An evidence of enhanced fitness by male remounting during the courtship in three species of *montium* subgroup of *Drosophila*

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Abstract: Male mating a second female after first mate (male remating) is a common feature in *Drosophila*, particularly in mass culture. On the other hand, female remating is not as frequent as that of male remating because the sperms introduced along with the semen into the spermatheca are stored there. However, the phenomena of female remating have been reported in few species. During sexual activity, the males and females are involved an elaborate courtship at the end of which the male mounts on to the female followed by mating. During each mating, the mounted male through its ejaculate, transmits the sperms into the females' genital tract where it is stored. The sperms are stored in the spermatheca. Usually, with one mount maximum sperms are transferred to female hence second mounting does not occur. In most *Drosophila*, when a male mounts a female after an elaborate courtship, the pairs remain in cupola for a fixed period then dismounts. No more courtship or mounting is performed by the male towards the same female. In *D.jambulina*, *D.nagarholensis* and *D.gangotrii* remounting occurs two or three times immediately after the first mount. The reason for remounting has been analyzed in the present study through measuring the width of the female reproductive tract and sperm count after each mount. The female reproductive tract was dissected out and width of uterus was measured. The result showed significant difference in the width after each mount. The sperm count of these species after each mount also showed significant variation. Remounting perhaps enhances the fitness of the mating pairs.

Keywords: Width of Uterus, Sperm Count, Male Remounting, Montium Subgroup

1. Introduction

Mating is the most important and fundamental process to select the best partner and to produce progeny. It is an important component of sexual behavior [1, 2] and transfer of sperm to female is the primary function of mating in sexually reproducing animals. Since each mating provides an opportunity to produce offspring, male can generally increase its fitness by mating with many females [3]. However female increases her fitness by increasing the number of viable eggs produced. This basic asymmetry between the sexes suggests that male fitness increases monotonically with increased mating rate, while single or a few mating are sufficient for females to maximize their reproductive success.

Fitness is the ability of an organism to produce the progeny and to leave the individuals of the species to the

next generation. Therefore it is one of the important aspects of organism's life activities. In the neodarwinian sense a successful species is the one which is able to reproduce successfully and leave more offspring to the next generation. An individual with higher fitness is said to be fit for survival than an individual with less fitness. Natural selection favors the organisms with higher fitness for survival. Fitness is a complex genetic trait which has a quantitative genetic control.

Since sexual behavior of male and female affects and modifies the contribution of different genotypes to the gene pool of succeeding generations, it becomes an important component to the fitness. In *Drosophila*, successful mating depends on male activity and female receptivity [4]. The fact that males can mate with more than one female forms one of the bases for sexual selection, especially because female generally accepts a limited number of mates [5].

Mating depends on a complex interaction between the sexes during which a male displays a repertoire of stimuli towards the female. If the female is receptive, mating can occur. The time elapsed until copulation is both a measure of female receptivity and male courtship efficacy and intensity.

Several studies have found that mate choice, mate number, or mating frequency influences components of offspring fitness [6-12]. The principal factors driving costs of mating to females are accessory gland proteins (Acps), which are produced by males and delivered to females in male seminal fluid [13]. Acps target tissues throughout the reproductive tract; some even enter the hemolymph of females and change female physiology [14, 15]. Maternal exposure to Acps also increases the early fecundity and population growth rate of daughters [16].

Wheeler [17] described the insemination reaction that prevents the female from mating immediately in many *Drosophila* species. Once a virgin female *Drosophila* has mated, she is usually unwilling to accept another male for some time. Because the female is usually the discriminating or limiting partner in the mating act, her activity of accepting or rejecting a courting male decides the fate of the second male in mounting the same female. Thus there would be a refractory period between the first and second mating. The length of the refractory period varies between species and in different strains of the same species [18]. After mating, behavioral and physiological changes are seen in *Drosophila* females, which include; decrease in attractiveness to male [19], less movement in response to males [20], decreased receptivity to further mating [21, 22], increasing of oogenesis, ovulation and oviposition rates [23], storage and utilization of sperm [24], and decreased life span [13,25]. These behavioral and physiological alternations in female after mating have short and long term effect. The short term effect, called the "Copulation effect" in *Drosophila* is due to seminal fluid components (Proteins), transferred during mating by males, causing an initial decrease in receptivity [26, 27], thus maximizing sperm usages and minimizing the sperm competition. However, the long term suppression of female remating in *Drosophila* has been indirectly linked to sperm load and termed the "sperm effect", [18, 24, 28]. Copulation and sperm effects would usually obscure any sperm effects within a day.

In many insects, mating is a onetime ritual and once the female is mated by a male, subsequently she will not receive another male. There are also instance of female remating with same or different male after a considerable gap of time. As it has been said earlier, in *Drosophila* female remating is a usual phenomena and a mated female can receive another male after sufficient gap of time. There are instances of second mount only if the first mount is unsuccessful and the pairs separate immediately after 2nd mount. Male remounting the same female for the second and third time and transfer the sperm to the female reproductive tract in each mount is an unusual behavior in *Drosophila*. The reason for male remating another female,

or female remated by another male had already been known. The phenomenon of male remounting the same female is unknown in *Drosophila*. The authors have shown that some species belonging to *montium* subgroup of *Drosophila* exhibit male remounting the same female [29]. The reason for this phenomenon is not known. It is hypothesized that the remounting enhances the fitness of these flies. This hypothesis has been tested in the present study by measuring the width of uterus after male remounting and through sperm count after each mount. Four species of *montium* subgroup, namely, *D.jambulina*, *D.nagarholensis*, *D.gangotrii*, *D.agumbensis* and one species of *D.bipectinata* were used to test this hypothesis.

2. Materials and Methods

D.jambulina, *D.gangotrii* and *D.nagarholensis*, *D.agumbensis* of the species of *montium* subgroup and *D.bipectinata* of *ananassae* subgroup of *Drosophila melanogaster* species group were obtained from *Drosophila* stock centre, Department of Zoology, University of Mysore, Mysore. The flies were cultured in a standard laboratory food (wheat cream agar yeast medium) at 22±2°C.

The virgin females and naive males were sorted by sex and aged for 7 days in separate food vials. These bachelor males and a virgin females were allowed to mate pair-wise separately in an Elens Wattiaux mating chamber (Observation chamber). Each pair was observed for 2 hours. When mating occurred the pair was allowed to complete copulation. The pair was allowed to stay together even after completion of mounting. The second and subsequent mounting and mating whenever occurred was noted.

Soon after each mating, mated females were isolated from males and the uteri of the female were dissected, the mated female were placed on a slide with a few drops of invertebrate saline (0.7%), the tergum and the fat bodies were removed carefully without disturbing the reproductive tract. Once the uterus was located it was carefully separated from rest of the tissues. The uterus was transferred on a clean slide with a drop of invertebrate saline. Width of uterus was measured in 10 x magnification and the image was captured using Olympus CX21 microscope with digital camera. The width of the uterus of virgin and mated females of four species of *montium* subgroup and one species of *D.bipectinata* were measured. One-way ANOVA and also Student T-test was carried out on the data using SPSS 11.5 program (IBM, www.ibm.com).

For sperm count, soon after each mating, reproductive organs of mated female were dissected out in 0.7% NaCl solution. Since there are chances of sperms to dissociate into the solution, 20µl of Lactoaceto oreicin was added to the slide, and then sperms were directly counted without draining the saline at 100x magnification using Olympus CX21. Sperm count of 10 individuals was taken for each mount. One-way ANOVA and also Student T-test was carried out on the data using SPSS 11.5 program (IBM, www.ibm.com)

3. Results

Table 1 shows the mean width of uterus in micrometers (μm) of female reproductive tract after each mount in *D.jambulina*, *D.gangotrii*, *D.nagarholensis*, *D.agumbensis* and *D.bipectinata*. In *D.jambulina*, width of uterus in virgin flies was 6.20 ± 0.44 . After first mount width of uterus was increased to 14.33 ± 0.95 , after second mount it was increased to 21.28 ± 0.55 and after third mount to 26.86 ± 0.636 . Mean difference was significant with $F=174.89$ (<0.05). In *D.gangotrii*, width of uterus in virgin flies was 6.44 ± 0.39 , after first mount it was 17.23 ± 1.19 and after second mount it was increased to $25.70 \pm 0.85 \mu\text{m}$ and

the mean difference was significant with $F=120.782$ (<0.05). In *D.nagarholensis* width of uterus in virgin flies was 6.33 ± 0.51 , after first mount it was 15.01 ± 0.0 , after second mount it was 23.70 ± 0.98 and after third mount it was 26.90 ± 0.78 . Mean difference was significant with $F=171.384$ (<0.05). In *D.agumbensis* width of uterus in virgin flies was 5.86 ± 0.21 . Even though *D.agumbensis* belonging to montium group this species did not show subsequent male mountings. The width of uterus in mated *D.agumbensis* females was 15.01 ± 0.0 . Even in *D.bipectinata* male remounting was absent and width of uterus in virgin flies was 4.12 ± 0.16 and mated female uterus width was 21.96 ± 0.51 .

Table 1. Showing the width of uterus of *D.jambulina*, *D.nagarholensis*, *D.agumbensis*, and *D.bipectinata* after different mounts. The values are Means $\pm$ SE represented in micrometers (μm). Values are significant at 0.05 levels by ANOVA for *D.jambulina*, *D.nagarholensis* and *D.gangotrii* it is by Paired *t* Test.

Species	N	Virgin	1 st mount	2 nd mount	3 rd mount	df	F	sig
<i>D.jambulina</i>	5	6.20 ± 0.44	14.33 ± 0.95	21.28 ± 0.55	26.86 ± 0.63	19	174.899*	0.001
<i>D.nagarholensis</i>	5	6.33 ± 0.51	15.01 ± 0.0	23.70 ± 0.98	26.90 ± 0.78	19	171.384*	0.000
<i>D.gangotrii</i>	5	6.44 ± 0.39	17.23 ± 1.19	25.70 ± 0.85		4	$t = -120.782^*$	0.003
<i>D.agumbensis</i>	5	5.86 ± 0.21	24.7 ± 0.31					
<i>D.bipectinata</i>	5	4.12 ± 0.16	21.96 ± 0.51					

Table 2 shows the sperm count after each mount. In *D.jambulina* after first mount number of sperm was 1017.5 ± 156.1 and after second mount sperm count was 2100.2 ± 135.5 and after third mount it was 4827.6 ± 194.1 . Mean difference was significant with $F=106.185$ ($P<0.05$). In *D.nagarholensis* after first mount number of sperm was 829.8 ± 30.4 and after second mount sperm count was 1806 ± 49.8 and after third mount was 3478.9 ± 110.8 . Mean

difference was significant with $F=343.22$ ($P<0.05$). *D.gangotrii* after first mount, number of sperm was 1690.3 ± 131.1 and after second mount sperm count was 3181.1 ± 101.1 . Mean difference with $t=-40.933$ ($P<0.002$). In *D.agumbensis* showed only one mount and the number of sperms in mated female was 5.673 ± 161.2 . Sperm count in *D.bipectinata* it was 4791 ± 131.2 .

Table 2. Showing sperm count in uterus of *D.jambulina*, *D.nagarholensis*, *D.agumbensis* and *D.bipectinata* (ANOVA significant at 0.05 levels) and for *D.gangotrii* (Paired *t* Test, value significant at 0.05 levels)

Sperm count Species	N	1 st mount	2 nd mount	3 rd mount	df	F	sig
<i>D.jambulina</i>	10	1017.5 ± 156.1	2100.2 ± 135.5	4827.6 ± 194.1	29	106.185	0.000
<i>D.nagarholensis</i>	10	829.8 ± 30.4	1806 ± 49.8	3478.9 ± 110.8	29	343.22	0.000
<i>D.gangotrii</i>	10	1690.3 ± 131.1	3181.1 ± 101.1		9	$t = -40.933$	0.002
<i>D.agumbensis</i>	10	5673 ± 161.2	-	-	-	-	-
<i>D.bipectinata</i>	10	4791 ± 131.2	-	-	-	-	-

Fig. 1. Showing width of uterus (in μm) of *D.jambulina* after each mount. A-Virgins, B – After 1st mount, C -After 2nd mount, D-After 3rd mount.

Fig 2. Showing width (in μm) of uterus of *D.nagarholensis* after each mount. A-Virgins, B – After 1st mount, C -After 2nd mount, D-After 3rd mount

Fig 3. Showing width of uterus of *D.gangotrii* after each mount. A-Virgins, B – After 1st mount, C -After 2nd mount

Fig 4. Showing the sperms in the genital tract of female flies. A- After first mount; B-After second mount

4. Discussion

In the present study of female *D.jambulina*, *D.nagarholensis*, *D.gangotrii*, shows significant variation in the of width of uterus after each unusual male remounting, which gives evidence that more and more sperm get transferred to female after each male remounting. The increase of width of uterus after each mount shows that there is a positive relationship between the size of the uterus and amount of semen and sperm transferred to female after every remounting. In the present study, sperm counting was also done from the reproductive tract of mated female after each male remounting in *D.jambulina*, *D.nagarholensis*, and *D.gangotrii*. We found significant increase in the sperm count after each male remounting. All these three species belong to *montium* subgroup of *D.melanogaster* species group. Contrary to this, *D.agumbensis* although belongs to the same *montium* subgroup male remounting was not found as much as *D.bipectinata* which belongs to *ananassae* subgroup.

In *D.jambulina*, *D.nagarholensis*, *D.gangotrii* perhaps the first mounting is incomplete, so to assure the enhancement of fitness the male mount again. But in *D.agumbensis* and *D.bipectinata* might be fitness is achieved at first mount hence they did not show remounting. Moreover in these two species, the sperm count reaches maximum level in the first mount itself. Whenever there is enough sperm accumulated in the female reproductive tract, then there is no remounting. When single insemination does not provide the female with sufficient sperm to insure high fertility during the female reproductive period then remating is necessary [30-32]. Hegde and Naseerulla [33] have demonstrated that the mating speed could also affect the transfer sperms from male to female during the courtship of *Drosophila* which

further supports the present finding that the remounting enhances the number of sperms transferred from male to female. A positive correlation between male mating activity and fertility has been found in *D.subobscura* and *D.ananassae* [34, 35]. Fulker [36] investigated the relation between time to begin mating, duration of copulation, the number of copulation resulting in fertilization, and the progeny produced in *D.melanogaster*. These appear to be general characteristics of male mating behavior and the male that mate quickly also copulate successfully and leave more progeny. The increased fitness of females may explain in part of the biological significance of the repeated copulations reported in *D.pseudobscura* [37-39]. Females are known to adjust their willingness to accept mating according to their state, for instance number of sperm in store [40, 24, 41] and nutrition [42-45].

Beckenbach [46] showed that fecundity was higher in females given continuous access to male than in singly mated females, even during the time when there was many sperm remaining for use in fertilization. Repeated mating seems to stimulate productivity long before the supply of stored sperm is exhausted, and a physiological basis for this stimulation is known for some *Drosophila* species [47]. Prakash [48] observed a relationship between first mating, repeat mating, and the increased number of offspring produced in *D.robusta*. Parsons [49] has suggested that the individual completing mating and copulation rapidly would most readily leave genes in subsequent generations and thus there is some selective advantage in completing mating and copulation rapidly. Friberg [50] is of the opinion that male *D.melanogaster* changes their copulatory investment quantitatively, rather than qualitatively, since manipulated males prolonged copulation and transfer higher sperm and subsequently increase their fertilization success. Thus prolonged copulation may have a positive effect on female fitness.

The cost of mating in *D.melanogaster* is mediated by seminal fluids [13]. A more recent finding suggest that a particular "Sex peptide" can increase female egg production [51,52], and causes releases of Juvenile hormone [53] it was previously known that juvenile hormone may positively affect *Drosophila* oogenesis and egg production process [54,55]. In the present study, as male shows re-mounting, availability of the sperm stored is more during second mount and third mate in *D.nagarholensis*, *D.jambulina* and *D.gangotrii* hence it gives an indirect evidence to increased fitness. Remating also can enhance the fitness, but of a different male. Therefore remounting the same female by a male enhances the fitness of both the partners.

The greater the quantity of sperm and protein transferred to the females, greater is the egg production [26]. In *D.bipectinata*, the size and number of the main cells in the accessory gland and the size of the accessory gland were important for the production of the protein. Females who mated with old males received more protein and sperm obtained fitness benefits by allowing them to have a higher fecundity and produce more progeny than females who mated with young and middle age males [56]. The presence of seminal fluid components with effects on female fitness may therefore make female fitness peak when some intermediate amount of seminal fluid is received. Males of many species of *Drosophila* and other insects provide nutrition to female in some way (nuptial gift, males secretion during courtship or nutrition through ejaculates), which may represent a form of paternal investment that increase female reproductive success. Nutrition role of male ejaculatory substances is strengthened by demonstrations that male-derived substance or their derivatives are incorporated into developing oocytes [57, 58]. Markow [59] found that these materials enhance female productivity over the first 10 days of life.

Once a virgin female *Drosophila* has mated, she is usually unwilling to accept another male for sometime because, after mating behavioural and physiological changes occur, including decrease in receptivity to further mating, male attractiveness and life span, increase of oogenesis ovulation and oviposition rates, storage and utilization of sperm [60,21,19]. In our previous study we have noticed that in few species of montium subgroup of *Drosophila*, the male performs all the courtship rituals, then mounts a female and copulates. After successful mating for considerable length of copulation duration the male dismounts and immediately remounts and mates the same female [29]. In *D.nagarholensis*, *D.gangotrii*, male mounts for subsequent times and produced more progeny [61]. In the present study, female *D.jambulina*, *D.nagarholensis*, *D.gangotrii*, show significant variation in the of width of uterus after each unusual male remounting and also significantly increase in the sperm count after each male remounting which is likely due to higher sperm transfer which is an indirect evidence to increase fitness.

Females that receive seminal fluid proteins, but no sperm, have a short-term post-mating response that lasts about a

day, with increased egg production and decreased receptivity to mating. Long-term post-mating changes, which last about a week, require receipt of both sperm and seminal fluid proteins [62, 63]. In the present study presence of sperm and significantly increase in the sperm number gives evidence that after each male remounting sperms get transferred along with seminal fluid to female. These seminal fluid proteins contribute to the post mating response including increasing ovulation rate (Acp26Aa, also called Ovulin-[64]) and sperm storage (Acp36DE – [27]). Additionally, CG33943 is required for full stimulation of egg laying, and four additional Acps (CG1652, CG1656, CG17575, and CG9997) are required for sustained egg laying, release of sperm from storage organs, and reduced receptivity to courtship [63].

Female flies can store sperm for up to two weeks after mating [65] and commonly store the sperm of two or more males simultaneously (e.g. [66]. In the examination of mated females, (1) the storage organs are normally filled to capacity after a single mating, leaving no available space for the female to accommodate in her receptacle and spermathecae a second store of sperm from another male; (2) the volume of sperm actually stored in the receptacle and spermathecae is only a small fractionize-possibly a fifth or so of the volume initially deposited in the uterus by the male; (3) the sperm-storage organs are non elastic and non contractile; (4) the absolute number of stored sperm is surprisingly small (500 to 700), of which about 80 percent are in the receptacle.

Thus in the present study it was found that the width of uterus and the number of sperm after each unusual male remounting increase significantly in *D.jambulina*, *D.nagarholensis*, *D.gangotrii*. The remounting the same female by the male is not just a mechanism of introduction of more sperms into the female's reproductive organs. Through remounting, the male enhances the fitness of himself as much as that of his partner.

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