Sexual perception does not modulate male short-term fitness components in *Drosophila melanogaster*.

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Abstract

Phenotypic plasticity in reproductive behaviour can be a strong driver of individual fitness. For example, in species with high intra-sexual competition, changes in socio-sexual context can trigger quick adaptive plastic responses in males. In particular, a recent study in the vinegar fly (*Drosophila melanogaster*) shows that males respond adaptively to perception of female cues in a way that increases their reproductive success, but we ignore the underlying mechanisms of this phenomenon. Here, we aimed to fill this gap by investigating the short-term effects of female perception on male pre- and post-copulatory components of reproductive success: a) mating success, b) mating latency and duration, c) sperm competitiveness, and d) ejaculate effects on female receptivity and oviposition rate. We found that brief sexual perception increased mating duration, but had no effect on the main pre- or post-copulatory fitness proxies. These results tie up with previous findings to suggest that male adaptive responses to sexual perception are not due to a short-term advantage, but rather to fitness benefits that play out across the entire male lifespan.

INTRODUCTION

Phenotypic plasticity is defined as the capacity of a genotype to produce alternative phenotypes depending on the environmental context it is exposed to (Gause, 1947; Levins, 1963; Bradshaw, 1965). In particular, adaptive phenotypic plasticity allows organisms to adjust their phenotype in order to cope with contrasting environmental conditions (Demmig-Adams *et al.* , 2008). Whether adaptive phenotypic plasticity can evolve or not depends on its associated costs and is contingent on a certain degree of environmental predictability (DeWitt, Sih and Wilson, 1998; Reed *et al.* , 2010; Botero *et al.* , 2015). More specifically, plasticity in reproductive behaviour and strategies (*reproductive plasticity*) is a central component of individual fitness, particularly so in the face of high spatio-temporal heterogeneity in socio-sexual contexts (Dewsbury, 1982; Gage, 1995; Kokko and Rankin, 2006; Rebar, Barbosa and Greenfield, 2019). For this reason, reproductive plasticity is often considered to be a key determinant of population responses to rapid environmental change (Agrawal, 2001; Charmantier *et al.* , 2008).

Across the animal kingdom, sexual selection is generally male-biased (Bateman, 1948; Janicke *et al.* , 2016), and because relatively high variance in reproductive success increases the adaptive value of phenotypic plasticity, males can be expected to display high plasticity in reproductive behaviour (Bretman, Gage and Chapman, 2011). Male plastic responses to intra-sexual competition are rather well documented across distant taxa (delBarco-Trillo and Ferkin, 2004; Aragón, 2009; Bretman, Gage and Chapman, 2011). For instance, males of different species have been shown to strategically adjust mating duration (Bretman, Fricke and Chapman, 2009; Mazzi *et al.* , 2009), mate guarding behaviour (Carazo et al. 2007), sperm transfer (Gage, 1991; Gage and Baker, 1991), and even seminal fluid protein transfer (Wigby *et al.* , 2009) in response to the socio-sexual environment (e.g. sperm competition risk and/or intensity cues; Shifferman, 2012). Ultimately, high intra-sexual competition can even lead to the evolution of adaptive alternative strategies in reproductive behaviour (Hurtado-Gonzales and Uy, 2010).

Recently an empirical study reported that, in *Drosophila melanogaster* , short-term perception of female cues ahead of access to reproduction (termed *sexual perception*) can increase male reproductive performance in a competitive environment (Corbel *et al.* , 2022). This plastic response to female cues seems to be adaptive, and may explain previously documented survival and reproductive costs linked with sexual perception in males (Gendron *et al.* , 2014; Harvanek *et al.* , 2017; García-Roa, Serra and Carazo, 2018). Interestingly, this study found that sexual perception benefits tended to be rapidly induced (noticeable as early as over the first 24 hours following access to females), and spanned across the lifespan of males. Given that male plastic responses to sexual perception can magnify the opportunity for selection (Carazo *et al.* , 2017; García-Roa, Serra and Carazo, 2018; Corbel *et al.* , 2022) and may help explain ageing in response to sensory stimuli (Gendron *et al.* , 2014; Harvanek *et al.* , 2017), identifying the mechanisms responsible for such male plasticity could provide valuable information.

Here, we aimed to investigated the short-term fitness consequences of sexual perception in detail. To this aim, we studied the effect of sexual perception on several short-term pre- and post-copulatory fitness components in *D. melanogaster* males. Similarly to other polygamous species with high intra-sexual competition, pre-copulatory fitness of males of this species is modulated by male-male competition and female choice, both of which contribute to determine male mating success in a competitive scenario (Dow and Von Schilcher, 1975; Andersson, 1994; Arbuthnott *et al.* , 2017). Post-copulatory male fitness, in turn, is largely driven by sperm competitiveness (mostly sperm-offense, see Fricke *et al.* , 2010; Simmons and Fitzpatrick, 2012). Additionally, male manipulation of female reproductive behaviour via the transfer of accessory gland proteins within the seminal fluid is known to benefit male post-copulatory fertilisation success (Chen*et al.* , 1988; Aigaki *et al.* , 1991; Chapman, 2001; Chapman*et al.* , 2003; Liu and Kubli, 2003; Fiumera, Dumont and Clark, 2005; Ravi Ram and Wolfner, 2007; Fricke *et al.* , 2009; Hopkins*et al.* , 2019). In fact, there is ample evidence that males strategically adjust their seminal fluid protein transfer depending on the socio-sexual environment context they experience (see for instance Wigby *et al.* , 2009; Hopkins *et al.* , 2019). Thus, in order to fully capture the effects of sexual perception on short-term fitness, we exposed virgin males to female cues for a period of 24 hours (while preventing mating) and subsequently measured the following male fitness components: a) mating success, b) mating latency and duration, c) sperm competitiveness, and d) ejaculate effects on female receptivity and oviposition rate.

METHOD

Fly husbandry and collection

In this experiment, we used laboratory adapted *Drosophila melanogaster* wild-type (*wt*) Dahomey flies. We also used*Drosophila melanogaster* sparkling poliert *(spa*) mutants flies in order to discriminate the paternity-share of focal *wt*males. The *spa* allele being recessive, individuals homozygous for this locus display the *spa* eye phenotype, whereas heterozygous*wt* /spa individuals display the *wt* phenotype. We kept stock populations at 24°C on a 12h light/12h dark cycle, with overlapping generations, and fed them with standard food weekly (solidified aqueous mix containing 60g.L^{-1} corn flour, 50g.L^{-1} white sugar, 40g.L^{-1} fresh baker's yeast, 10g.L^{-1} soy flour, 10g.L^{-1} industrial agar, 3g.L^{-1}Methyl 4-hydroxybenzoate, 10mL.L^{-1} 96 % EtOH, 5mL.L^{-1} 99% propionic acid). We collected eggs directly from stock populations using yeasted grape juice agar plates (FlyStuff grape agar premix, Genesee Scientific). We ensured a controlled density of ca. 200 larvae per 250mL bottle filled with ca. 75mL of standard food. Using ice anaesthesia, we isolated flies by sex 6 hours upon emergence in order to ensure virginity. We kept females by groups of 15 per vial and males by groups of 20 per vial. All vials used in this experiment contained a large amount of the same food the populations were fed with, both for adult feeding purposes and to provide an adequate egg-laying substrate to females.

Experimental design

Sensory treatment

We first exposed 3 day-old wildtype (*wt*) virgin males to females cues for 24 hours. To do so, we isolated standard males in a vial, and this vial was connected to either a) another vial containing three 3 day-old

virgin *wt* females (i.e. female-exposed male) or b) to an empty vial (control male). Importantly, interconnected vials were separated by a fine mesh partition, and this allowed exchange of female semio-chemicals (volatiles but also probably non-volatiles) as well as female visual cues across the chambers, while ensuring males would not mate (García-Roa, Serra and Carazo, 2018). Previous empirical research has determined that this methodology does not elicit any courtship behaviour in female-exposed males (Corbel *et al.* , 2022).

Mating success

Immediately following short-term (24h) exposure of experimental males to females cues (or control), we set up 317 triplets consisting of: a female-exposed *wt* male, a control *wt* male and a standard*wt* female. All three individuals were 4 day-old virgins at the start of the mating trials. To distinguish between female-exposed and control males, we marked both males with a dot of acrylic paint on the backside of their thorax using either of two easily discernable colours (Vallejo acrylic studio; cadmium red hue N°2 "PCKPCQL" or primary blue N°24 "PBN4CQK"). We haphazardly alternated assignment of colour to either treatment as a mean to balance any potential colour-induced bias in the behaviour of reproducing females and/or focal males. We used systematic scan sampling to record male mating success (which of the two males mated with the female), mating latency (time between the start of mating trials and the beginning of a successful copulation), as well as mating duration (length of a successful copulation by either male). We only considered a mating as successful if it lasted longer than 10 minutes (unpublished results show that this provides a conservative threshold for successful matings in this population). We ensured a one-minute resolution in the measurement of these variables by limiting the number of vials each of the two observers handled at the time. Observations were conducted following a blind protocol. After a successful mating, we discarded both males, but kept mated females alone in the vial for later experiments (see below). We gave females a total of 150 minutes to mate with either of the two males, after which we discarded all females that had not mated; a large proportion of the females mated with either of the two males (ca. 86%; 273 successful matings were recorded, out of 317 triplets set). Females that mated with a female-exposed male are hereafter called *treatment females* , whereas females that mated with a control male are called *control females* .

Post-copulatory fitness- mating effects on female receptivity and productivity

To test whether sexual perception could lead to altered female remating behaviour (mediated by the differential transfer of accessory gland proteins; Hopkins *et al.* , 2019), we monogamously housed 135 females (71 treatment females and 64 control females) with a standard virgin male (4 days old) and monitored remating latency over a period of 8 hours. This was done on the day following the initial mating. We discarded successfully remated females (i.e. at least 10 minutes long copulation) and isolated females that had failed to mate. The next day, we presented these unmated females to another standard virgin male, in a new vial, for up 8 hours. We ran remating trials for 4 successive days (i.e. starting 24h, 48h, 72h and 96 hours after the end of the first mating), after which a large proportion of the females had remated (110 out of 135, over 4 days). Females that did not remate following these 4 days were discarded, but accounted for in the remating latency analyses (right-censored, see below). When calculating remating latency over many days, the time between two remating trials was not included; i.e. maximum remating latency over 4 days was therefore 8 hours * 4 remating trials = 32 hours (1920 minutes).

We also monitored daily reproductive output of 135 focal females (71 treatment females and 64 control females) over the 7 days following the initial mating, in order to assess whether sexual perception could lead altered female immediate reproductive output (mediated via accessory gland proteins transferred within the seminal fluid). Following the initial mating, we flipped females into new vials every day in order to obtain a daily measure of female early-life reproductive output. Past seven days, we flipped females into new vials every 3-4 days until natural death. We incubated vacant vials for 15 days to allow F1 offspring emergence (average generation time being ca. 10 days), after which we froze them at ca. -20°C for later counting. Given that females only mated once, female lifetime reproductive success could serve as an indicator of the number of sperm transferred by experimental males (female-exposed or control males).

Post-copulatory fitness - sperm competition

Ahead of sperm competition assays, we created 13 *spa* inbred lines in order to obtain genetically uniform males to compete against our focal males. We did this by mating full-sibling *spa* originating from our stock population for three successive generations. We then selected the inbred line with the lowest inter-individual variance in reproductive behaviour (mating latency and mating duration; in a monogamous setting), with an average trait value most similar to the ancestral *spa* population, and with the strongest competitive abilities (i.e. low mating latency and high mating duration; Fig S1, S2). We then examined the effect of sexual perception on sperm-offense abilities (paternity share of a male mating second with a female;*P2)* , as it is the main sperm competition measure explaining male fitness in *D. melanogaster* (Fricke *et al.* , 2010). With this intent, we monogamously housed ca. 700 *spa* virgin females from stock populations with a genetically uniform *spa* virgin male for 150 minutes, in order for the couple to mate. After 150 minutes, we discarded *spa* males and kept females alone in the vial for 48 hours in order to provide a realistic time lag between the two matings (i.e. similar to what *D. melanogaster* my experience in the wild; Gromko and Markow, 1993; Harshman and Clark, 1998; Imhof *et al.* , 1998; Jones and Clark, 2003; Giardina, Clark and Fiumera, 2017; Soto-Yéber *et al.* , 2018; Dukas, 2020). Additionally, this 48h time lag permitted us to adequately assess whether *spa* females successfully mated with the *spa* male, via observation of eggs/first instar larvae in the egg laying substrate. We discarded all females that did not produce at least one egg from the pool of standard mated females used for sperm competition assays, which left us with 645 mated *spa* females. Following these 48 hours, we haphazardly set up 321 females to mate with control males and 324 to mate with female-exposed males, in fresh vials. Due to logistic limitations, we did this in two batches in which we balanced assignation the number of replicate of each treatment (female-exposed and control males). We recorded mating duration and only considered a mating as successful if it lasted longer than 10 minutes. Following a successful mating, males were immediately discarded to prevent remating, and females were left alone in the vial. Remating trials lasted 150 minutes, after which we discarded all females that did not remate. A total of 282 females remated with the male they were offered (136 female-exposed and 146 control males). We allowed isolated females to lay eggs for 4 days, during which we flipped them into fresh yeasted vials every day. We then incubated vials for 15 days to allow F1 offspring emergence (average generation time being ca. 10 days). We then froze them at ca. -20°C for later counting of offspring of each phenotype (*wt* vs *spa*). We pooled the offspring count from the 4 consecutive days in order to score sperm-offense abilities of the focal male. We discarded females that did not produce a single viable offspring during these four days (7 females) from further analyses, as no focal male paternity share could be computed. We also discarded 2 females from further analyses due to human error (e.g. escaped flies, erroneous sex determination following mating trial, etc.). Our final sample size was then 273 (n = 132 treatment females, n = 141 control females).

We computed sperm offense (P2) as the proportion of offspring sired by the focal (*wt*) male:

$$P2 = \frac{N\mathrm{wt}}{N\mathrm{spa} \ + \ N\mathrm{wt}}$$

Where N_{wt} is the absolute number of offspring sired by the focal (*wt*) male, and N_{spa} is the absolute number of offspring sired by the standard competitor (*spa*) male.

Statistical analyses

We analysed differences in mating success between female-exposed and control males using a one-sample Wilcoxon signed rank test with continuity correction; each female was assigned a binary response representing mate choice ("0" for control male, "1" for female-exposed male), with mu set at 0.5 as females should have no preference for either male under H_0. We analysed mating latency and duration of female-exposed versus control males using Kruskal-Wallis rank sum tests in which treatment (female-exposed vs control) was the sole categorical predictor. We analysed remating latency of treatment vs control females using a Cox proportional hazard model (Cox, 1972) with treatment as predictor, and we right-censored females that did not remate after the 4 days. We graphically and statistically verified the assumptions of the Cox proportional hazard

model using Schoenfeld residuals diagnostics (Schoenfeld, 1982). We analysed female early-life reproductive success (daily offspring production over 7 days) using a general linear mixed model ("lme4" R package; Bates *et al.* , 2015) with treatment, day and their interaction as categorical fixed effects, and female ID as random effect. We extracted the absolute values of the residuals-vs-fitted from an initial heteroskedastic model and used them as weights in order to meet the homoskedasticity assumption of the linear model (Midi, Rana and Imon, 2009, 2013). We analysed the effects of sexual perception on female lifetime reproductive success in a general linear model including treatment as the sole categorical fixed effect. Finally, we analysed sperm-offense data in a generalized linear mixed model with a beta-binomial error distribution using the "glmmTMB" R package (to deal with under-dispersion; Brooks *et al.* , 2017). We transformed this data in order to meet the beta distribution range (i.e. y'= (y*(N- 1)+0.5)/N ; Smithson and Verkuilen, 2006). Treatment was the sole fixed effect predictor included in this model, and batch was the only random effect.

We ran all statistical tests, and produced all figures in R studio 1.1.456 (R Core Team, 2020). For all tests, we set α=0.05, ran type III ANOVA and checked model assumptions using the "performance" R package (Lüdecke *et al.* , 2021). We corrected for multiple comparison using the Benjamini-Hochberg procedure (1995) for a false discovery rate of 0.05; outcome of this procedure is detailed when relevant (cases of false positive) in the result and discussion sections. We produced all figures using the "ggplot2" R package (Wickham, 2016)

RESULTS

Around 52% of the standard virgin females simultaneously presented to a female-exposed and a control male mated with the female-exposed male (143 out of 273 realized matings), with no evidence that exposure to female cues significantly affected mating success (i.e. deviation from the expected 50%; $V = 19800$, $P = 0.398$). We did not observe a significant difference in mating latency between female-exposed and control males (K-W $\chi^2 < 0.001$,$P = 0.996$; Fig. 1a). We did find that exposure to female-cues significantly increased mating duration (K-W$\chi^2 = 4.523$, $P = 0.033$; Fig. 1b), however the Benjamini-Hochberg (1995) procedure for multiple testing correction indicated that this result may represents a case a false discovery, given a significance threshold set at α=0.05. Average remating latency did not differ significantly between treatment females and control females (K-W $\chi^2 = 0.719$, $P = 0.397$; Fig. 1c). We found no significant effect of mating with either a female-exposed or a control male on female early-life reproductive success ($\chi^2_1 = 0.155$, $P = 0.694$; Fig. 2), and this was consistent across days ($\chi^2_1 = 7.290$, $P = 0.295$; Fig. 2). However, we found a significant effect of day ($\chi^2_1 = 164.359$, $P< 0.001$; Fig. 2). Similarly, we found no difference in average lifetime reproductive success between treatment females and control females ($F_{1,116}= 0.461$, $P = 0.498$; Fig. 3a). We found no significant difference in sperm competitiveness (sperm offense, *P2*) between female-exposed and control males ($\chi^2 = 0.015$, $P = 0.902$; Fig. 3b).

DISCUSSION

Overall, we found no conclusive evidence that the lifetime fitness benefits of sexual perception reported by previous studies are due to effects on male short-term fitness components. We found no differences in mating success or mating latency between control males and female-exposed males (Fig. 1a), showing that males do not derive pre-copulatory benefits from short-term sexual perception. Given that, in our assays, focal males of either treatment (female-exposed vs control) competed directly against each other over a female, these results represent the net outcome of simultaneous pre-copulatory male-male competition and female choice in a biologically relevant scenario (Dukas, 2020). We did find some evidence that sexual perception resulted in significantly increased mating duration (Fig. 1b), such that female-exposed males mated on average for 1'05" longer than control males (i.e. a 5.54 % increase). In *D. melanogaster* , mating duration is mainly driven by males (MacBean and Parsons, 1967), and longer matings often translate into higher reproductive success for males (Bretman, Fricke and Chapman, 2009; Wigby *et al.* , 2009). Thus, this difference could be biologically meaningful. However, this difference was flagged as a potential false discovery due to inflation of experiment-wise type I error rate (i.e. Benjamini and Hochberg 1995), and should hence be interpreted with extreme caution. Empirical evidence shows that, in *D. melanogaster* , sperm transfer is completed after only a few minutes, and that longer matings do not yield higher sperm transfer (Gilchrist and Partridge,

1997). With that regard, we did not find significant differences in lifetime reproductive success between treatment females and control males (Fig. 3a), which could imply that female-exposed males do not transfer more of sperm to females than control males (i.e. given that these females were only mated once, to either males). In fact, previous research has linked the fitness benefits associated to longer matings to the transfer of non-sperm components that increase immediate oviposition rate in females (see Chapman *et al.* , 2003; Chapman and Davies, 2004; Wigby *et al.* , 2009). However, we found no significant differences in daily reproductive output of females over the 7 days following mating with female-exposed vs control males (Fig. 2). In promiscuous species, female remating rate is often under high sexual conflict and, while males benefit from females not remating and thus utilizing all the sperm transferred during copulation (i.e. avoiding post-copulatory competition), females can benefit from remating with several males (e.g. through increased offspring genetic diversity; Yasui, 1998; Arnqvist and Rowe, 2005). We found no difference in remating latency between treatment females and control females (Fig. 1c). This suggests that males do not transfer higher amounts of seminal fluid protein mediating female receptivity as a consequence of sexual perception prior to access to mating. Altogether, the fact that the difference observed in mating duration did not result in a net fitness advantage supports the idea that this increase was not biologically relevant, or that it is a case of false discovery.

In polygamous species with high mating rates, sperm competitiveness can be an important driver of individual fitness (Singh, Singh and Hoenigsberg, 2002; Firman and Simmons, 2011; Schnakenberg, Siegal and Bloch Qazi, 2012). Sperm competitiveness is often measured as the paternity share that a male achieves when competing against another male, within the female reproductive tract. The paternity share of the first of two males to mate with the female will define his *sperm defense* abilities, whereas the paternity share of the second male will define his *sperm offense* abilities (Boorman and Parker, 1976). In*D. melanogaster,* sperm-offence abilities correlate more strongly with relative lifetime reproductive success than sperm-defense abilities (Fricke *et al.* , 2010). We thus investigated whether sexual perception could affect sperm-offense abilities, and found no significant difference between males shortly exposed to female cues prior to access to reproduction and control males (Fig.3b). It is worth noting that, given the very high baseline level of paternity share of control males (ca. 95.17% $\pm$ 0.85 SEM), our study may lack power to pick up any effects of sexual perception on *P2* , as that the magnitude of any such expected effect must be low.

The fact that the females over which males competed in our study were virgin could contribute to explain our results. In this species, virgin females are considerably less choosy than mated females (Bateman, 1948). As such, the use of relatively unselective females could have masked differences in male pre-copulatory competitiveness. Additionally,*D. melanogaster* males are known to adjust their ejaculate content depending on female mating status (Lüpold *et al.* , 2011; Sirot, Wolfner and Wigby, 2011). Consequently, female mating status could play a role in male plasticity in ejaculate content induced by sexual perception, and ultimately male post-copulatory performance.

In conclusion, we explored an array of pre- and post-copulatory short-term male fitness components and found no indication that any of the components measured are affected by brief sexual perception. While this means that the mechanisms leading to enhanced reproductive performance of males following sexual perception are still unidentified, our results suggest that these mechanisms do not involve improvement of males' short-term fitness proxies. This corroborates the previously mentioned idea that sexual perception benefits build up along the span of males' life (several weeks after initial perception of female cues) to yield a net lifetime fitness gain (Corbel *et al.* , 2022).

Authors contribution:

Q.C. and P.C. conceived the study; Q.C. and P.C. designed the study with help from C.L.N; Q.C., C.L.N and P.C. collected the data. Q.C. analysed the data and produced the figures. Q.C. and P.C. wrote the manuscript. C.L.N. reviewed the manuscript and provided useful comments.

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Data accessibility:

Reviewers and editors will be granted private access to the data, the R script and the associated README file upon request to the corresponding author. All these files will made available on the Dryad repository for public use upon acceptance of the manuscript.

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Figures 1: a) Mating latency and **b)** mating duration of control males (green filled circles) and female-exposed (orange empty circles) in reciprocal contest environment. Group means are displayed inwards relative to single observations, and overlapping vertical bars show one standard error around this mean. **c)** Females remating latency following a single mating with either a control male (solid green line) or a female-exposed male (dashed orange line). Females were given 8 hours to mate every day for 4 consecutive days following initial mating (summing up to 32 hours/ 1920 minutes of remating trials).

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Figures 2: Daily reproductive output of control females (green filled circles) and treatment females (orange empty circles). Group means are displayed inwards relative to single observations, and overlapping vertical bars show one standard error around this mean. Treatment females are females that initially mated with a 24h female-exposed male, whereas control females are females that initially mated with a control male (isolated for 24h).

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Figures 3: a) Lifetime reproductive success of control females (green filled circles) and treatment females (orange empty circles). Treatment females are females that initially mated with a 24h female-exposed male, whereas control females are females that initially mated with a control male (isolated for 24h). **b)** Sperm offense abilities of control males (green filled circles) and female-exposed males (orange empty circles). Sperm offense is calculated at the proportion of offspring by a focal male mating with a previously mated female. Group means are displayed inwards relative to single observations, and overlapping vertical bars show one standard error around this mean.

Appendix

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Figure A1: Mating latency of *spa* males originating from 7 genetically uniform lines, in a monogamous setting with a stock*spa* female. Group means are displayed inwards relative to single observations, and overlapping vertical bars show one standard error around this mean. The same number of monogamous matings were set up for each genetically uniform line (10); number of single observation thus indicates mating success of males from each line within 2 hours. Line 4 was selected to provide standard sperm donors for sperm competition assays.

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Figure A2: Mating latency of *spa* males originating from 7 genetically uniform lines, in a monogamous setting with a stock*spa* female. Group means are displayed inwards relative to single observations, and overlapping vertical bars show one standard error around this mean. Line 4 was selected to provide standard sperm donors for sperm competition assays.