Evidence of male mate choice for female age in *Drosophila nasuta*

Vijay Kumar B. K., M. S. Krishna*

Drosophila Stock Center, Department of Studies in Zoology, University of Mysore, Manasagangotri, Mysore –560006, Karnataka, India

Email address:

vijaym.phil2013@gmail.com (Vijay K. B K) drosokrish@gmail.com (M. S. Krishna)

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Abstract: In the present study experimental out bred population of *Drosophila nasuta* used, has been originated from progenies of 50 isolated female lines collected at Chamundi hill at Mysore, India. It was found that males of *D. nasuta* discriminated females on the basis of their age and prefers to mate more frequently with younger of the two competing females. Although middle aged females mated faster and copulated longer compared to young and old aged females. Males showed grater courtship activities such as tapping, scissoring, vibration, liking to middle aged female compared to young or old aged females. Middle aged females showed least rejection responses such as ignoring, extruding, and decamping to courting male than young or old aged females. Young aged females laid significantly greater number of eggs and produced more progenies than middle and old aged females. Further young aged females had greater number of ovarioles than those of middle and old aged females. Thus these studies in *D. nasuta* suggest that female age has significant effect on male mate preference.

Keywords: *Drosophila nasuta*, Female Age, Male Mate Preference, Ovariole Number, Wing Length

1. Introduction

Studies on sexual selection have largely concentrated on females, as they are often the selective sex that chooses from among males [1]. However when compared to female, mate choice is less common in males, being reported in only 58 insect species, distributed among 11 orders and 37 families [2]. There are now growing numbers of observations of male choice seen in a wide range of Taxes including insects, birds, and fish [1-5]. Those studies suggest that male mate choice is predicted to be adaptive when variance in female fitness is large and males experience costs of mating such that they cannot inseminate all females encountered [6-10].

Experimental evidences for occurrence of male mate choice are also available in many species because, males have a high cost of reproduction (mating) due to costs arising from factors such as energetically expensive courtship displays [11] and the productions of ejaculates [12-13]. If the cost of mating is low for males, in the currency of time lost and/or resources consumed, then low levels of male mate discrimination are predicted [14-15]. Individuals that are selective concerning mating partners

are likely to lose mating opportunities and energy in searching for more-attractive suitors [2]. For this behavior to be adaptive, these individuals have to benefit one way or another. On the one hand, benefits may come in the form of direct enhancement of survival or fecundity [6], as selection favors mating preferences towards mates that are more fertile [16], provide superior resources, offer more parental care or otherwise help to reduce reproductive costs. On the other hand, selective mating may be adaptive as a consequence of indirect benefits [17] and offspring may inherit genes that promote their survival or reproduction [18]. Specifically, mate choice may lead to production of offspring with genotypes that increase viability or that makes them more attractive to members of the opposite sex.

However, the empirical evidence suggest that limited Characters were used by males to select females are generally virginity, and her size, age, and gravid status [2]. The most-obvious character influencing the reproductive value of a female is her fecundity [2]. When mating opportunities are constrained, males that show a preference for more-fecund females will directly benefit by increasing the number of offspring they produce [19]. The most-compelling studies of male choice suggest that female mating success is often associated with traits that are

correlated with female fecundity [2-3], while in others, mate assessment may occur through display traits such as coloration, pheromones, or ornamental and morphological features [20-22]. However, models of the evolution of male choice suggest that male choice tends to break down when males target arbitrary female traits rather than those that reliably signal fecundity [15, 23-24]. Therefore, male preference for female traits may be an indirect way of assessing female fecundity. Thus, there is a need to understand how male choice is related to such traits. The fruit fly provides unique opportunities to test the predictions of male mate choice models. The status of *Drosophila* as one of the most important model genetic organism of the early 21st century is a consequence of the vision of early researchers who chose the fruit fly to study courtship behavior. Those early evolutionary biologists could easily maintain *Drosophila* in the laboratory, and they also recognized that the elaborate and complex courtship display was an ideal system to study aspects of sexual selection, i.e., to test predictions of male mate choice. Hence present study has been undertaken in *D. nasuta* sp. belongs to the *nasuta* subgroup of the *immigrains* species group of *Drosophila* to understand whether or not males of *D. nasuta* discriminate female on the basis of female age. If so, what is the adaptive significance of male mate choice on one hand and female age effect on female reproductive performance on the other hand in *D. nasuta*.

2. Materials and Methods

In the present study experimental stock of *D. nasuta* was originated from progenies of 50 naturally inseminated isofemale lines collected at domestic localities of Mysore, Karnataka, India was used. Progeny produced by each of the above fifty isofemale lines were mixed together and redistributed to 20 different culture bottles. In each generation 40 flies (20 males and 20 females) were transferred per quarter pint milk bottles (250 ml) containing wheat cream agar medium and were maintained them at $21 \pm 1^\circ\text{C}$ at a relative humidity of 70% using a 12:12 hour light: dark cycle. This procedure was continued for 3 generations to allow flies to acclimatize to the laboratory. At the 4th generation, synchronized eggs (± 30 min) were collected from parental stocks (5-6 d old) using Delcourt's procedure [25]. Eggs (100) were seeded in a vial containing wheat cream agar medium. When adults emerged, virgin females and unmated males were isolated within 3 hour of eclosion and were aspirated into a new vial containing wheat cream agar medium. These flies were aged as required for the experiment.

In order to provide uniform environmental conditions for females of different age classes, female flies collected first were assigned to 32-33 d (older); females flies collected next were assigned to 17-18 d (middle-aged). Following this, female flies collected were assigned to 2-3 d (younger). Before beginning the experiment, developmental times of flies collected at different times were also tested, and the

results indicated no significant variations. These females of different age classes were kept in groups of 4 flies each in culture vials containing wheat cream agar medium and were transferred to a new vial containing wheat cream agar medium once a week until they were used in the experiment. Male flies were aged for 5-6 d. These flies were also maintained using the above-described laboratory conditions until they were used in the experiment.

2.1. Male Mate Choice Experiment for Female Age Classes

To study female age influence on male mating choice, 2 females (younger vs. middle-aged, younger vs. older, or middle-aged vs. older) and a 5-6 d-old male were aspirated into an Elens-Wattiaux mating chamber [26] Indian ink was painted on the thorax of one of the females. The effect of paint was tested before commencing the experiment by painting young flies in 1 trial and middle-aged/older females in an alternate trial and allowing them to mate. Each pair was observed for 1 hr. When mating occurred, the copulating pair was aspirated out of the mating chamber and placed in a new vial containing wheat cream agar medium. In total, 50 trials were conducted for each combination of female ages. A Chi-square analysis was carried out to examine male mate choice data.

2.2. Female Age Effect on Courtship Activities and Fecundity

The influence of female age on mating activities and female fecundity one male with a female (younger, middle-aged, or older) were aspirated into an Elens-Wattiaux mating chamber [26] and observed for 1 h. Mating latency (time between introduction of the male and female together in the mating chamber and initiation of copulation by the pair) and copulation duration (time between initiation and termination of copulation by the pair) were recorded. We also quantified courtship acts such as tapping, scissoring, vibrating, licking, circling, ignoring, extruding, and decamping following the procedures of [27]. During tapping, the male initiates courtship with a foreleg motion, partially extends and simultaneously elevates 1 or both forelegs, and then strikes downward; thus, bringing the ventral surface of the tarsus into contact with the partner. During scissoring, a courting male, during the interval between wing vibrations, sometimes opens and closes both wings with a scissor-like movement. During vibrating, the wing movement of males involves expanding 1 wing laterally from a resting position and then rapidly moving 1 or both wings up and down. During licking, a courting male positions himself close behind the female, extends his proboscis, and licks her genitalia. During circling, the male, after posturing at the side or rear of a non-receptive female, faces her as he moves. Sometimes, he moves to face her and then retraces his path to the rear, while at other times, he moves completely about her in a full circle. During ignoring, a non-receptive female when courted sometimes

simply continues with whatever activity she is engaged in and obviously ignores the male's actions. During extruding, a non-receptive female presses her vaginal plates together, contracts certain of the abdominal muscles, and apparently relaxes other muscles. During decamping, a non-receptive female often attempts to escape by running, jumping, or flying away from the courting male. The behaviors of the male and female were simultaneously recorded but separately by 2 observers for 1 hour; the number of pairs that mated was also recorded. Soon after mating, the mated female was transferred to an individual fresh vial containing wheat cream agar medium and evaluated once every 24 hour to study fecundity, and this continued for 16 d. The total number of eggs laid by each female was recorded. In total, 50 trials were carried out for each of the 3 female age classes. One-way analysis of variance (ANOVA) followed by Tukey's honest post-hoc test was carried out on mating latency, courtship activities, copulation duration, and fecundity data using SPSS 10.1 software (SPSS, Chicago, IL, USA).

2.3. Female Age Effect on Ovarioles Number and Wing Length

The influence of female age on the number of ovarioles and wing length, Virgin young aged, middle-aged, and old aged females were individually sacrificed to count the number of ovarioles and determine the female wing length following the procedures of [27]. To count the number of ovarioles, each female was dissected in a drop of physiological saline using a binocular stereomicroscope; ovarioles of the left ovary were separated from one another with the help of fine needles. The number of ovarioles in each female was counted. From the same female, wing length was also measured using a 100x [microscope] following the procedures of [27]. The mean ovariole number and female wing length data were also subjected to one-way ANOVA followed by Tukey's honest post-hoc test. In total, 50 trials were separately conducted for each of the 3 female age classes.

3. Results

Table 1 shows that in 27 of 50 trials, middle-aged females mated, and in the remaining 23 of 50 trials older females mated ($\chi^2 = 0.32$; d.f. = 1; $p > 0.05$). Chi-square carried out showed insignificant differences suggesting that painting did not influence on the performance of the flies. Males generally prefer to mate with younger of the two females provided in the mating chamber (Table 1). Young-aged female success in crosses involving younger and middle-aged females was 40 (n = 50); young-aged female success in crosses involving young and old-aged females was 30 (n = 50). Middle aged female success in crosses involving middle and older females was 30 (n = 50). Chi-square carried out on above data showed significant variation in crosses involving young and middle aged females.

Time taken to initiate the copulation (mating latency) of males mated to females of different age classes is provided in figure 1. Mating latency data subjected to One-way ANOVA followed by Tukey's honest post-hoc test showed significant variations in the mean time taken to initiate copulation among males mated to females of different age classes. Tukey's honest post-hoc test showed that the mean mating latency of middle-aged females was significantly less than those of younger and older females. However, the time taken to initiate copulation of older females was found to be insignificantly greater than younger females.

Figure 2 shows courtship activities data of male mated with young, middle and old aged females. It was noticed that males showed of significantly greater courtship activities such as tapping, scissoring, vibrating, circling, and licking of middle-aged females compared to young aged and old aged females. On the other hand, middle-aged females showed fewer rejection responses toward males such as ignoring, extruding, and decamping than younger and older females. Mean data of courtship activities subjected to One-way ANOVA followed by Tukey's honest post-hoc test showed significant variations in courtship activities (Tapping, scissoring, vibrating, circling, and licking) among different female age classes. (Fig.2)

Copulation duration of males mated to females of different age classes are provided in figure 3. Longest duration of copulation was found in male mating with middle aged female while shortest duration of copulation was noticed in male mated with old female One-way ANOVA followed by Tukey's honest post-hoc test carried out on mean duration of copulation showed significant variations in copulation duration among females of different age classes. (Fig.3)

Mean data of eggs laid by male mated to young, middle and old aged females is provided in figure 4, It was noted that young-aged females had the highest mean fecundity and fertility while older females had the least mean fecundity and fertility. One-way ANOVA followed by Tukey's honest post-hoc test showed significant differences in mean fecundity among females of different age classes; Table Tukey's honest post-hoc test showed that young-aged females had significantly greater fecundity compared to middle and old aged females.

Figure 5 shows the mean number of ovarioles of young aged, middle-aged and old aged females of *D. nasuta*. The highest mean number of ovarioles was found in young-aged female, while the least mean number of ovarioles was found in older females. One-way ANOVA followed by Tukey's honest post-hoc test carried on mean ovarioles data showed significant variations in the mean numbers of ovarioles among females of different age classes. Table Tukey's honest post-hoc test also showed that young-aged females had a significantly greater mean number of ovarioles compared to middle and old aged females. The mean female wing lengths of younger, middle-aged, and older females of *D. nasuta* are given in figure 6. It was noted that differences in mean female wing lengths of

different female age classes were found to be insignificant.

Fig. 1. Female age effect on the mating latency of *D. nasuta*. [Different letters on bar graph indicate a significant variation by Tukey's honest post hoc test]. $F=21.11$; $d.f=2, 147$; $P<0.001$

Tapping $F=11.480$; $d.f=2,147$; $P<0.001$. **Scissoring** $F=3.500$; $d.f=2, 147$; $P<0.05$. **wingvibrate** $F=2.254$; $d.f=2,147$; $p>0.05$. **Circling** $F=1.211$; $d.f=2,147$; $p>0.05$. **Licking** $F=18.92$; $d.f=2, 147$; $P<0.001$. **Decamping** $F=17.31$; $d.f=2, 147$; $P<0.001$. **Extruding** $F=7.66$; $d.f=2,147$; $P<0.001$. **Ignoring** $F=18.33$; $d.f=2,147$; $P<0.001$

Fig. 2. The influence of female age on mating activities of *Drosophila nasuta*. [Different letters on bar graph indicate a significant variation by Tukey's honest post hoc test].

Fig. 3. Female age effect on the duration of copulation of *D. nasuta*. [Different letters on bar graph indicate a significant variation by Tukey's honest post hoc test]. $F=86.11$; $d.f=2,147$; $P<0.001$

Fig. 4. Female age effect on Eggs/Progeny production of *D. nasuta*. [Different letters on bar graph indicate a significant variation by Tukey's honest post hoc test]. $F=63.153$; $d.f=2$; $p<0.001$

Fig. 5. Female age effect on ovariole numbers of *Drosophila nasuta*. [Different letters on bar graph indicate a significant variation by Tukey's honest post hoc test]. $F=3.99147$; $d.f=2,147$; $P<0.05$

Fig. 6. Female age effect on wing length (mm) of *D. nasuta*. [The same letters on bar graph indicates an insignificant variation by Tukey's honest post hoc test]. $F=1.48$; $d.f=2$; $P>0.05$

4. Discussion

Table 1 reveals that males of *D. nasuta* prefer to mate with younger of the two females in trials of male choice experiment. This suggests that existence of male mate choice for female age classes in *D. nasuta*. Our result supports the work of *D. ananassae* [28]. They also found that males of these species discriminated females on the

basis of their age and males prefer to mate with middle aged females over young and old females. Thus these studies in species of *Drosophila* suggest that males do exercise choice in selecting females. This shows that like other insects, males of *Drosophila* also have high cost of reproduction due to cost arising factors such as energetically expensive courtship displays, the production of ejaculates etc [2]. It is assumed that males of *Drosophila* do exercise mate choice because, 1) Reproductive success of males of *Drosophila* is limited by more factors than simply the number of females mated, and 2) females also differ in quality, i.e., fecundity. These 2 criteria need to be in place for male *Drosophila* mate selectivity to be evolutionarily advantageous [29]. Thus, in species of

Drosophila it appears that female age is an important determinant of male mate choice. In other words, males of *D. nasuta* do not show the same levels of interest in females of different age classes they encounter. This confirms earlier studies of the existence of male mate choice for females in other insects [2]. Even in *D. melanogaster* in studies of male mate choice was also found for female characters [30] have also pointed out that males of *Drosophila* do not show the same level of interest in all females they encounter, and provided evidence that males have also evolved to selectively mate. Therefore, these studies suggest that in species of *Drosophila* male preference for female traits may be an indirect way of assessing female fitness.

Table 1. Female age effect on mating success in *D. nasuta*

Male					Female							
5-6 days	Young	Older	χ^2 value	p value	Young	Middle	χ^2 value	p value	Old	Middle	χ^2 value	p value
No	30	20	2	>0.05	40	10	18	<0.0001	20	30	2	>0.05
%	60%	40%			80%	20%			40%	60%		

It was assumed that in *Drosophila* studies that females control mating success, i.e., whether mating takes place and with whom [31]. In contrast to male mate choice, in female mate choice not only involves the female preference for males but also involves male-male competition [27, 16-17]. Those studies suggested that male-male competition can reinforce female mate preference rather than operating in an antagonistic fashion [32]. Even with male mate choice, it is difficult to separate among male choice, female-female competition, and differences in female motivations to mate [3].

Studies on mating behavior in *Drosophila* suggest that male mating success not only depends on male preferences, but successful copulation also requires female receptivity [31]. In *Drosophila*, sexually mature females show a range of behaviors to thwart unwanted advances of courting males, such as decamping, wing flicking, kicking, and ovipositor extrusion [31], while immature females do not show such rejection behaviors. This is because newly emerged (immature) females do not perform any of these rejection behaviors in *Drosophila melanogaster* (Manning, 1961). In *D. nasuta*, it was noted that females were unreceptive on the day of its eclosion (up to 36 h) thereafter, females were receptive, and they were found to be receptive for up to 32 d. This supports the work of [34] who while working on *Drosophila*, also found that most females are unreceptive on the day of eclosion. They are highly receptive in between 2 and 20 d, after which there is a general decline in receptivity. Those studies suggest the existence of species-species differences in female receptivity. In the present study, the different age classes used were all virgins and were found to be receptive. Therefore male preference for middle-aged females could not be accounted for differences in female life history.

Another important factor that known to affect male mate choice was the rearing of females of different age classes in groups before they were exposed to males is an important

factor that accounts for the experience of female interactions on female mating success [35]. It was noted that in the present study, females were kept individually separately for each of the 3 female age classes. Therefore, the observed greater mating success of young or middle-aged females in our study is unlikely due to females being kept with other females longer; instead, it was an effect of age. Thus, these studies on *D. nasuta* suggest that there are many other potential cues that males may use when making a choice between females of different age classes.

In species of *Drosophila* successful mating depends on male activity and female receptivity [33 and 36] In *Drosophila*, mating latency to courtship or copulation is a good estimate of sexual receptivity of females and sexual activity of males [37]. During this period, the male fly performs various courtship acts such as foreleg tapping, wing scissoring, wing vibrating, and circling to increase the receptivity of females [36 and 27]. Figure 1 reveals that middle-aged females took a shorter time to initiate copulation compared to young aged and old aged females, suggesting an influence of female age on mating latency. As speed is the reverse of time, flies which took more time were slow mated while those which took less time were fast mated. This suggests that in *D. nasuta*, middle-aged females were fast mated while older females were slow mated. This supports effect of age on mating activities as reported in *D. pseudoobscura* [38]. Since female receptivity is also related to the time of initiation of copulation, middle-aged females were more receptive than younger and older females of *D. nasuta*.

Our studies in *D. nasuta* also support the result of female age effect on mating latency in *D. ananassae*[28]. In the present study in order to understand the effect of female age on courtship activities during mating, both male courtship activities such as tapping, scissoring, vibrating, circling, liking and female courtship activity such as ignoring, decamping, and extruding of males mated with

different female age classes were recorded (Figure 2). It was noted that males of *D. nasuta* showed greater courtship activities toward middle-aged females compared to young and old aged females, suggesting an influence of female age on courtship activities in *D. nasuta*. This conforms earlier studies of male age, size female age and environmental influence on courtship activities in different species of drosophila [31, 36, 39]. Through these courtship activities, males of *Drosophila* better convey chemical, auditory, and visual signals to middle-aged females more quickly and try to convince the middle-aged females to mate than young and old aged females. This agrees with earlier studies of *Drosophila* that males which perform greater courtship activities are better mates and obtain greater mating success than those males which do not show high levels of courtship activities [27].

The duration of copulation, that is the time elapsed between initiation and termination of copulation, It is an important component of *Drosophila* mating behavior and it varies in different species of *Drosophila*. It varies from 30 s in *D. muller* to more than 1 hr in some species [31]. During copulation, sperm from the male is transferred to the female reproductive tract, and therefore the duration of copulation has a lot of significance in an animal's life. The duration of copulation is known to be influenced by genotype, environmental factors, size, age, etc. [40] Figure 3 reveals that in *D. nasuta* middle-aged females copulated longer than young and old aged females, suggesting an influence of female age on copulation duration. In *D. nasuta* an increased copulation duration increases the number of ejaculations, and it is advantageous for males with a limited number of mating opportunities during their lifespan [39].

It was suggested that males of a species exercise their choice to seek direct fecundity benefits, therefore they should discriminate among potential mating partners on the basis of traits which are reliable indicators of fecundity [41, 24]. Models of the evolution of male choice suggest that male choice tends to break down when males target arbitrary female traits rather than those that reliably signal fecundity [22-23].

In the present study fecundity and progeny number of male mated with young, middle aged old female was analyzed to asses that in *D. nasuta* male mate choice fecundity benefits. Figure 4 revealed that male mated with young age females had significantly greater fecundity compared to middle and older age. Our study also confirms that females of the same species show variations in reproductive potential across age. Earlier works in *D. melanogaster* also have suggested that female age effect on fecundity [2].

Prathibha and Krishna [28] who while working on *D. ananassae* has also found variation in fecundity of females of different age classes. They also found that male mated with middle aged female had laid greater number of eggs. Figure 5 shows that in *D. nasuta* young-aged females had a significantly greater number of ovarioles than middle an old age females, and follows a pattern of fecundity across

female aged. All these studies in species of drosophila female age have significant influence on reproductive success [2]. It was noticed that ovarioles number and fecundity are positively corrected in species of Insects including species of *Drosophila* too. Therefore in *D. nasuta* unmated females of different age classes to understand the relationship between ovarioles number and eggs laid (Fig 4). Thus it is advantageous for males to mate with young-aged females compared to middle and old aged females. However, it is not known why older female had fewer ovarioles than younger and middle-aged females. We do not know whether they reabsorb them as the female age.

In insects it was show that female size has significant position correlation with ovarioles numbers [42]. Even in *Drosophila*, studies of [43] found a positive correlation between female size and the number of ovarioles. Therefore, in the present study, flies which were sacrificed to study the ovariole number were also used to measure wing length to understand relationships among female age, wing length, and ovariole number. It was found that in *D. nasuta* insignificant difference was found in the mean female wing length among females of different age classes (Fig. 6). This suggests that in *D. nasuta* size of the female did not very with female age but had a significant influence on ovariole number. These studies suggest that females of *D. nasuta* differ in reproductive potential, i.e., fecundity and ovariole number across different female age classes, but they did not significantly differ in body size across the different female age classes. Therefore, one would expect that males of *D. nasuta* might exercise some degree of mate choice because of more factors that put an upper limit on male reproductive success. For this behavior to be adaptive, these individuals are expected to benefit by enhanced survival or fecundity. Therefore, it is important that males carefully select females to increase their fitness. The benefits may come in the form of direct enhancement of survival or in direct benefits in the form of fecundity [44]. Thus, these studies in *D. nasuta* suggest that young-aged females have greater mating success and fitness characters than middle and old aged females.

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