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Inter and intraspecific variation in female remating propensity in the cactophilic sibling species *Drosophila buzzatii* and *D. koepferae*

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ABSTRACT

Post-mating sexual selection by means of sperm competition or cryptic female choice occurs in species in which females remate before exhausting sperm supplied by previous mates. Thus, sperm competition is expected to be stronger when inseminated females remate more frequently or take longer to deplete sperm load. Previous studies comparing oviposition behavior in the pair of closely related species *Drosophila buzzatii* and *Drosophila koepferae* suggest that inseminated females of the latter deplete sperm load more rapidly. Here, we investigate female remating in *D. buzzatii* and *D. koepferae* by studying how female remating propensity changes after mating. Our study reveals that, after mating, female *D. buzzatii* recovers receptivity 14 times faster and remate more frequently than *D. koepferae*. Thus, we argue that *D. buzzatii* exhibits greater chances that sperm from different mates meet inside the same female suggesting more complex post-mating interactions than in its sibling. In addition, our results show that there is intraspecific genetic variation for the duration of female refractory period in both species.

1. Introduction

Drosophila females can store large quantities of sperm in a single tubular seminal receptacle and, in some species, in paired spherical organs called spermathecae (Fowler, 1973; Pitnick et al., 1999). The amount of sperm stored in these organs changes with the time elapsed since mating (Patterson, 1954). Two processes determine the dynamics of sperm load along the life of an inseminated female: the rate of sperm utilization and wastage, and the female remating frequency. Sperm supply is reduced when the female releases sperm from the storage organs to achieve fertilization as mature eggs coming from the ovaries pass through the uterus (Bloch Qazi et al., 2003). On the other hand, sperm supply is reloaded as the female remates, when the sperm deposited in the female uterus rapidly find their way into the storage organs of the female reproductive tract (Lefevre and Jonsson, 1962).

Together, female remating frequency and sperm releasing rate not only determine the amount of stored sperm but also the chances that sperm of different mates meet inside the reproductive tract of a female. If females remate before exhausting sperm supply, sperm from different males co-occur inside a female and her reproductive tract turns into an arena of post-mating sexual selection (Markow, 2002) that can include both male–male competition in the form of sperm competition and cryptic female choice (Birkhead and Pizzari, 2002). The risk that stored sperm encounter sperm of another male to compete with for fertilization of eggs is expected to raise as female remating frequency increases or the rate of stored sperm use decreases (Parker, 1970). Thus, both female remating rate and sperm use rate jointly determine the pressure of post-mating sexual selection.

Post-mating sexual selection can power sexually antagonistic coevolution by increasing the intensity of inter-sexual conflicts (Rice, 2000). For instance, sperm competition may favor male traits that alter sperm use, fertilization and female remating propensity by influencing female behavior and physiology (Birkhead and Pizzari, 2002). If reproductive interests of males and females differ, sexually antagonistic coevolution may be promoted through male coercion and female resistance (Arnqvist and Rowe, 2005). Thus, an evolutionary arms race for controlling sperm usage, fertilization and female remating propensity is expected to occur not only between competing males but also between males and females in species in which post-mating sexual selection is intense (Rice, 2000; Rice and Holland, 1997). These evolutionary arms races can impose intense selective pressures on sexual characters leading to a rapid inter-sexual coevolution which can trigger population divergence, reproductive isolation and, eventually, speciation (Arnqvist and Rowe, 2005; Birkhead and Pizzari, 2002; Haerty et al., 2007; Rice, 2000; Swanson and Vacquier, 2002). Therefore, studying the factors that determine female remating frequency or the rate of stored sperm usage (which together determine the

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intensity of sperm competition) may help to understand the causes of rapid evolutionary change that generates a startling diversity of morphological, behavioral and physiological adaptations.

Which are the factors that determine female remating frequency? *Drosophila* males can gain copulation by courting females but not through sexual coercion because females that refuse to mate can repel mating attempts (Manning, 1967). Thereby, female mating probability is determined not only by the frequency of courtship attempts but also by the female decision, which depends on her intrinsic physiological state (Dickson, 2008). Thus, the probability that a mated *Drosophila* female that is being courted assents to remate can be thought as a function of four parameters: (1) the effect (magnitude and duration) of mating on female sexual receptivity; (2) female receptivity before mating; (3) the effect of mating on female attractiveness and (4) female attractiveness before mating. In *Drosophila melanogaster*, both female receptivity and attractiveness are known to be negatively affected by mating (Fuyama, 1995; Tram and Wolfner, 1998). The evidence gathered so far indicates that sperm and accessory gland proteins (ACPs) that males transfer to females during copulation cause a latency time in which females refuse to remate (Wolfner, 1997; Wolfner et al., 2005). Regardless the ejaculate components, copulation itself may modify female receptivity probably by genital interactions (Eberhard, 1985). Such reduction of female receptivity or attractiveness after mating, known as the female refractory period, has been reported in many *Drosophila* species (Markow, 1996). The duration of female refractory period varies widely among the members of the genus *Drosophila* lasting from a few minutes, as in *Drosophila hydei* (Markow, 1985), to more than 10 days, as in *Drosophila biarmipes* (McRoberts et al., 1997).

Drosophila buzzatii and *Drosophila koepferae* are cactophilic species of the *repleta* group that have recently diverged in the arid lands of southern South America and coexist in vast areas of Argentina and Bolivia (Fanara et al., 1999; Fontdevila et al., 1988; Hasson et al., 2009). Oviposition experiments have shown in both species that inseminated females isolated from males lay eggs for at least three days after contact with males (Fanara et al., 1999; Fanara and Hasson, 2001). The oviposition schedule during that time, however, differs greatly between these species. Actually, Fanara et al. (1999) observed that the average proportion of the fertile eggs laid by *D. buzzatii* females was 38% on the first day and 31% on both the second and the third day after insemination, while *D. koepferae* females produce a massive reproductive output after contact with males laying on average 79% of the fertile eggs on the first day, 13% on the second day and only 8% on the last day. These results suggest that *D. buzzatii* females deplete sperm reserves more slowly, providing more chances for sperm competition in this species than in *D. koepferae*. However, the probability that sperm from different ejaculates exhibit temporal overlap inside the reproductive tract of a female depends not only on the time needed for sperm load depletion but also on the time elapsed until female remating. Thus, to determine whether *D. buzzatii* and *D. kopeferae* are subject to different post-mating sexual selection pressures, it is necessary to investigate female remating frequency, which has not been compared so far between these species.

In this paper we explore female remating frequency in *D. buzzatii* and *D. koepferae*. Particularly, we examine: (1) how female receptivity or attractiveness change with time after mating and (2) whether intraspecific variation in the duration of the refractory period is explained by male or female effects in both species. To investigate these questions we evaluate virgin female mating probability and the magnitude and duration of the effects of mating on female remating probability in different strains of both species.

2. Materials and methods

2.1. Drosophila stocks

Four isofemale lines of *D. buzzatii* (DB1, DB2, DB3 and DB4) and four of *D. koepferae* (DK1, DK2, DK3 and DK4) were established and partially inbred in the lab. Each line (strain from hereafter) was obtained after eight to ten generations of full-sib mating starting from the progeny of a wild inseminated female collected by bait trapping in northwestern Argentina in February 2010.

2.2. Time zero of remating assays

To obtain mated females, 5-day-old virgin females were placed together with two 5-day-old virgin males (“first males”) in a 3 cm^3 vial containing *Drosophila* culture medium, at 8 am until copulation. Therefore, 8 am was considered as time zero (T0) of the remating assays (see below). When copulation occurred, females were gently aspirated and released in individual vials with fresh *Drosophila* medium until remating assay. More than 90% of the females mated within an hour. Females that did not mate were discarded.

2.3. Remating assays

To investigate female remating propensity, two groups of twelve females each were simultaneously assessed at varying times after T0 for each strain. One group consisted of mated females (“mated group”) and the other of equally aged virgin females (“virgin group”). Each single female was released along with two 5-day-old virgin males (“second males”) in a 3 cm^3 vial containing fresh medium. These twenty-four trios were simultaneously assessed for mating occurrence during 15 min. Then, we recorded the proportion of females that acceded to mate and the proportion of females that were courted (chased, tapped, faced, circled or licked by a male) within the first 3 min, for each group. A more prolonged period would have implied too high proportions of courted females, and so differences between groups would have been undetectable.

Remating assays were performed at different times after first mating of the mated group (Times) in each species since preliminary assays revealed large differences in the duration of female refractory period: in *D. buzzatii* it was shorter than 24 h whereas in *D. koepferae* it was longer than 48 h. Therefore, remating assays were performed 1.5, 3, 6, 12 and 24 h after T0 in *D. buzzatii*, and 12, 24, 48, 72, 96 and 120 h after T0 in *D. koepferae*.

These experimental procedures were replicated three times for each strain and each Time, except for *D. koepferae* assays performed 12 h after T0, which were replicated only twice.

2.4. Factors and variables

2.4.1. Predictor variables

Our methods involved the following predictor factors or variables: (1) Species, with two levels: *D. buzzatii* and *D. koepferae*, (2) Time, with five or six levels depending on the species, (3) Strain, with four levels per species, (4) Assay, with three levels or replicates per Strain per Time and (5) Female Status, with two levels per Assay: virgin and mated.

2.4.2. Response variables

We estimated two variables in each group: (1) the proportion of females that were courted by any of the two males within the first 3 min of the remating assay and (2) the proportion of females that acceded to mate within 15 min (female (re)mating propensity). Although female attractiveness is a complex feature, it may be

thought of as the female's ability to induce male courtship. Thus, we believe that the proportion of courted females may be considered as a fair predictor of female attractiveness.

The experimental design allowed us to estimate an additional response variable called female Remating/Mating Odds Ratio (RMOR) that was defined as female remating propensity of the "mated group" divided by female mating propensity of the same aged "virgin group". This ratio depends on the effect of first mating on female receptivity and female attractiveness. Remating propensity should be lower than first mating propensity since copulation reduces both female receptivity and attractiveness (Gillot, 2003), Thus, RMOR is expected to be smaller than 1 and to increase with Time up to 1, when the effects of mating on female behavior are completely diluted, and then remating rate equals mating rate. For this reason, analyzing how RMOR varies with Time can reveal the duration of female refractory period.

2.5. Statistical analyses

2.5.1. Female attractiveness

To test whether Time or Female Status (virgin or mated) affects the proportion of courted females (female attractiveness), a Restricted Maximum Likelihood (REML) approach was applied for fitting a linear mixed effects model for each species in R (*lme* function of *nlme* package (Pinheiro et al., 2012)). The model included Time as a covariable, Female Status as a categorical fixed factor, Strain as a categorical random factor and Assay as a categorical random factor nested in Strain. Since interaction terms failed to increase model goodness of fit, they were not included in the model.

2.5.2. Female (re)mating propensity

We also applied a REML approach to test whether Time or Female Status affects female mating propensity by fitting a linear mixed effects model per species in R (*lme* function of *nlme* package). The model included Time as a covariate, Female Status as a categorical fixed factor, Strain as a categorical random factor and Assay as a categorical random factor nested in Strain. Since we detected an effect of the Time by Female Status interaction, another model was implemented for each level Female Status, separately. This model included Time as a covariable and Strain as a random factor.

Comparisons of female mating propensity between species could only be performed 12 and 24 h after T0 since Time levels of the other assays were not coincident across species. Then, we explored whether female (re)mating propensity differs between *D. buzzatii* and *D. koepferae* by fitting a linear mixed effects model with Species as a categorical fixed factor and Strain, nested in Species, as a categorical random factor.

2.5.3. Female refractory period

We aimed to estimate, for each strain, the time that mated females take to recover 50% of receptivity or attractiveness after mating. We called this time, which was taken as a surrogate of the duration of the refractory period, the R50. Since RMOR was supposed to be a monotonically increasing sigmoid function of Time, we approached this goal by a non-linear regression analysis, using the model:

$$\mathrm{RMOR} = 1/(1 + \exp\,(a^{*}(\mathrm{R50} - \mathrm{Time})))$$

where a stands for an adjustable parameter that indicates RMOR sensitivity to Time. This was called "sigmoid model". A non-linear least-squares approach applied in R (*nls* function of *stats* package (R Development Core Team, 2012)) was used to estimate the expected values of a and R50. To test whether R50 differs among strains within each species, we applied a deviation analysis in R (*anova* function of *stats* package). This analysis allowed us to compare the uncertainty of R50 prediction assuming a unique value for all strains with the uncertainty obtained without that assumption. To find strains with different duration of the refractory period, 0.95 confidence intervals of R50 for each strain were computed in R by interpolation in the profile traces of the parameter estimation (*confint* function of *MASS* package (Venables and Ripley, 2002)). This approach allowed us to identify a strain with a short refractory period ("fast strain") and another with a long refractory period ("slow strain") in each species.

We also aimed to identify whether the factors underlying genetic variation in female refractory period lied on the female, the first male or the second male in female remating assays. In other words, we aimed to test the extent to which female, first or second male strain of origin affected R50. For this purpose, we conducted additional remating assays in which males and females came from the "fast strain" or the "slow strain", in all possible combinations; for example, females of the "fast strain" first mated with males of the "slow strain" and tested with males of the "fast strain" as second males. The "sigmoid model" was applied again to represent RMOR as a function of Time. This time, R50 was considered to be linearly determined by the First Male Strain (FMS), the Second Male Strain (SMS) and the Female Strain (FS). Therefore, we replace R50 of the Sigmoid Model according to the following expression:

$$\mathrm{R50} = b + c^{*}\mathrm{FMS} + d^{*}\mathrm{SMS} + e^{*}\mathrm{FS}$$

where b stands for the minimum R50 value and c, d and e denotes the effect of FMS, SMS and FS on R50, respectively. FMS, SMS or FS equaled 1 when flies come from the "slow strain" and equaled 0 when flies come from the "fast strain". A non-linear least-squares approach computed in R (*nls* function of *stats* package) was used to estimate the model parameters (a, b, c, d and e).

To evaluate differences between species in female refractory period we compared the estimated R50 of *D. buzzatii* and *D. koepferae* strains by employing a two-sample Wilcoxon test in R (*wilcox.test* function of *stats* package).

An angular transformation was applied to all proportion data prior to statistical analyses.

3. Results

3.1. Female attractiveness

The proportion of courted females did not differ between virgin and mated females either in *D. buzzatii* ($F_{(59,\ 1)} = 0.374$, p-value < 0.543) or in *D. koepferae* ($F_{(73,\ 1)} = 1.433$, p-value = 0.235), suggesting that female status does not strongly affect female attractiveness (Fig. 1).

We could not perform between species comparisons of the effect of time elapsed since time zero (T0) on female attractiveness since the time scales employed in each species' assays were quite different. However, we found positive but weak linear relationships between the proportion of courted females and time after T0 (Time), irrespectively of the female status, in both species (Fig. 1). According to the applied model, mean proportion of courted females increased 0.28 per day after T0 (from 0.50 to 0.78 within the first 24 h) in *D. buzzatii* ($F_{(55,\ 1)} = 20.036$, p-value < 0.0001) and 0.07 per day after T0 (from 0.43 to 0.77 within 120 h) in *D. koepferae* ($F_{(69,\ 1)} = 30.025$, p-value < 0.0001).

3.2. Female mating propensity

Since the Time by Female Status interaction on female mating propensity was significant in both *D. buzzatii* ($F_{(58,\ 1)} = 23.422$, p-value < 0.0001) and *D. koepferae* ($F_{(72,\ 1)} = 128.441$, p-value < 0.0001), we decided to perform separate analyses for each female

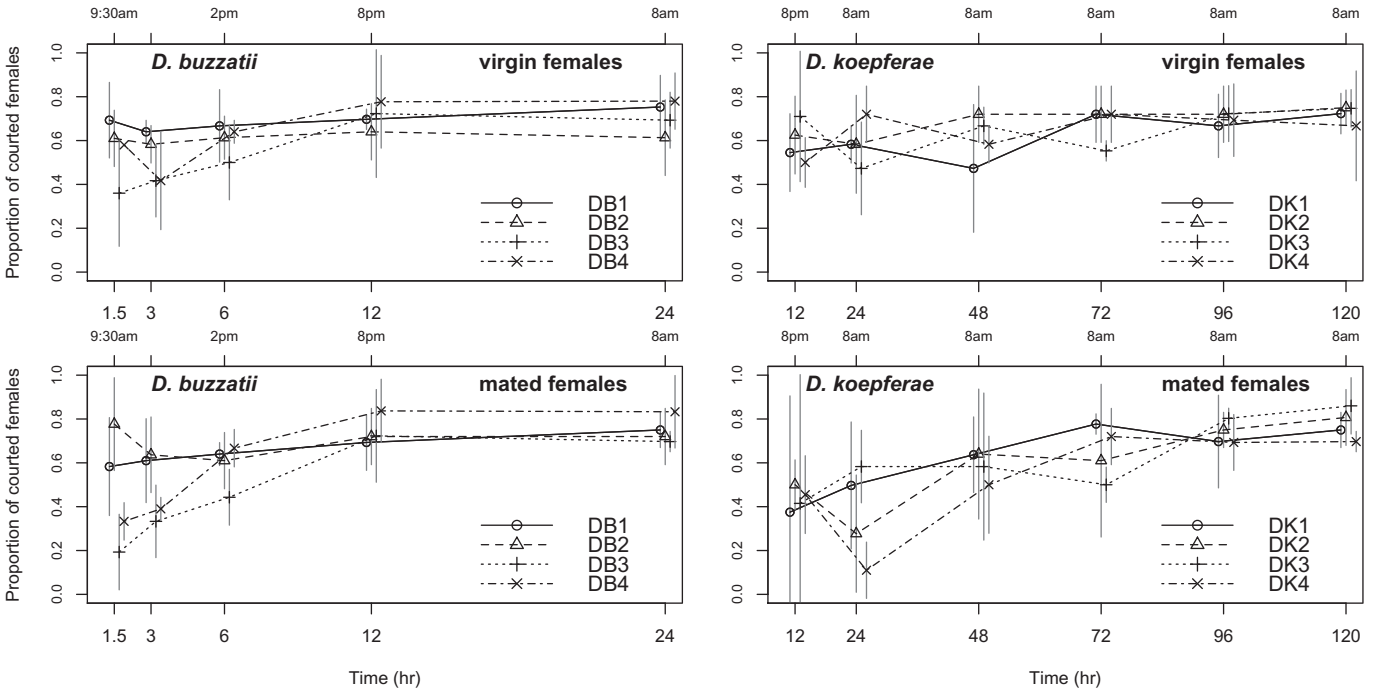

Fig. 1. Temporal variation of the proportion of females that were courted at least once in each strain. Mean values with their standard errors are displayed. *X*-axis states for the time elapsed since first mating of the mated females (Time). A different chart is shown for each species and female status. Daytime for each Time is included on top of the charts.

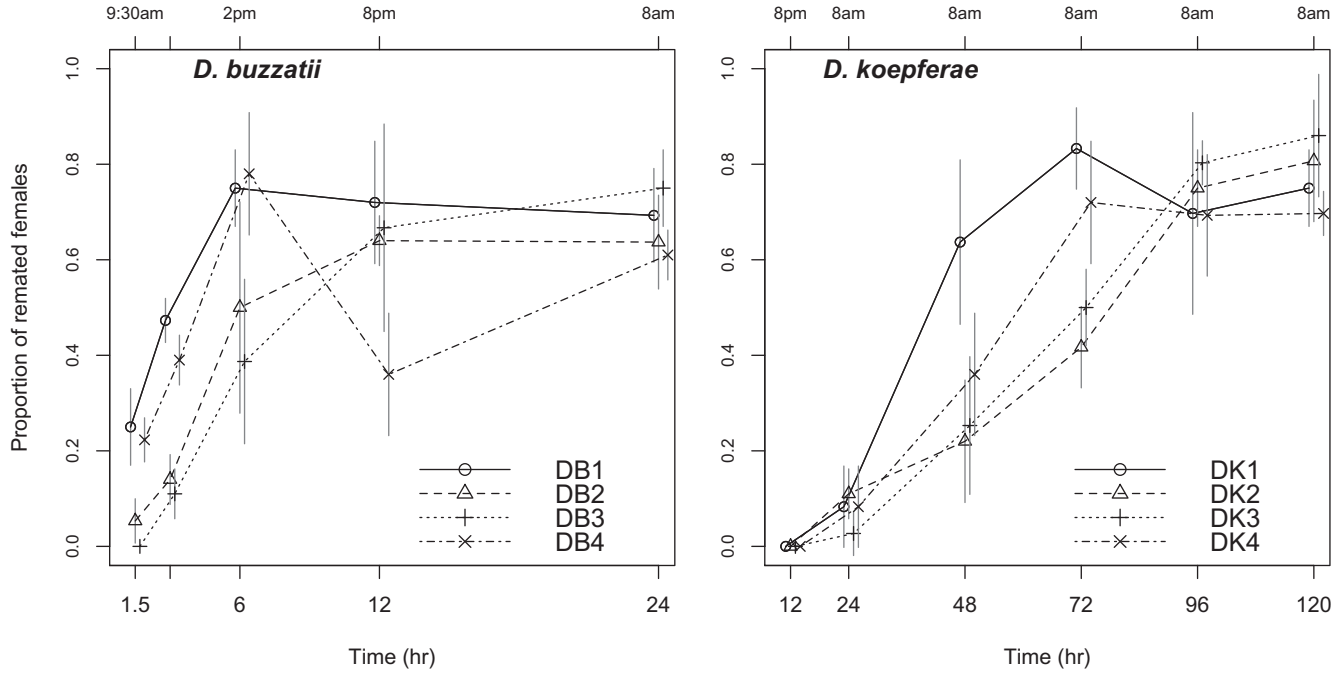

Fig. 2. Temporal variation of the proportion of mated females that acceded to remate (female remating propensity) in each strain. Mean values with their standard errors are displayed. *X*-axis states for Time. A different chart is shown for each species. Daytime for each Time is included in top of the charts.

status level to test the effect of time elapsed since T0. These tests showed that Time failed to predict virgin female mating propensity in *D. buzzatii* ($F_{(55,\ 1)} = 0.774$, p-values = 0.383) and *D. koepferae* ($F_{(69,\ 1)} = 0.029$, p-value = 0.866). In contrast, Time affected mating propensity in non-virgin females in both species; mated females were more willing to remate as Time increased (Fig. 2). Estimated Time effects revealed that remating propensity in *D. buzzatii* increased on average 0.54 per day after T0, from 0.23 to 0.77 within the first 24 h ($F_{(55,\ 1)} = 36.986$, p-value < 0.0001), while in *D. koepferae* female remating propensity increased on average 0.18 per day after T0, from 0.00 to 0.87 within the first 120 h ($F_{(69,\ 1)} = 175.993$, p-value < 0.0001). These results suggest that the negative effect of mating on female receptivity declines with time.

The proportion of females of the virgin group that acceded to mate 12 h or 24 h after T0 did not differ between *D. buzzatii* (68%) and *D. koepferae* (69%) ($F_{(6,\ 1)} = 1.740$, p-value = 0.235). In

contrast, the proportion of females of the mated group that accepted to remate with a second male 12 h or 24 h after the first mating was significantly greater in *D. buzzatii* (64%) than in *D. koepferae* (5%) ($F_{(6,\ 1)} = 147.386$, p-value < 0.0001).

3.3. Female refractory period

Remating/Mating Odds Ratio (RMOR) increased with Time from values close to 0 to nearly 1, in all strains (Fig. 3). Further analyses revealed that the time that a mated female takes to recover 50% of mating propensity (R50) depends on the strain in both *D. buzzatii* ($F_{(66,\ 3)} = 9.364$, p-value < 0.0001) and *D. koepferae* ($F_{(80,\ 3)} = 10.168$, p-value < 0.0001) indicating that the duration of female refractory period varies among strains. Estimates of R50 and confidence intervals for each strain are shown in Fig. 4. Neither in *D. buzzatii* nor in *D. koepferae* the 95% confidence intervals of the most divergent strains overlapped. Strains DB4 (R50 = 1.915 h) and DK1 (R50 = 33.245 h) exhibited shorter refractory periods than strains

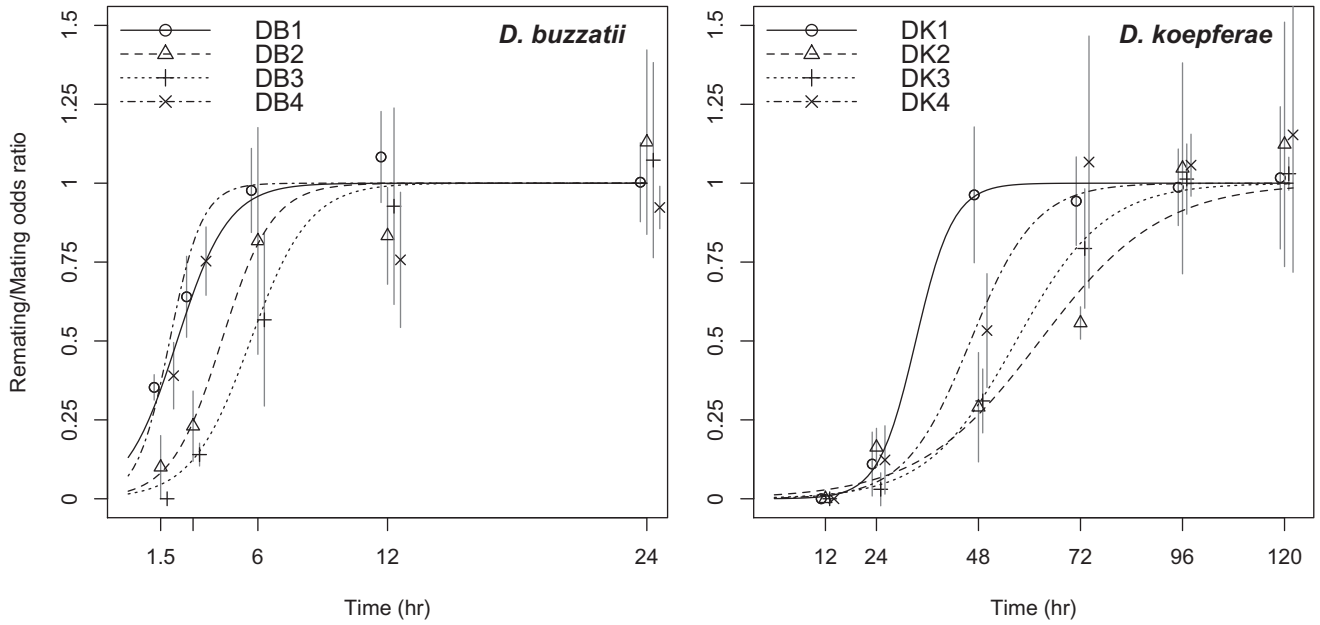

Fig. 3. Temporal variation of female Remating/Mating Odds Ratio (RMOR) in each strain. Mean values and their standard errors are displayed. A fitted sigmoid curve is shown per strain to illustrate how female remating propensity recovers with Time. A different chart is shown for each species.

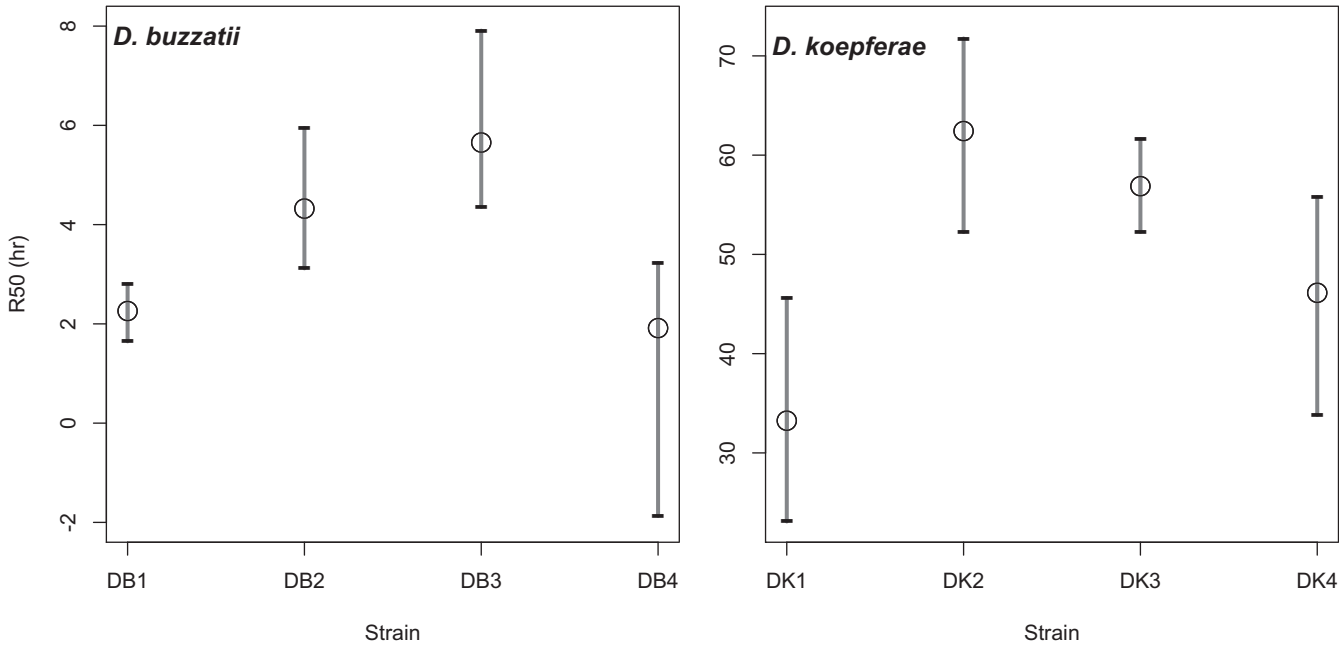

Fig. 4. Duration of the female refractory period. The mean time that females take to recover 50% of their mating propensity after a first mating (R50) is shown with its 0.95 confidence interval for each strain. R50 was estimated as a parameter of a sigmoid model that describes temporal variation of RMOR. 0.95 confidence intervals were computed by interpolation in the profile traces of the parameter estimation. A different chart is shown for each species.

Table 1
Effects of each male and female strain on the time that females take to recover 50% of their mating propensity after a first mating (R50). A sigmoid model of female Remating/Mating Odds Ratio (RMOR) as a function of time after first mating (Time) was applied per species to estimate the effects of "First Male" Strain (FMS), "Second Male" Strain (SMS) or Female Strain (FS) on R50. These effects were referred to the R50 of the "fast strain".

Species	Parameter	Estimate	*t*-Value	*p*-Value
D. buzzatii	Fast strain R50	1.800	5.915	3.486E−08
	FMS effect	0.494	1.559	0.122
	SMS effect	0.095	0.302	0.764
	FS effect	3.310	10.230	7.358E−18
D. koepferae	Fast strain R50	31.988	12.877	2.90E−26
	FMS effect	7.709	3.371	9.455E−04
	SMS effect	−0.091	−0.040	0.968
	FS effect	20.138	8.511	1.390E−14

DB3 (R50 = 5.654 h) and DK2 (R50 = 62.424 h), respectively. Thus, DB4 and DK1 were considered as "fast strains" and DB3 and DK2 as "slow strains".

Using the "fast strain" and the "slow strain" of each species in additional assays, we independently controlled the First Male Strain (FMS), the Second Male Strain (SMS) and the Female Strain (FS) as predictors of R50. In *D. buzzatii*, only FS affected significantly R50 while both FMS and FS affected R50 in *D. koepferae* (Table 1). These results suggest that differences in R50 between "fast" and "slow" strains can be accounted for by the female strain of origin in *D. buzzatii* whereas both first male and female origin can alter R50 in *D. koepferae*.

Finally, we also found conspicuous differences in the duration of the female refractory period between species ($W_8 = 0$, *p*-value = 0.029) since mean R50 was 14 times greater in *D. koepferae* (49.665 h) than in *D. buzzatii* (3.538 h).

4. Discussion

Our study shows that after mating females pass through a refractory period in which they tend to reject new mating attempts, resulting in a reduction of mating propensity as compared to virgin females of the same age. Our results also indicate that female receptivity decreases after mating whereas female attractiveness does not seem to be affected by the sexual status. We found evidence of genetic variation for the duration of the female refractory period in both species. In addition, we detected remarkable differences between species in the duration of the refractory period, which was quite longer in *D. koepferae*, pointing to a higher female lifespan remating rate in *D. buzzatii*. From these results, whereas previous results of oviposition behavior suggesting that female *D. buzzatii* store sperm for longer than *D. koepferae*, we can gather that *D. buzzatii* exhibit higher sperm competition chances. Thus, post-mating sexual selection is expected to be more intense in *D. buzzatii* than in its sibling.

We estimated the time that mated females take to recover 50% of remating propensity relative to virgin females of the same age using a composed variable that we called Remating/Mating Odds Ratio (RMOR). We found that RMOR increases as a function of the time elapsed since time zero (T0) when females of the mated group mated for the first time. Moreover, we can conclude that the temporal rise of RMOR (Fig. 4) might be only due to the increase or recovery of female remating propensity (Fig. 3) since mating propensity of virgin females did not change with time elapsed since T0 (Time). It may be argued that the recovery of female remating propensity depends on the recovery of both attractiveness and receptivity. Our results indicate, however, that mating does not seem to reduce female attractiveness since the proportion of courted females was not affected by female status (i.e. virgin and mated females were equally courted) (Fig. 1). Thus, despite the fact that we did not directly evaluate changes in female post-mating receptivity, our results suggest that the observed reduction of female mating propensity after first mating may be accounted for by a reduction of female receptivity. Interestingly, it has been observed that *D. melanogaster* females undergo dramatic alterations in reproductive physiology and behavior after mating, which have been shown to be initially induced by accessory gland proteins (ACPs) and to persist because of the presence of stored sperm (Aigaki et al., 1991; Wolfner, 2002). Thus, a possible explanation for our observations is that ACPs or sperm transferred during copulation modify the physiological state of the female exerting a negative effect on receptivity.

In response to mating, *D. melanogaster* females decrease the production of courtship-stimulating pheromones and release courtship-inhibitory pheromones that together decline their sex appeal for several days inducing less courtships attempts when males are present (Tompkins, 1984). Here, we did not find evidence of such effect. Our results show that female attractiveness, which was measured as the proportion of females that were courted by any male during the assays, was not affected by the mating status (i.e. virgin and mated females were equally courted). However, as each female was placed with two males in a 3 cm^3 vial, the conditions imposed during the assays are far from natural conditions. For instance, any female pheromone may saturate the atmosphere inside the vials more easily than in nature. Moreover, only one female was allowed per vial in the assays whereas the presence of potential "competing" females is very likely in nature. For these reasons, although female status failed to predict the proportion of courted females in *D. buzzatii* and *D. koepferae*, we cannot conclude that all aspects of female attractiveness are unaffected by mating. Nevertheless, our results suggest that there are at least some components of female attractiveness that are not affected by mating either in *D. buzzatii* or in *D. koepferae*.

Even though we did not find evidence that female attractiveness depends on sexual status, we observed that it depends on the time of the day in which the assays were performed or the female age. In *D. buzzatii*, female attractiveness changed along the day during the first 24 h after T0. Actually, females tended to be more attractive at dawn (8 am) and at dusk (8 pm) than at any other time (Fig. 1). Taking into account that attractiveness in this species was assessed near midday (9:30 am–2:00 pm) for the first three points assayed and at 8 pm and 8 am for the last two points, the observed effect of Time on female attractiveness may be due to the circadian rhythm of locomotor activity, which is known to peak at dawn and at dusk in *D. melanogaster* (Klarsfeld et al., 2003). Male courtship behavior and female mating propensity also exhibit circadian rhythms in *D. melanogaster* (Hardeland, 1972; Sakai and Ishida, 2001). These rhythms are governed by different clock neurons (Grima et al., 2004; Hamasaka et al., 2010) that could also be responsible for the relationship between Time and female attractiveness along the first 24 h after first mating observed in

D. buzzatii. In *D. koepferae*, instead, we evaluated how female attractiveness changed along a 5-day period after T0. In this case we observed a slight temporal increment of female attractiveness (Fig. 1). Since all the assays were performed at 8 am (with the exception of the assays performed at 12 h after T0 which were performed at 8 pm), the effect of Time on the proportion of courted females cannot be mediated by a circadian rhythm effect. Thus, the Time effect suggests that older females are scarcely more attractive for males at least in *D. koepferae*. Interestingly, a recent study has revealed that the composition of cuticular hydrocarbons, which act as sexual pheromones, is significantly affected by aging in *D. melanogaster* (Kuo et al., 2012). The authors showed that aging-related changes in cuticular hydrocarbon profiles are responsible for a significant reduction in sexual attractiveness. Thus, the temporal pattern of female attractiveness we observed in *D. koepferae* could be attributed to physiological changes associated with aging such as secretion of sexual pheromones.

As expected, RMOR increased with Time up to values close to 1 in all strains (Fig. 3). This indicates that, eventually, female remating propensity equals virgin female mating propensity as the effects of mating on female receptivity vanished, i.e. once the refractory period is over. We assessed the duration of the refractory period estimating the time needed by females of the different strains to recover 50% of mating propensity (R50) after the first mating. As the uncertainty of R50 prediction assuming a unique value for all strains was significantly higher than the uncertainty without that assumption in both *D. buzzatii* and *D. koepferae*, we inferred that the duration of the female refractory period varies among strains. Thus, genetic variation may be expected in these species in the time needed by inseminated females to recover mating propensity. To investigate whether the variation between the "fast strain" and the "slow strain" of each species may be explained by the strain of origin of the first-mating male, the second-mating male or the female, we estimated the effect of each fly strain on R50. The female strain accounted for the differences between the "fast strain" and the "slow strain" in *D. buzzatii*, while both the first male and female strains accounted for the pattern observed in *D. koepferae* (Table 1). These findings suggest that female genotype affects latency to remating among the strains of both species and that male genotype affects male ability to defer remating of their mates at least among *D. koepferae* strains. However, since the differences between two strains may depend strongly on the particular chosen pair of strains, our results do not necessarily have meaning at the species level. Males and female contribution to genetic variance of female mating latency had been assessed in the exhaustively studied *D. melanogaster*. In this species, selection for the duration of female mating latency was applied separately on females, on the first male and on the second male to mate with the female (Gromko and Newport, 1988). A rapid response was only observed when selection was applied on females, suggesting that female's, but not male's, genotype contributes to additive genetic variation of the duration of female refractory period in *D. melanogaster* (Gromko and Newport, 1988).

Beyond the differences observed among strains of the same species we found conspicuous interspecific differences in R50. *D. buzzatii* females (mean R50 = 3.5 h) recovered receptivity 14 times faster than *D. koepferae* ones (mean R50 = 49.7 h). This scene suggests a higher remating rate in *D. buzzatii*. Also, according to the results of a study of oviposition behavior, the proportion of the sperm load that remains available for egg fertilization 24 h after insemination is 62% in *D. buzzatii* and 21% in *D. koepferae* (calculated from the data reported in Table 4 of Fanara et al., 1999), suggesting that the rate of stored sperm usage or wastage is higher in *D. koepferae*. Taking into account all the evidence gathered so far, the chances that sperm from different mates meet inside the same female are expected to be greater in *D. buzzatii* than in its sibling. Thus, post-mating sexual selection by means of sperm competition or cryptic female choice is expected to be stronger in *D. buzzatii* than in its sibling.

Post-mating sexual selection has been proposed as the main candidate mechanism responsible for the rapid evolution of sexual characters such as ACPs or male genital morphology (Arnqvist, 1998). For instance, aedeagus size or shape may determine male influences on post-mating sexual selection such as delivery of ACPs, mechanical stimulation of the female reproductive tract or displacement of stored sperm of previous mates (Rowe and Arnqvist, 2011)). If post-mating sexual selection, arising from female promiscuity, were the major factor responsible for the rapid evolution of male genitalia, *D. buzzatii* would be expected to exhibit faster evolutionary rates. Yet, though rapid divergence of male genital morphology among natural populations has been detected in both species (Soto et al., 2007, unpublished results), forthcoming studies comparing evolutionary rates of male genitalia or "sexual genes" between *D. buzzatii* and *D. koepferae* may be a suitable complement to our present results in order to test the hypothesis that post-mating sexual selection drives the rapid evolution of reproductive traits.

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References

Aigaki, T., Fleischmann, I., Chen, P.S., Kubli, E., 1991. Ectopic expression of sex peptide alters reproductive behavior of female *Drosophila melanogaster*. Neuron 7, 557–563.
Arnqvist, G., 1998. Comparative evidence for the evolution of genitalia by sexual selection. Nature 393, 784–786.
Arnqvist, G., Rowe, L., 2005. Sexual Conflict. Princeton University Press, Princeton, New Jersey.
Birkhead, T.R., Pizzari, T., 2002. Postcopulatory sexual selection. Nature Review Genetics 3, 262–273.
Dickson, B.J., 2008. Wired for sex: the neurobiology of *Drosophila* mating decisions. Science 322 (5903), 904–909.
Bloch Qazi, M.C., Heifetz, Y., Wolfner, M.F., 2003. The developments between gametogenesis and fertilization: ovulation and female sperm storage in *Drosophila melanogaster*. Developmental Biology 256, 195–211.
Eberhard, W.G., 1985. Sexual Selection and Animal Genitalia. Harvard University Press, Harvard.
Fanara, J.J., Fontdevila, A., Hasson, E., 1999. Oviposition preference and life history traits in the cactophilic sibling species *Drosophila koepferae* and *Drosophila buzzatii* in association to their natural host. Evolutionary Ecology 13, 173–190.
Fanara, J.J., Hasson, E., 2001. Oviposition acceptance and fecundity schedule in the cactophilic sibling species *Drosophila buzzatii* and *D. koepferae* on their natural hosts. Evolution 55, 2615–2619.
Fontdevila, A., Pla, C., Hasson, E., Wasserman, M., Sanchez, A., Naveira, H., Ruiz, A., 1988. *Drosophila koepferae*: a new member of the *Drosophila serido* (Diptera-Drosophilidae) superspecies taxon. Annals of the Entomological Society of America 81, 380–385.
Fowler, G.L., 1973. Some aspects of the reproductive biology of *Drosophila*: sperm transfer, sperm storage, and sperm utilization. Advances in Genetics 17, 293–360.
Fuyama, Y., 1995. Genetic evidence that ovulation reduces sexual receptivity in *Drosophila melanogaster* females. Behavior Genetics 25, 581–587.
Gillot, C., 2003. Male accessory gland secretions: modulators of female reproductive physiology and behavior. Annual Review of Entomology 48, 163–184.
Grima, B., Chelot, E., Xia, R., Rouyer, F., 2004. Morning and evening peaks of activity rely on different clock neurons of the *Drosophila* brain. Nature 431, 869–873.

Gromko, M.H., Newport, M.E.A., 1988. Genetics basis for remating in *Drosophila melanogaster*. II. Response to selection based on the behavior of one sex. Behavior Genetics 18, 621–632.

Haerty, W., Jagadeeshan, S., Kulathinal, R.J., Wong, A., Ravi Ram, K., Sirot, L.K., Levesque, L., Artieri, C.G., Wolfner, M.F., Civetta, A., Singh, R.S., 2007. Evolution in the fast lane: rapidly evolving sex-related genes in *Drosophila*. Genetics 177, 1321–1335.

Hamasaka, Y., Suzuki, T., Hanai, S., Ishida, N., 2010. Evening circadian oscillator as the primary determinant of rhythmic motivation for *Drosophila* courtship behavior. Genes to Cells 15, 1240–1248.

Hardeland, R., 1972. Species differences in the diurnal rhythmicity of courtship behavior within the melanogaster group of the genus *Drosophila*. Animal Behaviour 20, 170–174.

Hasson, E., Soto, I.M., Carreira, V.P., Corio, C., Soto, E.M., Betti, M.I., 2009. Host plants, fitness and developmental instability in a guild of cactophilic species of the genus *Drosophila*. In: Santos, E.B. (Ed.), Ecotoxicology Research Developments. Nova Science Publishers, Hauppauge, pp. 89–109.

Klarsfeld, A., Leloup, J.C., Rouyer, F., 2003. Circadian rhythms of locomotor activity in *Drosophila*. Behavioural Processes 64 (2), 161–175.

Kuo, T.-H., Yew, J.Y., Fedina, T.Y., Dreisewerd, K., Dierick, H.A., Pletcher, S.D., 2012. Aging modulates cuticular hydrocarbons and sexual attractiveness in *Drosophila melanogaster*. The Journal of Experimental Biology 215, 814–821.

Lefevre, G.J., Jonsson, U.B., 1962. Sperm transfer, storage, displacement, and utilization in *Drosophila melanogaster*. Genetics 47, 1719–1736.

Manning, A., 1967. The control of sexual receptivity in female *Drosophila*. Animal Behaviour 15, 239–250.

Markow, T.A., 1985. A comparative investigation of the mating system of *Drosophila hydei*. Animal Behaviour 33, 775–781.

Markow, T.A., 1996. Evolution of *Drosophila* mating systems. Evolutionary Biology 29, 73–106.

Markow, T.A., 2002. Perspective: female remating, operational sex ratio, and the arena of sexual selection in *Drosophila* species. Evolution 56, 1725–1734.

McRobert, S.P., Adams, C.R., Wutjke, M., Frank, J., Jackson, L.L., 1997. A comparison of female post-copulatory behaviour in *Drosophila melanogaster* and *Drosophila biarmipes*. Journal of Insect Behavior 10, 761–770.

Parker, G.A., 1970. Sperm competition and its evolutionary consequences in the insects. Biological Reviews 45, 525–568.

Patterson, J.T., 1954. Fate of the sperm in the productive tract of the *Drosophila* female in homogamic matings. University of Texas Publication 5422, pp. 19–37.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D.The R Development Core Team, 2012. Nlme: Linear and Nonlinear Mixed Effects Models. R Package Version 3.1-104. R Foundation for Statistical Computing, Vienna.

Pitnick, S., Markow, T., Spicer, G.S., 1999. Evolution of multiple kinds of female sperm-storage organs in *Drosophila*. Evolution 53, 1804–1822.

R Development Core Team, 2012. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna.

Rice, W.R., 2000. Dangerous liaisons. Proceedings of the National Academy of Sciences of the United States of America 97, 12953–12955.

Rice, W.R., Holland, B., 1997. The enemies within: inter-genomic conflict, interlocus contest evolution (ICE), and the intra-specific red queen. Behavioral Ecology and Sociobiology 41, 1–10.

Rowe, L., Arqvist, G., 2011. Sexual selection and the evolution of genital shape and complexity in water striders. Evolution 66–1, 40–54.

Sakai, T., Ishida, N., 2001. Circadian rhythms of female mating activity governed by clock genes in *Drosophila*. Proceedings of the National Academy of Sciences of the United States of America 98, 9221–9225.

Soto, I.M., Carreira, V.P., Fanara, J.J., Hasson, E., 2007. Evolution of male genitalia: environmental and genetic factors affect genital morphology in two *Drosophila* sibling species and their hybrids. BMC Evolutionary Biology 7, 77.

Swanson, W.J., Vacquier, V.D., 2002. Rapid evolution of reproductive proteins. Nature Reviews Genetics 3, 137–144.

Tompkins, L., 1984. Genetic analysis of sex appeal in *Drosophila*. Behavior Genetics 14, 411–440.

Tram, U., Wolfner, M.F., 1998. Seminal fluid regulation of female sexual attractiveness in *Drosophila melanogaster*. Proceedings of the National Academy of Sciences of the United States of America 95, 4051–4054.

Venables, W.N., Ripley, B.D., 2002. Modern Applied Statistics with S, fourth ed. Springer, New York.

Wolfner, M.F., 1997. Tokens of love: functions and regulation of *Drosophila* male accessory gland products. Insect Biochemistry and Molecular Biology 27, 179–192.

Wolfner, M.F., 2002. The gifts that keep on giving: physiological functions and evolutionary dynamics of male seminal proteins in *Drosophila*. Heredity 88, 85–93.

Wolfner, M.F., Applebaum, S., Heifetz, Y., 2005. Insect gonadal glands and their gene products. In: Gilbert, L., Iatrau, K., Gill, S. (Eds.), Comprehensive Insect Physiology, Biochemistry, Pharmacology and Molecular Biology. Elsevier, Amsterdam, pp. 179–212.