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Original Article

Does the response of *D. melanogaster* males to intrasexual competitors influence sexual isolation?

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The evolutionary consequences of phenotypic plasticity are debated. For example, reproductive barriers between incipient species can depend on the social environment, but most evidence for this comes from studies focusing on the effects of experiencing heterospecific individuals of the opposite sex. In *Drosophila melanogaster*, males are well known to invest strategically in ejaculate components and show different courtship behavior when reared in the presence of male competitors. It is unknown whether such plasticity in response to same-sex social experience influences sexual isolation, so we tested this using African and cosmopolitan lines, which show partial sexual isolation. Males were housed in social isolation, with homopopulation, or with heteropopulation male partners. We then measured their mating success, latency, and duration, their paternity share, and female remating success. Isolated males copulated for a shorter duration than males housed with any male partners. However, we found no difference in any measure between homopopulation or heteropopulation treatments. Our findings suggest that the male intrasexual competitive social environment does not strongly influence sexual isolation in *D. melanogaster*, and that plastic effects on reproductive isolation may be influenced more strongly by the experience of social isolation than by the composition of individuals within different social environments.

Lay Summary: The strength of reproductive isolation between diverging populations may depend on the social interactions experienced by individuals. We used partially isolated populations of fruit flies, *Drosophila melanogaster*, and showed that whether males had previously interacted with homopopulation or heteropopulation male partners did not affect the strength of premating or postmating sexual isolation. Thus, although male sexual traits are highly labile, this flexibility does not seem to affect the strength of sexual isolation in this system.

Key words: **behavioral isolation, postmating sexual isolation, premating sexual isolation, social learning, speciation, species recognition.**

INTRODUCTION

The role of phenotypic plasticity in evolution is intensely debated. Organisms can adjust phenotypic traits within a generation, but whether and how this plasticity impacts longer-term evolutionary change is less clear (Price et al. 2003; West-Eberhard 2005; Ghalambor et al. 2007; Scoville and Pfrender 2010; Parsons et al. 2016; Schmid and Guillaume 2017; Bailey et al. 2018). One factor to which animals show considerable phenotypic plasticity is the social environment. Socially mediated plasticity can allow individuals to cope with variation in demography and social interactions within generations, but whether it influences evolutionary processes across generations is poorly understood and challenging to study empirically. One route by which socially mediated plasticity could affect trait evolution or speciation dynamics is if the expression of traits involved in mate recognition and choice is sensitive to the social environment (Rodríguez et al. 2013). Theory suggests that evolutionary effects of socially mediated plasticity might accelerate or decelerate the evolution of reproductive isolation, depending on whether individuals encounter conspecifics or heterospecifics, the fitness consequences of the encounters, and the genetics of plasticity (Servedio et al. 2009; Servedio and Dukas 2013). For instance, a recent study has found that bird songs diverged faster in songbird species with innate songs than in species with socially learned songs (Freeman et al. 2017), suggesting that socially mediated phenotypic plasticity can slow down the evolution of traits involved in reproductive isolation.

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Although reproductive barriers between species have usually been assumed to be relatively canalized traits, theoretical and empirical data both challenge this view (Irwin and Price 1999; Servedio and Noor 2003; Servedio et al. 2009; Verzijden et al. 2012; Kawecki 2013; Verzijden et al. 2013; Servedio and Bürger 2014; Yeh and Servedio 2015). The social environment experienced by individuals has repeatedly been found to influence traits with roles in reproductive isolation, such as mating rates (e.g., Billeter et al. 2012), sexual signals (e.g., Krupp et al. 2008; Groot et al. 2010), mating preferences (e.g., Bailey and Zuk 2009; Danchin et al. 2018), courtship behavior (e.g., Lehtonen et al. 2016), aggressive behavior (e.g., Carazo et al. 2014), and ejaculate allocation (e.g., Wigby et al. 2009). Female preferences can be modified according to experience with heterospecifics (Li et al. 2018), and it is well established that postmating prezygotic reproductive interactions evolve rapidly and are an early acting component of reproductive isolation (Alipaz et al. 2001; Manier et al. 2013; Jennings et al. 2014; Turissini et al. 2018). But is the response to the male social environment tuned to the identity of the competitor? Is it population specific? And does it influence premating and postmating reproductive success?

Here, we evaluate the impact of socially mediated plasticity on sexual isolation between diverging populations of *Drosophila melanogaster*, focusing on male responses to the presence of potential sexual competitors in their social environment. In *Drosophila*, many plastic responses of male reproductive traits are adaptive responses to the perceived likelihood of intrasexual competition. For example, males may produce more competitive behaviors or ejaculates, which increase sperm competition success when they experience rivals during development (Bretman et al. 2009) as predicted under classic models of strategic investment in sperm competition (Parker 1970; Parker and Pizzari 2010). Moreover, young male flies court each other on the first day after eclosion (Gailey et al. 1982), which is thought to potentially contribute to courtship learning (Griffith 2014). Interestingly, the genetic makeup of other males encountered in the social environment may influence the expression of both premating and postmating reproductive traits (reviewed in Bretman et al. 2011; Schneider et al. 2012; Griffith 2014; Schneider et al. 2017). For instance, the expression of key cuticular pheromones and male mating behavior are affected by the genetic composition of male social partners (Kent et al. 2008; Krupp et al. 2008). The degree of familiarity and genetic relatedness among males impacts female reproduction and female lifespan in that males exposed to familiar or related males seem to be less harmful to females (Carazo et al. 2014; Hollis et al. 2015; Le Page et al. 2017). Such an effect is presumably mediated by the ejaculate transferred to females, which is known to be highly flexible. Males adjust the transfer of sperm and seminal fluid proteins when they are exposed to rivals (e.g., Bretman et al. 2009; Wigby et al. 2009) presumably as part of a flexible strategic investment strategy influenced by the likelihood of sperm competition and mating opportunities. Despite what is known about flexibility in male *D. melanogaster* ejaculate characteristics, relatively little is known about how this might translate to flexibility in sexual isolation.

In this study, we take advantage of African and cosmopolitan populations of *D. melanogaster*, which show incomplete sexual isolation at both the premating and postmating stages (Hollocher et al. 1997; Alipaz et al. 2001). We used multiple lines from these two populations to test if plastic responses of males to intrasexual competitors influence the strength of sexual isolation in the early stages of evolutionary divergence. We manipulated the male social environment by housing focal males in social isolation or with either five homopopulation or five heteropopulation males for 5 days. We assessed the effects of this treatment on premating isolation by measuring mating latency, mating success, and mating duration with heteropopulation females. To assess effects on postmating isolation, we measured remating rates of the females with second males and, when copulations occurred, we quantified the focal males' paternity share. We test several predictions about how the male social environment may influence sexual isolation. First, *D. melanogaster* males are known to plastically increase the mating duration or ejaculate components in the presence of other males (e.g., Bretman et al. 2009). If males only perceive homopopulation males as sexual competitors, then we would expect that males exposed to heteropopulation males would show a similar strength of sexual isolation as previously isolated males. Second, *D. melanogaster* males can plastically modify the expression of cuticular pheromones according to the genetic composition of the other group members (Kent et al. 2008; Krupp et al. 2008). If such a plastic response allows males to better match the pheromones profiles of their male social partners, then males exposed to heteropopulation male social partners would show a lower strength of sexual isolation than males previously exposed to homopopulation males. Such effects can influence both the premating (e.g., mating success) and/or the postmating (e.g., mating duration and sperm precedence) episodes of selection.

MATERIAL AND METHODS

Line establishment

We used six lines of *D. melanogaster* (three African and three cosmopolitan; Supplementary Table S1) into which we inserted markers allowing parentage scoring. We backcrossed two dominant fluorescent markers, a green fluorescent protein (GFP), and a red fluorescent protein (RFP) into these strains.

In the first generation, we pooled five virgin wild-type females with five males from the marker lines (parental individuals), replicated three times per line. We then grouped five of the resulting F_1 virgin females with five males of the corresponding wild-type line, replicated three times per line. We continued backcrossing for 10 generations by sampling the females carrying the marker, that is, expressing the green or red fluorescent protein. In every generation, we crossed females from the backcross to males from the wild-type line to allow recombination. Because the first generation did not yield any offspring in some lines, we made the reverse cross (i.e., female from marker line × wild-type male) in the first generation but used wild-type females subsequently (F_2 or F_3) so that the mitochondrial DNA was correctly introgressed into all newly established lines.

To create homozygous lines for the introgressed marker, we made $F_{10} \times F_{10}$ crosses within each line and selected homozygous individuals by eye based on the intensity of the fluorescent signal using a fluorescence microscope (Tritech Research, Inc). Hence, the backcross breeding program yielded 12 newly established lines (2 markers × 2 populations × 3 lines), with genetic backgrounds from African or cosmopolitan populations and stable expressions of GFP or RFP (Supplementary Figure S1). These lines are expected to share more than 99.9% of their genome with the initial wild-type lines (Hartl and Clark 1997) and to contain on average 10-cM DNA segments from the marker line on each side of the locus of the introgressed markers (Hospital 2001).

During the backcrossing, we assayed the fitness of individuals carrying the markers using two tests. First, we sampled 566 and 870 F_4 offspring in the GFP and RFP backcross, respectively, and counted the number of offspring carrying the markers of interest vs. wild-type offspring. We tested for viability effects of the markers by calculating heterogeneity and pooled G tests. There were no significant deviations (see Supplementary Information). Second, in the fifth generation, we

sampled males that did and did not carry markers and tested their reproductive success in a competitive mating situation. We grouped two males (one of each type) with two wild-type females in vials for 10 days, which we replicated 20 times per marker, and assessed the status of 40 resulting offspring per replicate. The observed proportion of offspring expressing the markers was tested against an expectation of 0.25 using *G* tests. There was significant heterogeneity but, for the GFP marker, no overall difference from expectations. For the RFP marker, there was again significant heterogeneity but individual comparisons were inconsistent in direction, so there was no consistent evidence for an excess of wild type as would be expected if the marker was less competitive in these assays (see Supplementary Information).

Experimental design

Rearing and social environment manipulation

All flies were maintained at 23 °C on a 12:12 light:dark cycle and we standardized stock densities to 12 males and 12 females per vial (25 × 95 mm, Scientific Laboratory Supplies) for two generations before sampling flies used in experiments. To manipulate the social environment of males, we raised virgin focal males for 5 days either in isolation or with five homopopulation or five heteropopulation virgin male partners in small vials (15 × 95 mm, Sarstedt) containing food and yeast (Figure 1). The five male partners were from the same line, which either matched the line of the focal male (i.e., homopopulation treatment) or the line of the female (i.e., heteropopulation treatment; see below for line combinations). Focal males carried markers (GFP or RFP) and male partners were wild type.

Line combinations for reproductive isolation tests

We crossed African and cosmopolitan *D. melanogaster* populations as follows: Chipata1.1 × IT-IV-69; LZV3.4 × FIN-I-15–17; and Zim30 × Canton-S. Each cross was performed in both directions (i.e., ♀ cosmopolitan × ♂ African and ♀ African × ♂ cosmopolitan). However, we observed few copulations in ♀ LZV3.4 × ♂ FIN-I-15–17 and ♀ Zim30 × ♂ Canton-S, confirming that these African females discriminate strongly against cosmopolitan males (Hollocher et al. 1997), so we discarded these two crosses from subsequent analyses. Our initial sample size was 18 replicates per treatment and cross (i.e., 324 samples). However, we lost replicates over the course of the experiment due to unsuccessful first or second mating trials, fly death, or handling mistakes (see Figure 2 and Supplementary Table S2 for final sample sizes).

Premating isolation

To test premating isolation among lines and evaluate the effect of male social environment manipulation upon it, we exposed focal males to virgin heteropopulation females (i.e., 1-day old) in small vials containing food. We first sampled all males—without anesthetization—and then distinguished focal males from male partners by momentarily exposing flies to epifluorescence illumination using a fluorescence compound microscope. We observed all male–female pairs for 2 h and recorded mating success, mating duration, and room temperature. Importantly, pair formation and mating observation were done by two different experimenters to ensure that the data were recorded blind with regards to the fly lines and the treatment. We then kept females in isolation for 5 days. Note that we confirmed at this stage that focal males were homozygotes for the marker by verifying that all offspring produced during these 5 days expressed the marker.

Postmating isolation

To assess postmating isolation, we exposed females from the procedure above that had been isolated for 5 days after their first mating

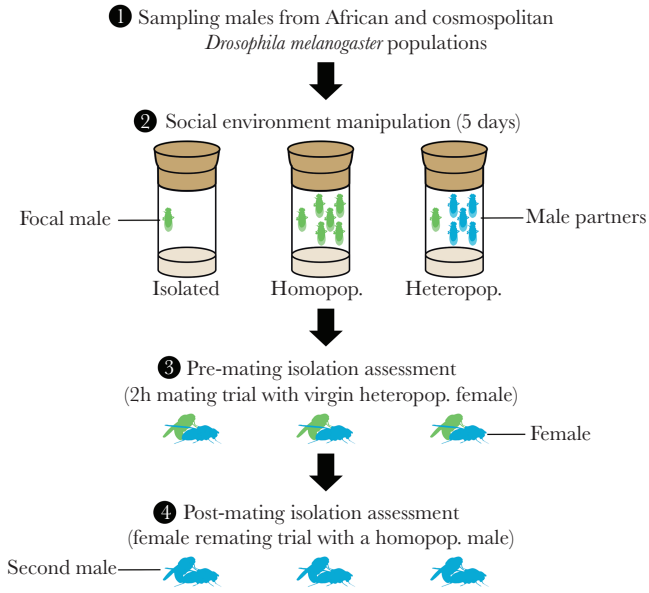

Figure 1
Experimental setup used to manipulate male social environment and assess premating and postmating isolation. First, males were sampled from lines of either African or cosmopolitan populations. Second, we experimentally manipulated the social environment experienced by focal males by raising them for 5 days either in isolation, or with five homopopulation or heteropopulation male partners. Third, we exposed focal males for 2 h to a virgin heteropopulation female and scored mating success and mating duration. Fourth, females had a second mating opportunity with a homopopulation male, and we scored mating success and mating duration, as well as the resulting paternity share. Fly color denotes fly population.

to a second male that came from the same line as the female. We observed the pair for 2 h and recorded mating success, mating duration, and room temperature. Again, mating observations were blinded. We kept the twice-mated female in isolation for a further 5 days and counted all resulting offspring and scored the marker, allowing quantification of offspring sired by focal males.

Data analysis

We measured premating isolation using three response variables (mating success, mating latency, and mating duration), and postmating isolation using two (remating success and paternity share). We tested whether these responses were influenced by the male social environment (isolated, homopopulation male partners, and heteropopulation male partners), by line, and by a male social environment × line interaction. We included room temperature as a covariate in all data analyses. We used binary nominal logistic regressions for mating success and remating success, analyses of covariances for mating latency and mating duration, and a binomial generalized linear model with logit link function for paternity share. Note that when we found a significant interaction effect, we tested for male social environment effect within each line. Similarly, when we found significant male social environment effects, we ran post hoc pairwise comparisons to determine which treatment explained the overall effect. All statistical analyses were carried out in JMP (SAS Institute Inc., Cary, NC).

RESULTS

The male social environment did not affect the mating success or mating latency (Table 1; Figure 2A,B). The significant interaction

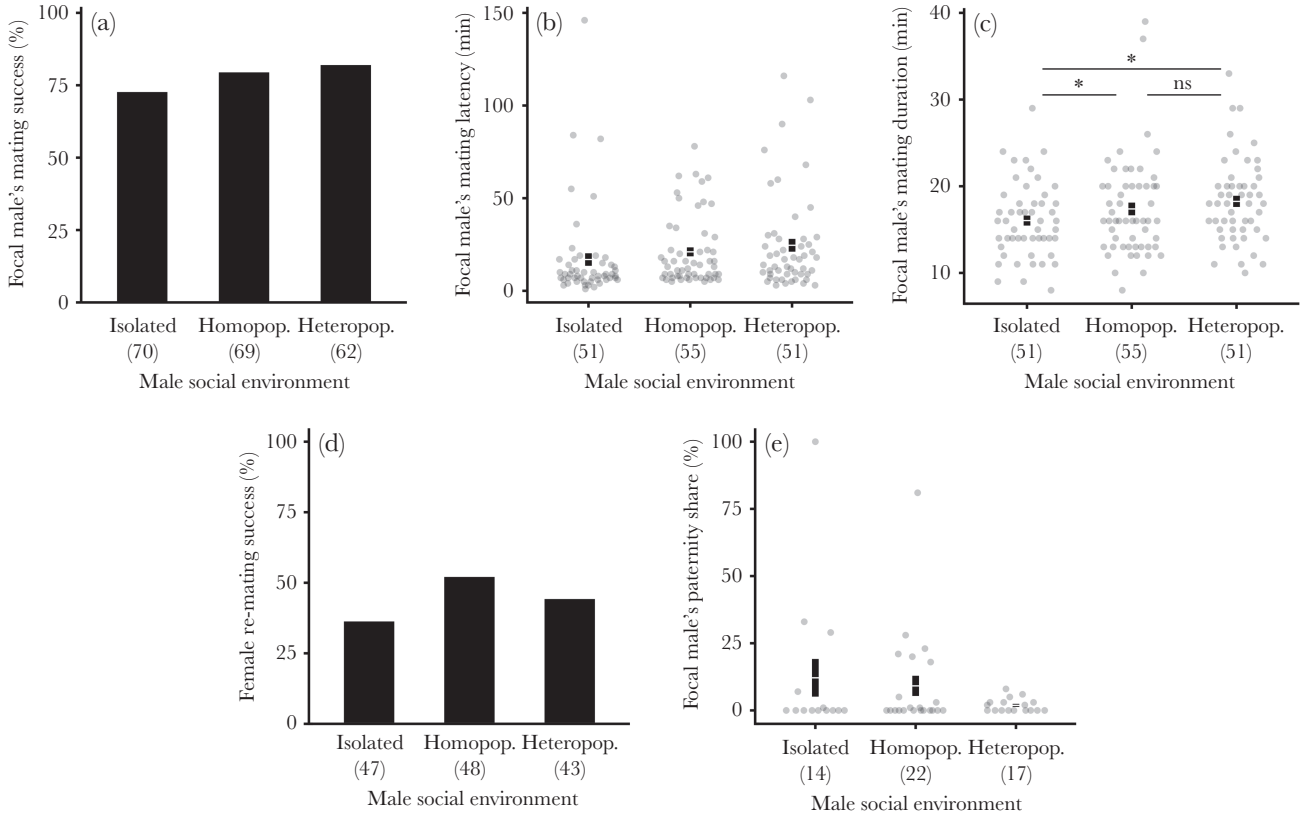

Figure 2

The effects of male social environment on premating and postmating sexual isolation. We manipulated the social environment of focal males, and then measured mating success (A), mating latency (B), and mating duration (C) with heteropopulation females. Females were then exposed to a second male, and we measured female remating success (D) and focal male's paternity share (E). Stars and ns stand for significant and nonsignificant pairwise differences, respectively. In panels B, C, and D, all data points are shown jittered, thick black bars indicate standard errors, and the white gap between them the means for each comparison. Sample sizes are indicated under brackets. See Results for statistics.

Table 1

The effect of male social environment, line, social environment × line social environment, and temperature on measures of premating isolation (mating success, mating latency, and mating duration) and postmating isolation (remating success and paternity share). See Methods for details

		Mating success		Mating latency		Mating duration		Remating success		Paternity share	
	df	χ^2	*P*	*F* ratio	*P*	*F* ratio	*P*	χ^2	*P*	χ^2	*P*
Social environment	2	0.0	1.000	0.2	0.847	3.6	0.030	3.5	0.1701	0.4	0.838
Line	3	53.1	<0.001	18.0	<0.001	33.2	<0.001	16.3	0.001	0.1	0.995
Social environment × line	6	13.5	0.035	1.1	0.355	1.8	0.103	4.0	0.671	5.5	0.486
Temperature	1	0.3	0.597	0.8	0.364	2.7	0.105	0.7	0.672	0.0	0.841

observed for mating success × line suggested that social environment affected mating success differently among lines. However, we did not find significant social environment effects on mating success in follow-up analyses conducted within each line (all $P > 0.05$; binary nominal logistic regressions accounting for multiple testing), so any effect was weak. The only significant effect of social environment we found was on mating duration (Table 1; Figure 2C). Post hoc analyses showed that males previously raised in isolation copulated for a shorter duration than males raised with either homopopulation male partners (2.04 ± 0.81 min [0.43–3.65], $t = 2.5$, degrees of freedom [df] = 144, $P = 0.013$; mean duration difference ± standard error [lower and upper confidence limit]; post hoc Student's *t* test) or with heteropopulation male partners (1.72 ± 0.80 min [0.13–3.31], $t = 2.1$, df = 144, $P = 0.033$). Males raised with homopopulation or heteropopulation male partners did not significantly differ in copulation duration (0.32 ± 0.79 min [−1.24–1.87], $t = 0.4$, df = 144, $P = 0.688$; Figure 2C). We did not find significant effects of male social environment on either measure of postmating isolation, female remating success, and paternity share (Table 1; Figure 2D,E). Note that the exclusion of the two outliers on male paternity share (>0.75) does not qualitatively change the statistical outcomes (all ns).

DISCUSSION

The strength of sexual isolation between animal species can depend on whether individuals have previously experienced

heterospecific individuals of the opposite sex (e.g., Magurran and Ramnarine 2004; Fincke et al. 2007; Dukas 2008; Kujtan and Dukas 2009). Here, we test if male experience of other males could also influence isolation. For example, strategic allocation of courtship effort or ejaculate components could influence sexual isolation, both in terms of mating success and postmating fertilization success. However, we found that the male social environment had little influence on sexual isolation between African and cosmopolitan *D. melanogaster* populations. Whether males experienced homopopulation or heteropopulation males did not affect the strength of sexual isolation despite examining both premating and postmating reproductive barriers. The only significant difference we found was on mating duration. Previously isolated males copulated for a shorter duration than males that had (any) social partners. Thus, despite the fact that many premating and postmating reproductive traits are known to depend on the male social environment in *D. melanogaster*, our findings suggest that plastic responses in these traits might have limited effects on sexual isolation.

If males can alter their reproductive strategy due to the likelihood of sperm competition intensity, how phylogenetically related must males encountered in the social environment be for focal individuals to perceive them as sexual competitors? In this study examining intraspecific, but population-level, variation in social experience, we found that males showed similarly longer mating durations in response to the presence of either homopopulation or heteropopulation males, suggesting that focal males perceived both as sexual competitors. In a previous study examining interspecific variation in social experience, we found that *D. melanogaster* and *Drosophila simulans* males produce longer courtship songs after being raised with other males, regardless of whether social partners were *D. melanogaster* or *D. simulans* (Marie-Orleach et al. 2019a). In contrast, Bretman et al (2017) found that *D. melanogaster* males increase their mating duration in response to the presence of heterospecific males but not as might be predicted based on genetic distances between species. Responses to other species may be related to phenotypes rather than genetic distance per se. *D. melanogaster* males increase their mating duration in response to the presence of *D. simulans* and *Drosophila pseudoobscura* males (though not to the same extent as to the presence of *D. melanogaster* males) but not of the closely related *Drosophila yakuba* or *Drosophila virilis* males. More surprisingly, such a response to the risk of sperm competition is also seen in monandrous populations of *Drosophila subobscura* (Fisher et al. 2013), and such responses may have evolved in the context of direct male–male competition rather than (or alongside) sperm competition to maximize strategic investment (Lizé et al. 2014). Altogether, our data and these previous findings suggest that plasticity mediated by male competition may be a general response to interactions with other males but not in a manner that is generally predicted by phylogenetic distance, suggesting that such plasticity may not be instrumental in influencing subtle levels of sexual isolation.

Our results suggest that phenotypic plasticity mediated by the male social environment is unlikely to play a role in accelerating population divergence, which is important in the light of current debates about how socially mediated phenotypic plasticity affects trait evolution and speciation processes (Price et al. 2003; West-Eberhard 2005; Ghalambor et al. 2007; Scoville and Pfrender 2010; Parsons et al. 2016; Schmid and Guillaume 2017; Bailey et al. 2018). In contrast, it is clear that male–male competition itself is a strong agent of selection and likely responsible for rapid evolutionary change in multiple phenotypes. For instance, accelerated evolutionary rates are observed in gonadal and genital traits (e.g., Civetta and Singh 1998) and in ejaculate proteins (Swanson et al. 2001). Similarly, closely related species are often found to have higher levels of diversification in sperm traits and in genital morphology (e.g., Pitnick et al. 2003), as well as in sperm precedence traits (e.g., Manier et al. 2013). Our study suggests that this accelerated evolutionary rate of male traits is not reflected in species-specific plasticity in their expression or that any such plasticity in traits is not effective in influencing sexual isolation.

Our data confirm that previously isolated *D. melanogaster* males engage in shorter copulations than males previously housed with (any) types of social partners do. This is consistent with previous studies showing that males respond to the risk of sperm competition by copulating for longer. This is usually thought to increase the number of sperm transferred and offspring sired (Bretman et al. 2009; Garbaczewska et al. 2013). However, in our study, this effect did not translate to subsequent increases in offspring production as we did not find that the social environment influenced paternity share. This discrepancy is surprising. Perhaps any influence is relatively subtle and not detected in our experiment. Because paternity share can only be assessed on the subset of females that remate, our sample size decreased over the course of the experiment. Nevertheless, our findings indicate that there are no large effects of the male social environment on postmating sexual isolation, despite our observation of increased copulation duration when reared in the presence of rivals.

Any phenotypic plasticity mediated by the male social environment is not likely to accentuate the population divergence seen here, and such plasticity seems to be relatively broadly tuned to the identity of interacting partners. Additional experiments investigating more diverse components of the social environment, and premating and postmating sexual isolation, at different stages of evolutionary divergence, are required to fully address how the social environment affects speciation processes in general.

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Conflict of interest: The authors declare no conflicts of interest.

Data accessibility: Analyses reported in this article can be reproduced using the data provided by Marie-Orleach et al. (2019b).

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