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Exposure to rivals and plastic responses to sperm competition in *Drosophila melanogaster*

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Responses by males to the level of sperm competition have been documented across a wide range of taxa. Recent work in *Drosophila melanogaster* shows that males respond adaptively to the presence of other males by making facultative adjustments to mating duration, resulting in increased transfer of ejaculate proteins, direct effects on postmating responses in females, and, ultimately, increased male competitive reproductive success. Here, we investigated how males detect the presence of rival males. We tested the effect of the length of male-to-male exposure, male age at first exposure, time since initial exposure to rivals and density. We found that the longer the males were exposed to rivals prior to mating (from 0 to 101 h of exposure), the longer their subsequent mating duration. There was no detectable effect, however, of increasing the number of rivals above 1. Increasing the density (hence encounter rate) in which males were kept had no effect on a male's response to rivals and there was also no evidence that responses to rivals could be evoked by a brief (2 h) time window of exposure to males at various times prior to mating. The age at which males were first exposed to other males did not affect their ability to respond to rivals. Taken together, our findings show that it is the absolute length of exposure to rivals and not the number of rivals that is critical in determining male plastic responses to the potential level of sperm competition in *D. melanogaster*. *Key words:* accessory gland proteins, Acps, fruit fly, mating duration, sexual selection, sperm competition. *[Behav Ecol 21:317–321 (2010)]*

Sperm competition has driven the evolution of morphology, physiology, and behavior across organisms from a huge diversity of taxa (Birkhead and Møller 1998; Simmons 2001). Ejaculates are often costly and in limited supply, hence, males should allocate them prudently (Hihara 1981; Dewsbury 1982; Wedell et al. 2002). Theory makes clear predictions about how males should invest in matings when faced with sperm competition (Parker 1993; Parker et al. 1997). Sperm competition can be split into risk (probability that a female has mated) and intensity (number of ejaculates in competition) and the importance of making this distinction has been highlighted (Engqvist and Reinhold 2005). Males from diverse species have been shown to respond to the presence of rivals by ejaculating more sperm (e.g., Gage and Baker 1991; Gage and Barnard 1996; Wedell and Cook 1999), producing more viable sperm (Thomas and Simmons 2007) and by mating for longer (Bretman et al. 2009). In *Drosophila melanogaster*, males show plastic responses to the level of sperm competition, which is signaled by both female mating status (Friberg 2006) and the number of rival males present before and during mating (Bretman et al. 2009). In both cases, males adjust mating duration: they mate for longer when 1) females have, or are perceived to have, mated (Friberg 2006) or 2) they have been exposed to rival males prior to mating (Bretman et al. 2009). These responses have profound fitness effects. Longer matings by males exposed to rivals prior to mating lead to increased transfer of ejaculate proteins (Wigby et al. 2009), increased female refractoriness, fecundity and egg to adult survival (Bretman et al. 2009), and, ultimately, higher paternity success under competitive conditions (Bretman et al. 2009).

Evidence from moths (*Plodia interpunctella*, Gage 1995) and armyworms (*Pseudaletia separata*, He and Miyata 1997) suggests that the larval environment can affect adult male ejaculate allocation strategies. This is believed to occur because differences in larval density communicate in some way the future level of sperm competition that is likely to be experienced. In adults, the mechanisms by which males detect the presence of rivals are likewise not yet known; for example, what is the minimum length of time or number of males necessary to trigger adaptive responses in a male's subsequent mating duration? Our current study focuses on these questions. If males respond to the potential level of sperm competition by allocating existing ejaculate resources, then males should be able to respond maximally and instantaneously to rivals through increased mating duration and ejaculate transfer. However, if the response to the potential level of sperm competition involves a physiological process such as increased production of seminal fluid accessory proteins (Acps) or sperm, males may require time to respond adaptively. Our previous work (Bretman et al. 2009) suggests that it is primarily exposure of males to rivals prior to mating that is important; males that have no prior exposure to rivals do not (or cannot) respond adaptively when they meet rivals for the first time in the mating arena. This implies that the response to rivals is the initiation of physiological processes that take time to come into effect. Hence, we predict that a minimum period of exposure to other males is necessary in order for males to be able to make adaptive responses to the presence of rivals. It is not yet known, however, whether mating duration continues to increase with increasing time of exposure to males, with increasing numbers of males, or whether male age is important. In addition, the encounter rate with rivals (i.e., density rather than absolute number) may also have an effect. An alternative possibility is that there is a critical time window, where exposure to rivals triggers a male's response to sperm competition, regardless of the continual length of exposure.

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We addressed these issues in this study by a systematic investigation of the effect of different male-to-male exposure regimes on a male's subsequent mating duration. We manipulated the length of exposure to rivals by exposing males to 3 rivals for periods of between 101 and 5 h prior to mating. In this way, we could determine whether there was a binary response—a minimum threshold of exposure time below which males do not increase mating duration—or whether mating duration continues to increase with longer exposure times. We also examined, using a constant exposure time, whether mating duration continued to increase after exposure to increasing numbers of rival males (1, 2, 4, 8, or 16 rivals) with and without controlling for density. We then investigated whether there was a critical time window of exposure by measuring the effect of exposing males to rivals for 2 h at various times prior to mating, thus testing if a short initiating "signal" is all that is necessary to start a response that takes time to come into full effect. Finally, we tested whether the ability of males to respond to the presence of rivals depends on male age (with males aged 0, 4, 10, and 19 days post adult eclosion).

MATERIAL AND METHODS

Fly rearing and all experiments were conducted in a 25 °C humidified room, with 12:12 h light:dark cycle, on standard sugar yeast agar media (100 g brewer's yeast powder, 100 g sugar, 20 g agar, 30 ml nipagin (10% solution), and 3 ml propionic acid per liter of distilled water) with additional live yeast granules. Wild-type flies were from a large laboratory population originally collected in the 1970s in Dahomey (Benin) and were the same strain as used in our previous work (Bretman et al. 2009). Larvae were raised at a standard density of 100 per vial. At eclosion, flies were collected, and sexes were separated using ice anesthesia. Virgin females were kept at a standard density of 10 per vial for 5 days and males as mentioned below. All flies were mated at 5 days post-eclosion, unless otherwise stated. All trials within each experiment were conducted on the same day. For mating assays, all males were aspirated singly into a vial containing a single female. Vials that contained dead males were discarded. Introduction time, start and end of mating were recorded. Flies were discarded if they did not mate within 2 h.

Effect of length of exposure to rivals on a male's subsequent mating duration

Males were kept singly from eclosion and then placed randomly together in groups of 4 at 13 time points prior to mating. We aspirated males into groups at 09.00, 14.00, and 18.00 each day for 5 days from the day after eclosion until 09.00 on the day of mating, corresponding to 101, 96, 92, 77, 72, 68, 53, 48, 44, 29, 24, 20, 5 h of male–male exposure prior to mating. We also had a group of males that were never exposed to rivals (the 0 h treatment), which acted as a baseline against which to compare the other treatments (final sample sizes per treatment n = 21–31).

Effect of the number and density of rivals on a male's subsequent mating duration

After collection, males were kept in groups of 1, 2, 4, 8, or 16 and were assigned to 1 of 2 density treatments. In one treatment, we varied density with group size by keeping the volume of the vials constant (by placing the cotton wool bung at the same height). For the other treatment, density was made constant by moving the cotton wool closure to different heights within the vial to equalize the volume available per fly. Final samples sizes were n = 36–39 for each treatment/density combination. In all cases in this experiment, males were exposed to rivals for 4 days prior to matings.

Effect of 2 h window of exposure to rivals prior to mating

To test whether there was a critical window of exposure, we kept males singly or with 3 rivals for a period of 2 h, commencing at 96, 72, 48, 24, and 2 h before mating (final sample sizes per treatment n = 29–34). The groups of males randomly assigned to each time point were separated after 2 h of exposure and held singly until mating.

Effect of male age at exposure

To test whether young and old males can make facultative adjustments to mating duration, males were collected at eclosion and maintained singly until randomly assigned to treatments, in which they were kept singly or grouped with 3 rivals for 2 days commencing at 0, 4, 10, and 19 days post-eclosion. Although male flies may live for 40–50 days, at 19 days old males have completed the most productive part of their reproductive life span. After 2 days of exposure to rivals, males were given the opportunity to mate; hence, males were mated at 2, 6, 12, and 21 days old (final sample sizes per treatment n = 34–40).

Analysis

Statistical analysis was performed using R v2.6.1 (Ihaka and Gentleman 1996) and SPSS v14 (SPSS Inc., Chicago, IL). All raw data and residuals were tested for normality using Kolomogorov–Smirnov tests and for homogeneity of variance using Flingen Killeen tests. For the length of exposure to rivals experiment, an analysis of variance (ANOVA) was performed by nesting treatment (number of hours with rivals) within time of day of introduction (09.00, 14.00, or 18.00) to rivals. This was then simplified to an ANOVA of treatment on mating duration, with post hoc linear contrasts and least square difference (LSD) tests. Six extreme values (of a total of 399 data points) from 4 treatments (identified in SPSS as outside 3 interquartile range lengths) were found to be highly significant outliers (Grubbs' test, all $P < 0.001$; Grubbs (1969)) in the length of exposure data set and excluded from further analysis. These data points represented "pseudocopulations" (extremely short matings where genitalia were not engaged) or copulations where genitalia became stuck (extremely long matings with failure to disengage from mating). The same procedure was applied to the density data set where 4 (of 372 data points) highly significant outliers (Grubbs' test, all $P < 0.001$) across 3 treatments were removed, and an ANOVA was performed with post hoc LSD tests. Data on the window of exposure experiment did not conform to normality despite transformation; and hence, the effect of treatment duration of mating was assessed using a Kruskal–Wallis test. This was also the case for the age of exposure experiment, and a general linear model with poisson errors was performed and the models tested using analysis of deviance.

RESULTS

Effect of length of exposure to rivals on a male's subsequent mating duration

The number of pairs mating ranged from 75% to 95% per treatment and was not significantly different between treatments (data not shown). There was no evidence that the time of day at which males were exposed to rivals affected their subsequent mating duration (ANOVA treatment nested within time of day; time of day $F_{3,399} = 1.54$, $P = 0.21$, time of day

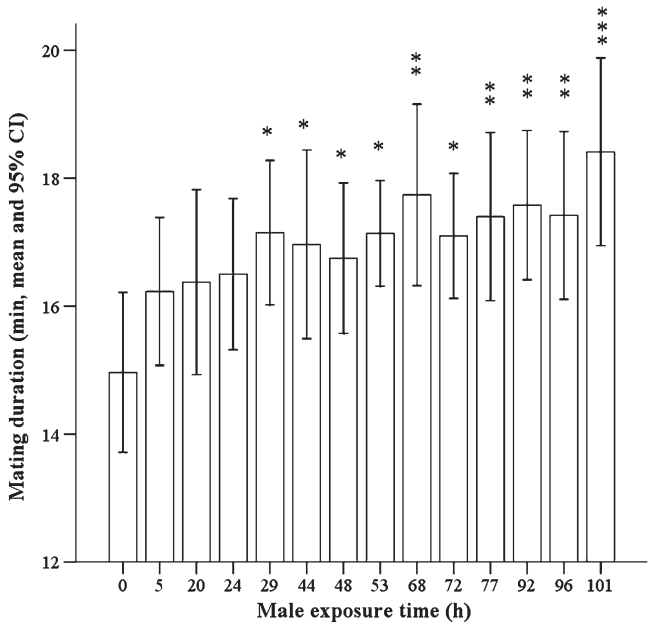

Figure 1
Mating duration in response to varying lengths of exposure of males to rivals prior to mating. Males were collected singly and then placed in groups of 4 at 13 time points from 101 to 5 h prior to mating and then placed singly with females for matings. (A) Mean mating duration (min and 95% confidence interval) for males exposed to other males for 5 to 101 h prior to mating. Treatments identified with asterisks mated for significantly longer than the 0 h treatment (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

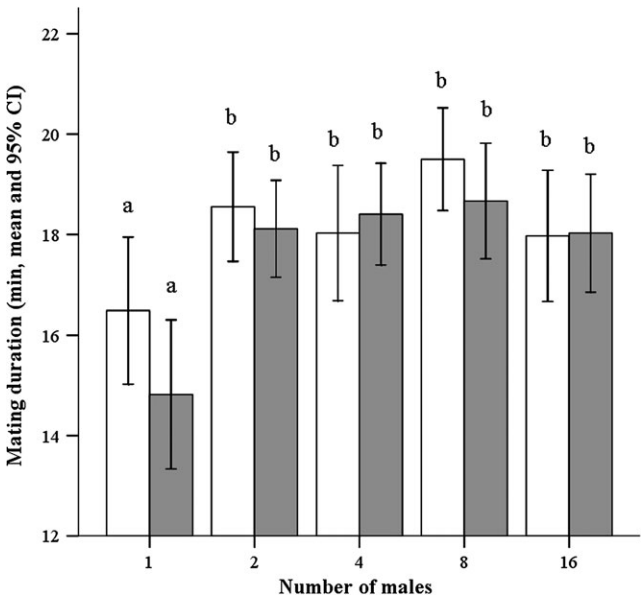

Figure 2
Mating duration in response to varying density and number of rivals prior to mating. Males were maintained in groups of 1, 2, 4, 8, and 16 males in the 4 days prior to mating and held at either constant (white bars) or variable densities (gray bars). Mean (and 95% confidence interval) mating duration in response to group size and density treatment. Males kept with rivals (group size 2 and above) mated for significantly longer than males kept alone. Bars identified with the same letter are not significantly different from each other; those with different letters are significantly different ($P < 0.005$).

(treatment) $F_{10,399} = 0.48$, $P = 0.903$); hence, we adopted a simplified univariate ANOVA for the rest of the analysis for this experiment. There was a significant positive effect of the length of male-to-male exposure on subsequent mating duration (ANOVA treatment $F_{13,392} = 1.86$, $P = 0.033$: Figure 1). Post hoc LSD tests revealed significant differences between 0 h and all other treatments ($P < 0.05$) except for the 5, 20, and 24 h groups, though the difference between 0 and 24 h is marginally nonsignificant ($P = 0.07$). There was a strong positive association between exposure time to rivals and a male's subsequent mating duration (post hoc linear contrasts $F_{1,13} = 18.42$, $P < 0.0001$: Figure 1). Hence, over the exposure times tested, the longer the exposure of males to other males, the longer the subsequent mating duration of those males, with males having the longest exposure mating for more than 2 min longer than males that had the shortest exposure to rivals.

Effect of the number and density of rivals on a male's subsequent mating duration

There was no effect on a male's mating duration of whether males were kept at constant or variable densities, but there was a significant effect of the number of rivals on a male's subsequent mating duration (ANOVA density treatment $F_{1,368} = 1.75$, $P = 0.187$; group size $F_{4,368} = 8.77$, $P < 0.0001$; interaction $F_{1,368} = 0.79$, $P = 0.533$: Figure 2). Post hoc tests showed that this was driven by the difference between group size 1 versus all other group sizes (all $P < 0.005$), with matings approximately 3 min longer when males were previously exposed to rivals, with no significant differences between the other group sizes. Hence, exposure to 1 rival significantly increased duration, but the addition of further rivals did not significantly increase (or decrease) duration.

We are confident that this result is not driven by males that are kept together acting in a more similar manner as the variance between the 1 male and 16 males treatments did not differ. Hence, it is unlikely that our main effects are vial effects rather than treatment effects.

Effect of window of exposure to rivals on a male's subsequent mating duration

There was no effect of the 2 h window of exposure of males to other males on their subsequent mating duration (Mean [± standard error] duration in minutes per treatment, where treatment name is the time before mating at which the 2 h time window of exposure was imposed; 0 h exposure 18.41[1.63], 2 h 16.55 [0.73], 24 h 16.50 [0.47], 48 h 16.21 [0.49], 72 h 16.31 [0.55], and 96 h 16.80 [0.73]. Kruskall–Wallis $\chi^2 = 0.49$, degrees of freedom [df] = 5, $P = 0.99$). Therefore, there was no evidence that there is a 2 h critical time window that initiates responses to rivals that take time to develop fully. Instead these data, combined with the results above, suggest that it is the absolute length of time of exposure to rivals that determines a male's subsequent mating duration.

Effect of male age at exposure to rivals

Males of all ages tested made the same facultative adjustment and significantly increased their mating duration after exposure to rivals. There was no significant interaction effect between a male's age and treatment; hence, a male's age did not affect his ability to lengthen mating duration after exposure to rival males (analysis of deviance; treatment deviance = 293.56, $\chi^2 = 46.06$, df = 2, $P < 0.0001$; age deviance = 294.42, $\chi^2 = 1.92$, df = 2, $P = 0.38$; treatment × age deviance = 248.09, $\chi^2 = 0.59$, df = 1, $P = 0.44$; Figure 3).

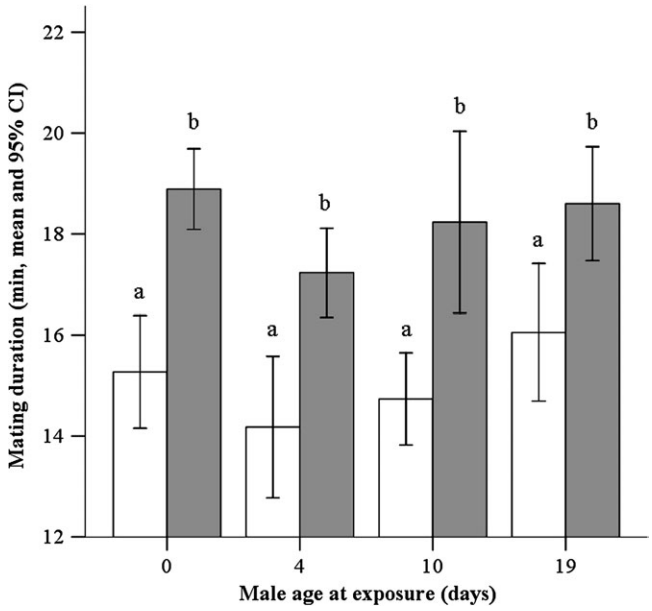

Figure 3
Mating duration (mean and 95% confidence interval) in response to exposure to male rivals of different ages. Males were kept singly (white bars) or exposed to 3 rivals (gray bars) at 4 different ages (0, 4, 10, and 19 days old). Bars identified with the same letter are not significantly different from each other; those with different letters are significantly different ($P < 0.0001$). Age at exposure had no effect on duration of mating; however, exposure to rivals at any age tested significantly increased mating duration.

DISCUSSION

Our results show that the length of exposure to rivals was critical in determining the subsequent mating duration responses by males to the potential level of sperm competition. We detected no significant difference in mating duration until males had been exposed to rivals for a period of 29 h. We found that a male's subsequent mating duration increased after longer exposures to rival males and that there was no effect of the time of day of exposure. Length of exposure to rivals was the most important determinant of a male's subsequent mating duration; increasing the number of rivals did not have an additive effect beyond 1 rival. There was also no detectable effect of encounter rates as the response to increasing numbers of males was similar regardless of whether we controlled for density or not. There was no evidence for a critical 2 h time window of exposure at any of the times we tested prior to mating, and male age was not important in determining the degree of the response to rival males.

There was no evidence that the younger a male was when exposed to rivals, the greater the increase in mating duration, or that the critical factor was the time since initial exposure, rather than absolute length of exposure. We explicitly tested these possibilities in our experiments and excluded them as important factors. Manipulation of male age at exposure to rivals also had no effect on the pattern of mating duration that we had previously observed; males across the age range we tested all responded in a similar way to 2 days of exposure to rivals, that is, by increasing their mating duration. Males exposed to rivals for 2 h at various times prior to mating made no adjustment in mating duration. This suggests there is instead a minimum exposure time for adaptive responses to occur. As noted above, the most important determinant of the response to rivals was absolute exposure time, and this was supported by our observation that any exposure of less than 24 h (and 2 h in the "time window" experiment) did not result in a significant adjustment in mating duration in the presence of rivals. The magnitude of male responses to rivals increased gradually with increasing time of exposure, and hence so did our ability to detect significant differences. Hence, if we had used larger sample sizes throughout we might have detected significant differences at earlier exposure times (especially as there was a positive relationship with increasing exposure time). However, increasing the sample sizes (and so possibly detecting significant differences at shorter exposure times) is not likely to increase the effect sizes or dilute the positive relationship between length of male–male exposure and subsequent mating duration. Overall, our results suggest that facultative adjustments to mating duration are costly because males do not react to transient signals of the potential level of sperm competition.

Density of flies had no effect on mating duration. It may be that the density at which we kept males was already high, and therefore, we were testing for differences between high and very high densities. However, the presence of more than one rival did not increase the magnitude of male responses to rivals, which further supports the conclusion that density of males (or encounter rate) is not an important cue, and this is in agreement with our previous study (Bretman et al. 2009). This finding also implies that density does not increase the strength of the signal that males receive, whether visual, tactile, or pheromonal. In terms of the cues that males use to detect the presence of other males, olfaction seems likely to be important (Kurtovic et al. 2007; Kent et al. 2008; Krupp et al. 2008), though visual and auditory cues also need to be explored. Clearly the identification of the cues by which males sense sperm competition levels is a valuable topic for future study.

Interestingly, we did not detect increased male responses to increased numbers of rivals above one, either by increasing or decreasing their investment in mating duration. This does not fit with existing models of sperm competition risk or intensity, which predicts increased investment (where "investment" is an arbitrary value which can reflect sperm number or ejaculate investment) with increased mean intensity but decreased investment with more than one rival (Parker et al. 1997; Engqvist and Reinhold 2005). This reinforces our previous assertion (Bretman et al. 2009) that existing theory does not adequately predict the pattern of male investment in systems such as *Drosophila*, in which there is nonlinear sperm transfer with mating duration, and strong second male precedence. Nevertheless, we have found that males show larger responses to increased exposure time, which suggests that the critical information for males about the potential level of sperm competition is the time spent with rivals and not the number of rivals present.

Our findings are important because they suggest that males cannot respond instantaneously to the presence of rivals by simply allocating more from their current pool of resources, because any shifts in mating duration that do occur in response to the current number of males in the mating arena do not result in significantly increased reproductive success (see Bretman et al. 2009). Rather, our data indicate that males allocate extra resources over time as they find themselves together with rivals. Over the exposure times we tested, we found no evidence for a leveling off in terms of a maximum mating duration. We suggest that males require time to increase the representation of component(s) of the ejaculate, in response to the presence of rivals. These increased components of the male ejaculate can then be transferred to females in higher quantities in longer matings. The relevant ejaculate components concerned could be sperm and/or seminal fluid proteins including Acps. Our data from another study show that at least 2 key Acps which affect egg production and female receptivity are transferred in higher quantities to females when males are maintained together with rival males (Wigby

et al. 2009). Further work is required to examine the full set of ejaculate components that can be modified in this way, and this work should also include tests of variation in sperm numbers transferred.

Our study demonstrates the importance, as recognized by Engqvist and Reinhold (2005), of specifying clearly what is being measured in investigations of sperm competition. In particular, these models make the distinction between "immediate" risk/intensity (at the time of mating) and "mean" risk/intensity (Parker et al. 1996, 1997; Engqvist and Reinhold 2005). We did not see a clear switch point in the length of male exposure experiments between males that responded to the presence of other males and those that did not, instead we found that mating duration increased gradually with exposure time. This suggests that the immediate level of sperm competition should only be used to describe processes that occur between rival males in the mating arena. As Engqvist and Reinhold (2005) point out, many studies employ an experimental design where immediate levels of sperm competition are tested by placing males together some time before mating (e.g., Gage and Barnard 1996; Pizzari et al. 2003). Our data show that variation in the period that males spend with rivals prior to mating can precipitate very different outcomes and should be taken into account in future studies.

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