible explanation for these results is that sperm displacement or incapacitation, as adaptations promoted by strong SC, are stronger in *D. buzzatii* (Price *et al.* 1999). Nevertheless, further investigations are needed to understand the causes of the observed patterns of sperm precedence.

Given the documented costs associated with remating in *Drosophila* females, it remains enigmatic why females mate with multiple males (Singh *et al.* 2002). Female remating is therefore particularly difficult to explain in species in which the number of ova produced by the female can be fertilized by a single ejaculate. However, we found that remating causes an increase in female fecundity in both *D. buzzatii* and *D. koepferae*. Seminal fluid proteins from the second mating might cause the boost in female fecundity by inducing, for instance, oogenesis (Heifetz *et al.* 2001). In such case, females may just remate to receive the seminal proteins that enlarge their reproductive success. Alternatively, the number of functional sperm transferred during mating may be thought of as the constraining factor for female fecundity. In such case, the boost in female fecundity, caused by remating, shows that a single ejaculate is insufficient to fertilize all eggs. Thus, reloading nonfull sperm supply to increase reproductive output is a feasible explanation for female remating in these species. If reloading sperm supply was the main cause of female remating, species with higher SUR should exhibit higher FRR. However, we found the opposite: *D. buzzatii*, which shows higher FRR than *D. koepferae*, exhibited a lower SUR. Then, causes different from sperm reloading must enhance female remating in these species. For instance, females may remate to obtain nuptial gifts or indirect benefits via SC or cryptic female choice (Singh *et al.* 2002).

Rapid evolution of reproductive proteins and male genital morphology is one of the most intriguing observations in evolutionary biology that remains poorly understood. Postmating sexual selection via SC has been proposed as a potent trigger of rapid evolution of sexual characters (Arnqvist 1998; Hosken & Stockley 2004). According to this hypothesis, the evolutionary rate of male genital morphology would be higher in species exposed to stronger SC. Thus, taking into account our findings, the SC hypothesis predicts that male genital morphology should evolve more rapidly in *D. buzzatii* than in *D. koepferae*. Yet, though rapid divergence of male genital morphology among natural populations has been detected in these species (Soto *et al.* 2007, 2013; unpublished results), forthcoming studies comparing evolutionary rates of male genitalia between *D. buzzatii* and *D. koepferae* may be a suitable complement to our present results in order to test the hypothesis that postmating sexual selection via SC drives the rapid evolution of reproductive traits.

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J. Hurtado contributed to all aspects of the work including initial design, sample collection, genotyping, laboratory experiments performance, data analyses and manuscript preparation. P. P. Iglesias contributed to genotyping work and laboratory experiments performance. P. Lipko assisted with genotyping work. E. Hasson participated in the design, sample collection, laboratory experiments and in the drafting of the final version of the the manuscript.

Data accessibility

Data sets that are not fully presented in the paper, which include those of paternity analysis, sperm usage assays, female fecundity estimations and sperm precedence experiments, are provided as online supplementary material. Histograms of α and β posterior for the laboratory-based broods, can also be found as online supplementary material.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Histograms of the posterior distribution of α and β for the laboratory-based broods of *Drosophila buzzatii* (left) and *Drosophila koepferae* (right). Charts are based on 10 000 samples obtained from the joint posterior produced by the Monte Carlo Markov Chains.

Table S1 Multilocus microsatellite genotypes for all flies included in the paternity analysis. Missing data are denoted as 0.

Table S2 Number of fertile eggs laid per day since mating by all single-inseminated females included in the sperm usage analyses.

Table S3 Total reproductive output of once and twice-mated females included in the analysis of sperm competition cost.

Table S4 Number of offspring, obtained from the sperm precedence assays, sired by the first and the second-mating male.