Influence of mating histories and age on female remating behaviour in a few closely related species of *Drosophila nasuta* subgroup

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Female remating with more than one male leads to coexistence of sperm from different males in the same female, thus creating a selection pressure on sperm. To understand the extent of divergence in the reproductive behaviour among closely related species, in the present study, the influence of first mating histories like mating latency, duration of copulation and age of flies have been analysed on female remating behaviour in closely related *Drosophila nasuta* subgroup species with varying levels of reproductive isolation. The time taken for the once mated females to remate varied from 7 days in *D. s. sulfurigaster* to 19 days in *D. s. neonasuta* after first mating. The female remating frequency varied from a minimum of 29% in *D. s. neonasuta* to a maximum of 95% in *D. s. sulfurigaster*. The younger flies, which had remating latency of three times less than aged flies, show 100% remating frequency. In addition, it was observed that the duration of copulation in the first mating influences the remating behaviour among the *nasuta* subgroup members. The results revealed that *D. nasuta* subgroup members despite being closely related differ in their reproductive behaviour.

**Keywords:** Copulation duration, *Drosophila nasuta* subgroup, Female remating, Reproductive isolation, Sexual selection

During reproduction in insects, in a multiple mating scenario, successful copulation does not ensure paternity as competition among sperm from different males to fertilize the ovum continues even within the female reproductive tract. Hence, male has evolved various mechanisms to alter female’s physiology and behaviour to ensure his paternity. However, females counteract the influences of male leading to sexual conflict¹. In *Drosophila*, females that are once mated can store sperm in her storage organs namely, a pair of spermathecae and seminal vesicle and utilize them to fertilize her eggs. However, she often remates even in the presence of stored sperm in her reproductive tract²,³ creating a competitive milieu for sperm coming from different males. The female remating though ensures fertility, diversity and fitness of her offspring, it is of course disadvantageous for males as their sperm are at risk of displacement by sperm of other males. In this conflict between interests of males and females during reproduction, female remating acts as a key determinant of the pattern of sexual selection⁹ leading to the genetic heterogeneity and divergence in populations.

The time needed for female to be receptive after mating once varies from a few minutes to weeks among different species of *Drosophila*¹⁰. Further, the female remating is influenced by various factors such as density, availability of food and age of flies¹¹-¹⁷. Most of these findings come from the studies on *D. melanogaster* and a few other species, which are either pre-zygotically or post-zygotically isolated from each other. The *D. nasuta* subgroup comprises morphologically identical species and subspecies with varying levels of reproductive isolation among them. The subgroup has been an excellent model system for evolutionary biology studies including biochemical differentiation, chromosomal differentiation, incipient sexual isolation, sexual selection and genome introgression in hybrids¹⁸. However, the absence of knowledge on the pattern and importance of female remating in driving sexual selection among these closely related species having varied levels of reproductive isolation prompted to undertake the present study. It has been shown that even though the members of the subgroup are closely related and few among them share open genetic system, they differ

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from each other in their mating and remating behaviour and their mating and remating performances are influenced by the age of flies.

**Materials and Methods**

*Fly stocks*—Members of *D. nasuta* subgroup included in the present study namely *D. nasuta nasuta* (Coorg, India; Stock No. 201.001), *D. n. albomicans* (Okinawa, Japan; Stock No. 202.001) and *D. n. kepulauana* (Sarawak; Stock No. 203.001), from frontal sheen complex; *D. sulfurigaster sulfurigaster* (Queensland, Australia; Stock No. 205.001) and *D. s. neonasuta* (Mysore, India; Stock No. 206.001) from orbital sheen complex of the subgroup were obtained from Drosophila Stock Center, University of Mysore, Mysore, India. These stocks were maintained in a vivarium on standard wheat cream agar medium at 22°±1ºC, 70–80% RH and 12:12 h L:D cycle. Flies (50) of either sex in equal numbers were introduced into each culture bottle. These flies were transferred to fresh culture bottles once in every 3–4 days to maintain uniformity in population density and food conditions. From these cultures, virgin females and unmated males were collected by isolating males and females within 3 h of their eclosion. These flies were maintained in the vivarium to generate populations of two age groups namely, 3 days old (young) and 7 days old (aged) flies.

*Remating assay*—Following the periodic confinement method,8,9 pair mating was conducted in the culture vials (8 cm × 2 cm) at room temperature (22°–25°C) using 7 days old unmated males and virgin females. Mating experiments were performed during 0700–1100 h. Time required for initiation of copulation after introducing male and during 0700–1100 h. Time required for initiation of copulation using 7 days old unmated males and females mating for the second time. The duration of copulation during remating was also recorded as described above.

*Influence of age on remating behaviour*—To analyze the influence of age on regaining receptivity, similar remating experiments were conducted using 3 days old flies. Female remating was recorded for 15 days since most of the young flies remated within 15 days after mating once.

*Influence of mating histories on remating behaviour*—To analyze the influence of mating histories on remating behaviour, the sexual behaviour parameters such as mating latency and duration of copulation during first mating were correlated with remating latency and duration of copulation during remating.

*Statistical analysis*—The data obtained were subjected to one-way ANOVA followed by Tukey’s HSD test to determine the significance of differences in the parameters observed. The influence of age on female mating and remating behaviour was assessed by comparing the components of mating and remating behaviour observed between the two age groups using two-way ANOVA in which the interaction between species (factor 1) and age group of the flies (factor 2) was considered. To assess the differences in the percentage of female mated for first time (mating frequency) and for the second time (remating frequency)9 as well as to compare differences in mating and remating frequencies between the two age groups, the data were analyzed using Chi-square ($\chi^2$) test. To assess the influence of mating histories on female remating behaviour, Pearson correlation coefficient test was carried out. All the above statistical tests were carried out using SPSS software (Version 16.0).

**Results**

In the present study, the remating behaviour among 7 and 3 days old flies and the mating history traits such as mating latency, frequency of mating and copulation duration during first mating in both the age groups (Fig. 1) have been analyzed. To begin with, these parameters were compared between 5 species/subspecies within the age group to analyze the differences in the mating and remating behaviour. Subsequently, these results were compared between the two age groups to analyze the influence of age on the mating and remating behaviour (Fig. 2).

*Mating latency*—The mating latency ranged from a minimum of 23.52 min (*D. n. albomicans*)
to a maximum of 36.09 min (D. s. sulfurigaster) among 3 days old flies. In flies of 7 days age group, it ranged from 29.79 min (D. s. neonasuta) to 54.02 min (D. n. kepulauana). No significant difference in the mating latency of young (P = 0.122) and aged (P = 0.081) flies was observed in any of the species/subspecies analyzed in the present study. Hence, members of the subgroup show similar level of receptivity for mating in both age groups (Fig. 1A).

**Mating frequency**—The mating frequency ranged from 50.82% (D. s. sulfurigaster) to 87.72% (D. n. kepulauana) in young flies and they differed significantly (χ² = 20.031, df = 4, P = 0.0001). In aged flies, mating frequencies ranged from 46.51% (D. s. sulfurigaster) to 77.78% (D. s. neonasuta) and the difference was significant (χ² = 14.558, df = 4, P = 0.0001) (Fig. 1C).

**Copulation duration during first mating**—The copulation duration ranged from 17.58 min (D. s. sulfurigaster) to 30.44 min (D. n. kepulauana) in young flies, whereas in aged flies it ranged from

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**Fig. 1**—Components of female mating behaviour. D. n. n. - D. n. nasuta; D. n. a. - D. n. albomicans; D. n. k. - D. n. kepulauana; D. s. s. - D. s. sulfurigaster; D. s. ne.-D. s. neonasuta. (A) mating latency, (B) copulation duration during mating [(A) and (B) show values of mean ± SE] and (C) mating frequency (%). Species with different alphabet differ significantly at 0.05 level according to One-way ANOVA followed by Tukey’s HSD.

**Fig. 2**—Components of female remating behaviour. (A) remating latency, (B) copulation duration during remating [(A) and (B) show values of mean ± SE] and (C) remating frequency (%). Species with different alphabet differ significantly at 0.05 level according to One-way ANOVA followed by Tukey’s HSD.
It was observed that the copulation duration during first mating differs significantly among young \((P = 0.0001)\) and aged \((P = 0.0001)\) flies. Further, Tukey’s HSD test among the young \(D. n. nasuta\) and \(D. n. albomicans\) belonging to frontal sheen complex did not show significant variation in their copulation duration during first mating. However, \(D. n. kepulauana\) varies significantly from \(D. n. nasuta\) and \(D. n. albomicans\). Similarly, among young flies belonging to orbital sheen complex namely \(D. s. sulfurigaster\) and \(D. s. neonasuta\), the copulation duration did not differ significantly. However, significant differences were observed in the copulation duration between members of frontal and orbital sheen complexes.

Similar study on copulation duration in aged flies revealed that \(D. n. nasuta\) differs significantly from \(D. n. albomicans\) and \(D. n. kepulauana\) but not from \(D. s. sulfurigaster\) and \(D. s. neonasuta\) (Fig. 1B).

**Remating latency**—Remating latency in young flies ranged from 2 days (\(D. s. sulfurigaster\)) to 5 days (\(D. n. nasuta\)), whereas in aged flies it ranged from 7 days (\(D. s. sulfurigaster\)) to 19 days (\(D. s. neonasuta\)). Significant difference was observed in the female remating latency among young \((P = 0.0001)\) and aged flies \((P = 0.0001)\). Young \(D. n. nasuta\) flies differed significantly only from those of \(D. n. kepulauana\) and \(D. s. sulfurigaster\), whereas \(D. s. sulfurigaster\) differed from all the other members except \(D. n. kepulauana\). Further, among aged flies \(D. n. albomicans\), \(D. n. kepulauana\) and \(D. s. sulfurigaster\) differed significantly from \(D. n. nasuta\) and \(D. s. neonasuta\). However, \(D. n. nasuta\) and \(D. s. neonasuta\) did not differ significantly from each other (Fig. 2A).

**Remating frequency**—Young females of all the species/subspecies under study showed 100% remating frequency and hence did not differ in their remating frequency \((\chi^2 = 0.000, \text{df} = 4)\) (Fig. 2C). In \(D. n. nasuta\), 16% of females remated at 48 h after mating once and the remaining females remated within 10 days after first mating. Similarly, 40% of \(D. n. albomicans\) and 54% of \(D. n. kepulauana\) females remated at 24–48 h after first mating and the remaining females remated within 9 and 8 days after first mating, respectively. In case of \(D. s. sulfurigaster\), 70% of the mated females became receptive for remating in 24–48 h after first mating whereas 31.25% of \(D. s. neonasuta\) were receptive after 48 h of mating and remaining females remated within 12 days after the first mating (Fig. 3A). The remating frequency of aged females ranged from 29% in \(D. s. neonasuta\) to 95% in \(D. s. sulfurigaster\) and they differed significantly in their remating frequency \((\chi^2 = 55.706, \text{df} = 4, P = 0.0001)\). In the aged category, majority of \(D. n. nasuta\) females remained non-receptive until 11th day after first mating and by 20th day 83.7% females remated and the rest remated within 23 days after first mating. Interestingly, 13.3% of \(D. n. albomicans\) females were receptive 24 h after mating, whereas 60% of them remated within 6 days after mating and the remaining females remated within 22 days after mating once. In \(D. n. kepulauana\), 8.1% females were receptive at 24 h after mating, however, 59.4% of them remated in 6–14 days after first mating and rest of them remated within 19 days after first mating. In case of \(D. s. sulfurigaster\), 8.1% of females became receptive to remating in 3 days whereas
peak remating frequency of 35.1% was observed on the 6th day after the first mating and remaining females mated within 17 days after first mating. D. s. neonasuta females were receptive for remating only after 15 days after mating once. While 50% of them remated on 19th day after first mating and remaining females remated within 22 days after mating once. Figures 3A and 3B show the cumulative percentage of female remating on successive days after mating once. Figures 3A and 3B show the cumulative percentage of female remating on successive days after mating once in young and aged groups, respectively, that reflects the pattern of regain of receptivity and the time required for the same.

Influence of age on female remating—Influence of age on the female remating was assessed by comparing the components of the mating and remating in young and aged flies. Members of D. nasuta subgroup showed similar levels of receptivity for mating in both the age groups (P = 0.124). However, significant differences were observed in the mating frequencies between the age groups (\(\chi^2 = 3.980, \text{df} = 4, P = 0.046\)). Similarly, significant influence of interaction was observed between species and the age on duration of copulation during the first mating (P = 0.0001). Young flies copulate longer during first mating and remating when compared to aged flies. Significant interaction was observed between species and the age on remating latency (P = 0.0001). As the age advances, remating latency increases by three folds. Both the age group flies differed in their remating frequency (\(\chi^2 = 89.383, \text{df} = 4, P = 0.0001\)). However, there was no effect of interaction between species and age on duration of copulation during remating (P = 0.082).

Discussion

During mating, males compete for their mates and females always choose a better mate for mating. However, mating does not ensure paternity of a male as females can store sperm from different males and use them differentially for fertilization. In this scenario of reproduction, male has evolved strategies to monopolize his mates so that he can sire more number of offspring. However, females counteract to fast evolving male traits to increase the fitness of her offspring by means of remating with more than one male even in the presence of sperm load in her

| Table 1—Correlations between various mating and remating parameters |
|------------------|------------------|------------------|------------------|------------------|
|                  | Young flies      |                  | Aged flies       |                  |
|                  | Pearson Correlation | Significance | Pearson Correlation | Significance |
| First copulation duration Vs remating latency | -0.153 | 0.025∗ | -0.199 | 0.032∗ |
| First copulation duration Vs remating copulation duration | 0.354 | 0.0001∗∗ | 0.152 | 0.103 |
| Remating latency Vs remating copulation duration | -0.095 | 0.168 | 0.062 | 0.505 |

*Significant at 0.05 level; **significant at 0.01 level
storage organs. This act of female increases the heterogeneity of a population and also facilitates selection of sperm from different males. These sexual selections act as driver of reproductive isolation.

Although there have been preliminary investigations on female remating in a few other species such as *D. pseudoobscura*, *D. ananassae*, *D. pavani* and *D. gaucha*, bulk of the information on female remating in *Drosophila* comes from the experiments involving *D. melanogaster*. It is necessary to extend such a study to taxonomically closely related species with varying levels of reproductive isolation to understand the role of sexual selection among various species of *Drosophila* and to get an overview of male and female contributions to the process of raciation/speciation during evolution. In this context, the *D. nasuta* subgroup of *immigrans* group serves as an ideal system as it comprises a cluster of siblings that have varying levels of reproductive isolation. Hence, present study on *D. nasuta* subgroup was undertaken to dissect out the extent of sexual selection by analyzing female remating behaviour in order to understand the pattern and process of reproductive differentiation due to female remating. Among *D. nasuta* subgroup members employed for the present study, *D. n. nasuta* and *D. n. albomicans* belong to frontal sheen complex of the subgroup but the former two have an open genetic system. *D. n. kepulauana* is reproductively isolated from *D. n. albomicans* but shows cross-fertility with *D. n. nasuta* to a certain extent. *D. s. sulfurigaster* and *D. s. neonasuta* of orbital sheen complex are reciprocally inter-crossable but their hybrids are sterile.

The remating behavioural studies in various species of *Drosophila* have shown that after first mating, the time required for female to be receptive for remating and the frequency of remating varies. *D. mercatorum* females readily accept males in 3 min after first mating and have 82% remating frequency. Interestingly, *D. buzzatii* females remate in 4 h after the first mating and their remating frequency is about 90%. In *D. melanogaster*, the mated female is reluctant to accept males until 1 week, after which more than 80% of the once mated females remate. Similarly *D. ananassae* females show remating latency of 7.17 days but only 39.4% remating frequency. *D. biarmipes* females show receptivity for remating in 10 days after first mating with very low frequency of only 26%. These studies suggest that there is a huge variation in the remating time and the remating frequency across *Drosophila* species. In the present study, *D. s. sulfurigaster* remating pattern was similar to that of *D. melanogaster*: Females of *D. s. sulfurigaster* were receptive within 7.16 days after first mating and have shown a very high remating frequency of 95%. Similarly females of *D. n. kepulauana* show 9.08 days of remating latency with 93% remating frequency. Whereas females of *D. n. albomicans* show remating latency of 8.8 days but their remating frequency is only 50%. *D. n. nasuta* and *D. s. neonasuta* females show very high remating latency of 16.73 and 19.50 days, respectively, after first mating their remating frequencies were 45 and 29%, respectively. Thus, similar to the trend observed in Drosophilids mentioned above, differences exist with respect to remating latency as well as remating frequency even within the *nasuta* subgroup of *Drosophila*. The *D. nasuta* subgroup members differ among themselves despite being closely related and even though a few among them share open genetic system. Hence, like any other sexual traits in different species of *Drosophila*, the female remating behaviour in *D. nasuta* subgroup also shows high divergence.

The female remating behaviour is influenced by various factors such as sperm load received during first mating, the quantity of seminal proteins received during mating, size of the mating plug, the nutritional status of female, density of population and the age of flies. In the present study, the influence of nutritional status of female and density of population can be ruled out as all males and females employed for the experiments are obtained from the populations that were raised under uniform conditions of temperature, humidity, nutritional status and population density. The previous mating histories like successful copulation during first mating, which is an indirect measure of optimum amount of sperm and seminal fluid received, influences the receptivity of the female for further matings. However, studies showing the influence of mating histories on female remating behaviour have been scanty. Hence, a few components of mating behaviour such as mating latency and duration of copulation during first mating were analysed to check their influence on remating. Also the influence of age on mating and remating behaviour among the members of the *D. nasuta* subgroup was analyzed. The age of flies to attain maximum sexual maturity varies in different species.
of *Drosophila*. Here, to investigate the influence of age on mating and remating behaviour, two age groups of flies that are 3 and 7 days old were selected. Perusal of literature revealed that in most mating and sperm competition experiments involving *Drosophila*, 3-5 day old flies have been employed. In view of this, 3 days old flies were used and considered them as young to analyze and compare the age related differences in reproductive behaviour. In *D. nasuta* subgroup species/subspecies, males that are 7 days old have maximum quantity of accessory gland proteins and hence have chosen 7 days old flies and considered them as aged flies. Such an analysis revealed that the members of the subgroup although show similar level of latency for first mating, they differ significantly in their mating frequency. Young flies show shorter mating latency with high level of mating frequency compared to aged flies.

The duration of copulation is a trait subjected to high degree of sexual conflict and in *Drosophila* it always exceeds the time required to transfer sperm. This excess time serves as mate guarding mechanism to reduce the likelihood of female remating. The duration of copulation varies from few seconds in *D. acanthoptera* to an hour in *D. enigma*. Van-Vianen and Bijlsma have shown that 1-2 days old *D. melanogaster* flies are reluctant for both mating and remating compared with those of 4-6 days old. The present study revealed that the copulation duration during both first mating and remating vary significantly among members of the *D. nasuta* subgroup wherein young flies show longer copulation duration than the aged flies. Also, the young flies show 100% remating frequency with much shorter remating latency (about three folds less).

To understand the influence of mating history on the remating behaviour, the correlation, if any, between the copulation duration during first mating and the remating latency was analyzed. The observed negative correlation between duration of copulation during first mating and remating latency in both the age group of flies is in contrast to prevalence of positive correlation between copulation duration during first mating and remating latency in *D. montana*. In the present study, it was observed that irrespective of the species/subspecies, the young females show similar remating frequency. However, aged flies differ significantly in their remating frequency. It is also evident that the copulation duration during second mating is shorter than the first in both the age groups and this is in agreement with earlier observation in various species of *Drosophila*. Further, there is a significant positive correlation between first and second copulation duration in young flies; however, no such significant correlation in aged flies exist. Furthermore, there is no influence of remating latency on second copulation duration in both the age groups.

Present study has enabled us to get an insight into the pattern and extent of differences in the mating and remating behaviour and hence the extent of differentiation and sexual selection among the cluster of sibling species analyzed.

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