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Mating success and copulation duration in *Drosophila melanogaster* flies having different mating experience: a brief experimental note

ABSTRACT

Mating behavior is one of the most complex behaviors in repertoire of fruit flies. In this paper, effects of previous mating experience of *Drosophila melanogaster* on their mating success and duration of copulation were investigated. Flies having different mating status were used in three mating assays. In the first mating assay, virgin females did not discriminate between virgin and recently mated males. In the second mating assay, males having prior mating experience three days before testing were more successful in mating than males mated two times during the same morning, when they were offered to experienced females. In the third mating assay, virgin females obtained significantly more copulations than experienced females. In all mating assays, duration of copulation did not differ between couples in which flies having different mating experiences took part.

Key words: *Drosophila melanogaster*, mating experience, mating success, copulation duration

Introduction

A large amount of information is available today showing the genetic determination for a wide range of behaviors in different *Drosophila* species. Mating behavior is considered as one of the most complex behaviors. Courtship sequences, mating latency, mating speed, duration of copulation, remating time and frequency, and other specific courtship/mating activities have been tested in both laboratory and field conditions and extensive analyses have been carried out using mutants, inbreed lines, different strains and species (Spieth, 1952; Gromko et al., 1984; Hall, 1994; Terzić et al., 1996; Yamamoto et al., 1997; Greenspan & Ferveur, 2000; Markow, 2002; Singh et al., 2002). Beside, many aspects related to sexual selection were investigated, including cryptic female choice (Eberhard, 1996), sperm size evolution (Joly et al., 2004), then genetic basis of sexual isolation (Takahashi & Ting, 2004). Further progress in chemical, molecular-genetic and neurobehavioral methods, as well as genomic research has given new insights in many aspects of *Drosophila* mating behavior (Jallon, 2007; Ellis & Carney, 2010; Ferveur & Cobb, 2010; Immonen & Ritchie, 2011; Fan et al., 2013).

Sexual selection in fruit flies was thoroughly studied over time (Markow, 2002; Singh et al., 2002; Snook et al., 2013). Numerous studies have shown that mating success may be influenced by many factors, including morphological characteristics (size, shape and symmetry of certain traits, Markow & Ricker, 1992; Vishalakshi, 2011; Menezes et al., 2013; Pavković-Lučić & Kekić, 2013; Snook et al., 2013; Trajković et al., 2013), physiological ability (qualitative and/or quantitative differences in the production and/or reception of different types of stimuli during courtship) and behavioral patterns (including various types of experiences). Environmental enrichment also may improve their mating success (Dukas & Mooers, 2003). Beside, prior mating experience, social experience with other flies, as well as adaptive role of learning in the context of sexual selection, have been the subject of many research (Mehren et al., 2004; Dukas, 2005, 2005a; Polejack & Tidon, 2007; Ellis & Carney, 2010). In *D. melanogaster*, it was found that flies of both sexes can learn in the context of courtship behavior and this learning is adaptive (Dukas, 2005a). Loyau et al. (2012) assumed that visual signals may contribute in a way that females avoid mating with males they just watched copulating.

Drosophila melanogaster, species under this study, has a multiple mating system, i. e. flies of both sexes mate repeatedly during their lives. However, remating time significantly varies between the sexes. Males mate more often, since multiple copulations were observed in the same day (Marinković et al., 1970), while females fall in infrequent remating category (Markow, 2002), and their remating time lasts about 7 days (Singh et al., 2002). Whether inseminated females carry amount of sperm sufficient for fertilization of hundreds of eggs, they remate through their reproductive lives, because they may have direct (non-genetic) and indirect (genetic) benefits of multiple mating (see for review Singh et al., 2002). Successive copulations in males may result in their temporary sterility (Markow et al., 1978). In females, mating results in a number of physiological and behavioral changes due to "seminal" and "sperm" effects (see for review Singh et al., 2002). Changes occur also in pheromonal profiles (Everaerts et al., 2010), as well as in hormonal status (Harshman et al., 1999).

Duration of copulation is one of behavioral traits often discussed in sexual selection literature. Mean duration of copulation significantly varies among *Drosophila* species having different reproductive strategies (behavioral tactics during courtship, remating time, remating frequency, different time of sexual maturation, Markow, 2002). In some species, the length of copulation is very short (Polejack & Tidon, 2007; Mazzi et al., 2009), while it may last for two hours, as in case of *D. acanthoptera* (Castrezana et al., 2013). In natural conditions, it may be expected that many factors promote brief copulation, whether, in some species, copulation is relatively long, which also may have some advantages (for review see Eberhard, 1996). Copulation duration is a trait also subjected to sexual conflict, as sexes may have different interests (Mazzi et al., 2009). In different *Drosophila* species, one sex or another, as well as their interactions, can make control over duration of copulation (Lefranc & Bundgaard, 2000; see for review Mazzi et al., 2009). In addition, in many species, significant difference in duration of copulation between first and successive female mating was observed (Singh & Singh, 2004; Pavković-Lučić & Kekić, 2006). In *D. melanogaster*, duration of copulation may be affected by prior mating experience (Singh & Singh, 2004; Pavković-Lučić & Kekić, 2006; Pavković-Lučić & Kekić, 2009) and by female body size (Lefranc & Bundgaard, 2000). Beside, longer copulation duration was recorded in males exposed to rivals during sexual maturation compared with males matured in isolation (Taylor et al., 2013). Recently, different neuronal populations controlling neural basis of copulation duration was also investigated (Crickmore & Vosshall, 2013).

Males may improve their mating success if they are more experienced, because of better courtship performing (Ellis & Carney, 2010). On the other hand, given that females invest more in producing gametes than males, it can be assumed that females must choose wisely among present males, especially during second and subsequent mating, if they have more offspring with the last mated male (see Gromko et al., 1984). Having this in mind, we performed three mating assays, using males and females with different mating status (mated vs. non-mated), wherein males varied in time elapsed since the last mating. Those flies were tested for mating success and copulation duration in relation to their previous mating experience. Since the age and mating status of flies, both of which are known to affect mating success, cannot be controlled in natural conditions, the present study was designed to partially simulate the social surrounding in natural populations.

Materials and Methods

Fly stock

Mating experiments were performed on 3-4 days old flies from laboratory population of *Drosophila melanogaster*, originated from natural population collected in the area of New Belgrade, Serbia (44° 49' 6" N / 20° 28' 5" E). Flies were maintained on standard cornmeal-sugar-agar-yeast medium, at 25 °C, relative humidity of about 60%, and 12h:12h = light : dark cycle. Flies used in mating experiments were collected as virgins and sorted according to sex every 4 – 5 hours after hatching, without using anesthesia.

Experimental design

Flies having different mating status (previously mated or not) were mated in three mating assays and scored for *mating success* (number of copulation achieved) and *copulation duration* (time from beginning to terminating mating). All experiments were set up during morning hours.

Experienced flies were obtained according to the following procedure. Virgin males and females were marked using UV dust 24 hours prior to the experiment. Fluorescent powder, as commonly used marking method in *Drosophila*, does not alter mating success (Terzić et al., 1994). In the "mating bottle", 20 virgin males and 20 virgin females (*per* replica) were placed. After copulation terminated, flies were separated. Mated (experienced) males (EM), as well as mated (experienced) females (EF), were placed in separate bottles containing food. Those flies were stored until they were tested again, according to the procedures described below.

Mating assay I

During the same morning, experienced males (EM) were tested in a presence of other virgin males (VM) and virgin females (VF), according to the procedure described below. Males were labeled with different fluorescent powder.

10 EM (mated during the same morning) + 10 VM + 20 VF

Mating assay II

Experimental group was formed using experienced males (EM) and females (EF), according to the following scheme. Designation given in the brackets indicates time after first mating. Males were labeled with different fluorescent powder.

10 EM (mated during the same morning) + 10 EM (3 days) + 20 EF (7 days)

Mating assay III

Experimental group was formed according to the following scheme, using virgin males (VM) and females (VF), as well as experienced males (EM) and females (EF), labeled with different fluorescent powder. For experienced flies, time after first mating is shown in the brackets.

10 VM + 10EM (3 days) + 10 VF + 10 EF (7 days)

Mating success and duration of copulation were scored in ten replicates *per* mating assay. Mating observation lasted 60 minutes *per* replica and 400 flies were tested *per* mating assay. When copulation began, every single pair was carefully removed and placed in 50 ml vials containing a small amount of substrate, where flies terminated copulation. Duration of copulation was recorded using a stopwatch. Later, labeled virgins or experienced flies were identified under UV light. Flies that did not mate within 1 hour of observation were treated as unsuccessful.

Statistical analyses

Mating success was tested using Chi-square test, calculated on the basis of 1:1 null hypothesis. Differences in number of homogamic *vs.* heterogamic matings in the third mating assay were tested also using Chi-square test. Copulation duration between different mating types was compared using t-test, in statistical software Statistica 5.0.

Results

In mating assay I, virgin and recently mated males (those mated during the same morning) were offered to virgin females. In this experimental group, 78.5% of possible copulations occurred. Virgin females obtained 53.5% of copulations with virgin males and 46.5% with experienced males. Virgin males and males previously mated during the same morning did not significantly differ in mating success (χ^2 = 0.771, df =1, P >0.05). Duration of copulation was compared between couples in which virgin and previously mated males took part. Copulation lasted between 19 - 20 min and did not significantly differ between virgin and experienced males (Table 1).

Table 1. *Copulation duration* (mean ± S.E., in min) *in mating assay I. Abbreviations: SM – males mated two times during the same morning.*

Type of males	Mean ± S.E.	t	df	P
Virgin males	20.139 ± 0.290	-1.738	151	0.084
Experienced males (SM)	19.352 ± 0.353			

In mating assay II, females having mating experience 7 days before testing were used. Out of possible two hundred mating, 53% of mating occurred. Out of 106 mating, experienced females achieved 35 copulations with males mated during the same morning and 71 with males mated 3 days before (Figure 1). Males mated 3 days before testing obtained significantly more copulations than those recently mated (χ^2 = 12.226, df =1, P<0.001). Mean duration of copulation compared between couples in which two male types took part did not significantly differ (Table 2).

In mating assay III, out of 200 possible copulations, 68.5% occurred. Virgin males and males experienced mating 3 days before testing did not significantly differ in number of achieved copulations (χ^2 = 1.233, df =1, P >0.05). However, virgin and experienced females significantly differed in number of copulations (χ^2=13.496, df =1, P <0.001) in which they took part (90 vs. 47, respectively), i. e. virgin females were more successful in mating (Figure 2). In this mating assay, mean duration of copulation also did not significantly differ in all possible mating combinations (P >0.05).

A significantly greater number of copulation (χ^2=7.015, df=1, P<0.01) was recorded between individuals with the same experience than between individuals having different mating experience. Virgin flies (both males and females) achieved significantly greater number of copulations with each other than with the individuals of the opposite sex having previous mating experience (Table 3).

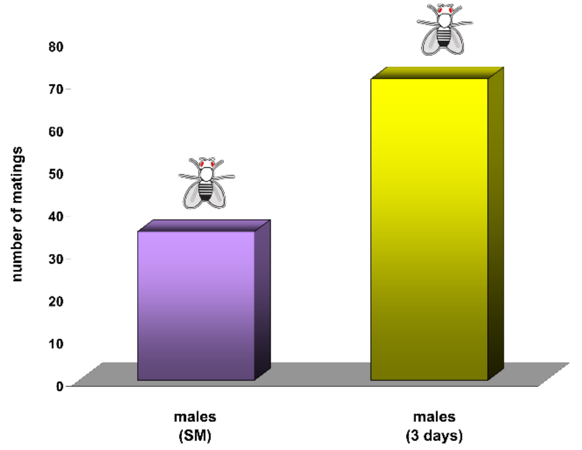

Figure 1. *Number of mating achieved by experienced males in mating assay II. Designation given in the brackets indicate time after first mating (SM – mated during the same morning; 3 days – mated 3 days before testing).*

Table 2. *Copulation duration* (mean ± S.E., in min) *in mating assay II. Abbreviation: SM – mated during the same morning; 3 days – mated 3 days before testing.*

Type of males	Mean ± S.E.	t	df	P
Experienced males (SM)	21.053 ± 0.610	-0.139	104	0.890
Experienced males (3 days)	21.139 ± 0.316			

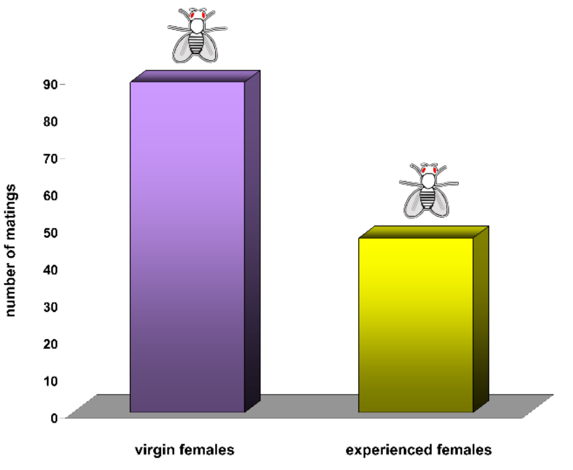

Figure 2. *Number of mating achieved by virgin and experienced females in mating assay III.*

Table 3. *Number of copulations achieved by individuals having different mating experience. Abbreviations: VF – virgin females; EF – experienced females; VM – virgin males; EM – experienced males.*

Type of comparing	χ^2value	df	P
Virgin males (mated with VF *vs.* EF)	18.253	1	<0.001
Experienced males (mated with VF *vs.* EF)	0.5806	1	>0.05
Virgin females (mated with VM *vs.* EM)	5.3778	1	<0.01
Experienced females (mated with VM *vs.* EM)	1.7234	1	>0.05

Discussion

Choosing a mate with high quality performances increases the chooser's fitness, and such selectivity should be favored by natural selection (Halliday, 1978). In *D. melanogaster*, female accepts or rejects male on the basis of different traits: previous mating experience may be one of them. Whether courtship behavior represents a sequence of fixed action patterns (Greenspan & Ferveur, 2000; Jallon, 2007), flies can learn from their prior mating experiences. Also, they may adjust their behavioral patterns in different environments, including social ones.

In this work, we tested mating success and copulation duration of flies having different mating experience, which were available as potential mates. This situation is considered

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to be common in natural conditions, since flies with different mating status usually feed, mate and lay eggs in the same feeding/breeding habitats.

According to the "sperm competition" hypothesis, sperm predominance (usually that of the last mated male) is the result of competition between the sperm of different origin in the female storage organs (Barbadilla et al., 1991). In *D. melanogaster*, the proportion of offspring sired by the last mated male is large, having been estimated over 80% (Gromko et al., 1984). Having this in mind, we assumed that females should be more selective in the second mating, as well as that males having different mating experience may differ in mating success. There is evidence that previous mating experience and exposure to other males and females can influence mate choice in *D. pseudoobscura* (Ehrman, 1990) and *D. paulistorum* (Kim et al., 1996). In *D. melanogaster*, virgins are more ready to mate with strangers than with previously encountered flies (Ödeen & Moray, 2008). Also, in this species, courtship is shorter in experienced males, due to reduced duration of courtship sequence designed as "following" (Stanić, 1995). Furthermore, mating may change different type of stimuli involved in subsequent courtship activities; the most literature data are available for changes in olfactory profiles (Everaerts et al., 2010).

According to results obtained in the first mating assay, virgin females did not make difference in accepting virgin and previously mated males as their mates. This result may be, at least partially, explained by the fact that 3-4 days old virgin females were highly sexually motivated at the time of testing. Also, previous copulation in recently mated males probably did not result in significant sperm depletion, since the temporary sterility in *D. melanogaster* occurs only as a result of multiple ejaculations (Markow et al., 1978). Since females can accept or reject males on the basis of different male characteristics (morphological, physiological, and behavioral), their choice may be also achieved by perceiving some other male traits, not studied here.

Contrary to virgin females, experienced females in the second mating assay made difference between recently mated males and males having mating experience 3 days before testing. They rejected recently mated males in most of the cases, probably because of recognizing some changes in their physiology and behavior (lower vigor or modification of courtship signals, for example). Experienced females were rather mated with males having prior mating experience 3 days before testing. Those males were highly sexually motivated, possible as much as virgins, and probably they were able to initiate courtship toward females more quickly than recently mated males. In such males, effects of former courtship learning may also be involved.

In the third mating assay, when flies having different mating status were placed together, virgin females obtained significantly more mating than experienced females. As noted above, virgin females were highly motivated for mating, while fertilized females may not be so. This result confirms some previous findings of lower receptivity of mated females (Singh et al., 2002; Pavković-Lučić & Kekić, 2009). According to the previously published data (Singh et al., 2002), prior mating in female is associated with numerous physiological and behavioral changes. Also, there is variability in time of restoring receptivity, so it is possible that some of our females were not receptive yet at the time of testing. Beside, as males decide to "whom to court" (Dickson, 2008), changes in female chemical cues after mating (involving those with anti-aphrodisiac properties) and visual cues may further reject males to court them (see Jallon, 2007 and references herein). In the same mating assay, we noticed that flies participated significantly more in homogamic over heterogamic mating, related to their mating status. The great number of copulations was recorded among virgin males and females, which was expected, since both sexes were highly sexually motivated. These results are consistent with findings obtained by Markow et al., 1978, when virgin males were highly preferred by virgin females, since they apparently recognized them as males with potentially larger amount of sperm available. Also, when comparing mating assays I and II, virgin females have achieved more copulations than experienced ones (~ 80% *vs.* 50%). Previously mated females also had longer copulation latencies, i. e. they need more time to evaluate males during the second mating (Pavković-Lučić & Kekić, 2009).

Previous mating experience did not influence length of copulation, since mean copulation duration did not differ within all mating assays. These results are opposite to our previous data, obtained in different strains and experimental designs, when naive females copulated significantly longer than previously mated females (Pavković-Lučić & Kekić, 2006; Pavković-Lučić & Kekić, 2009). Namely, in previous experiment, single female had opportunity to choose between only two males (Pavković-Lučić & Kekić, 2009). In such case, female has more time to evaluate the quality of

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available males, while, in higher density of flies, such possibility is quite reduced and choice is complicated by presence of many different males and with interactions among individuals of the same sex, as well. Also, in previous experiment, copulation duration was scored twice in the same females, while, in this experiment, it was measured in two groups of females which differed in terms of prior experience. Furthermore, testing of duration of copulation between repeated mating (mating with the same male) and remating (mating with the different male) were previously carried out, which was not the case in this experiment.

In experiment provided herein, virgin females were more ready to mate than experienced females. However, after females become receptive again, they may "redefine" their mate choice. The first and subsequent mate decisions may depend on many different factors, including female physiological and behavioral state, quality and quantity of available males, and local environment (physical, social) in which courting and mating occurs. In nature, flies are effectively exposed to the numerous and complex stimuli that are not clearly "separated" and defined, such that flies must change their behavior, giving the priority to the most relevant recent experience. Criteria used in mate choice may also vary throughout the female life cycle, especially if females have some direct and/or indirect benefits from mate selection.

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