International Journal of Zoology Studies
www.zoologyjournals.com
ISSN: 2455-7269
Received: 27-07-2021, Accepted: 11-08-2021, Published: 27-08-2021
Volume 6, Issue 4, 2021, Page No. 32-35

Female mating with middle aged males obtains greater accessory gland proteins and sperms in *Drosophila malerkotliana*

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Abstract
It was widely suggested that for species in which mating is resource independent female receive only accessory gland proteins and sperms in such species female should give concentration to age of mate to obtain benefits. *D. malerkotliana* on is one such species. In this species age effect of male on quantity of accessory gland proteins, sperm and post mating fitness benefits to Mated female has been studied. Outbred population of *D. malerkotliana* was used. It was noticed that females mated with Middle aged males obtains greater quantities of accessory gland proteins and sperm than females mated either with old or young males. As a result, females mated with middle aged males produced more eggs and progeny than those mated with older males. These studies indicate that in *D. malerkotliana* females obtain direct fitness benefit from mating with intermediate aged males.

Keywords: male age, male accessory gland proteins (ACPS), sperms, fertility

Introduction
Female of a species often use a variety of male phenotypic cues that serve as indicators female mating with preferred male phenotype can receive a variety of benefits which includes material benefits that affects female fitness or material benefits that affect offspring fitness or genetic benefits that affect offspring fitness (Andersson. 1994) [1]. Therefore, the success of males in achieving mating is often linked to the reproductive benefits which female derive (Jennions and Petrie. 1997) [10]. In animals with internal fertilization, male mating with female receive not only genes but also sperms and a variety of seminal fluid products in male ejaculates thereby post copulatory mechanism play an integral role in promoting variation in male reproductive success (Eberhard 1996; Simmons 2001; Arnqvist, 2005) [7, 15, 2]. The effect of male age on reproductive success, female mate preference, and female and offspring fitness is a long-standing controversy in the field of evolutionary biology (Beck and Powel, 2000). Numerous theoretical and empirical evidence of female preference for male age has demonstrated preference for old, young and even intermediates aged males in species of many taxa, (Johnson and Gemmell, 2012) [11]. Most of these studies have been devotes to understanding the benefits females derive from mating with preferred male age. These studies raise the questions 1) If male age is an honest indicator of male quality, then why females of many species belonging to same genera show difference in preference for male age. 2) Does male quality vary with increasing of male age in different species? 3) Does male quality remain constant throughout the age? 4) Does female have the capacity to bias mate preference for male age classes? Therefore, more studies involving sperm and Acps traits are needed to study the quantity of gametes produced by males of different male age classes. The re- examination of models relating to age based female mate preference is very much essential particularly studies embodying accessory gland and sperm traits. Therefore, present study has been undertaken in *D. malerkotliana* 2) What is the association between male age, duration of copulation and quantity of accessory gland proteins transferred to mated females on one hand the relationship between male age, duration and quantity of sperm transferred to the mated female on the other. 3) Whether females obtained benefits by mating with particular male age class.

Materials and methods
Establishment of *experimental stock*
Progenies of 150 naturally inseminated iso female lines of *D. malerkotliana* collected from Mysore region of Karnataka, India was used to establish experimental stocks. In each generation, progeny obtained from each experimental stock were mixed together and redistributed to 20 different culture bottles containing wheat cream agar medium each with 20 males and 20 females. These culture bottles were maintained them at 21 ± 1 °C at a relative humidity of 70% using a 12: 12 h light: dark cycle. At the 3rd generation, synchronized eggs were collected separately from each of these experimental stocks. Virgin females and unmated males were isolated within 3 hours of their eclosion.

Assigning of age classes to males
For obtaining males of different age classes before the start of experiment, longevity of male *D. malerkotliana* was studied by transferring unmated males into a vial containing wheat cream agar medium once a week and maintained them in above lab condition. This process was continued until their death and longevity was recorded. A total of 50 replicates was made and mean longevity was found to be 62±2days. Mysore population. In addition to this, mating activities of males were also studied from day 1 of their inclusion until 55th day. Results showed that showed least male courtship activities at 1st day whereas from 2 day and

onwards day male showed least courtship activities and after 50th day male rarely mated with the female. Hence age classes assigned to males were 2-3 days for young, 24-25 days for middle and 46- 47 days for older males. The first set of flies emerged were allowed to age for 46-47 day (to obtain old males). When these flies reached 20th day the next set day the next set of new flies were isolated and allowed to age for 24-25 days (to obtain intermediate aged males). When the second set of flies reached 20th day and the first set of flies reached 47th day, then the new set of flies was isolated and was aged for 2-3days (to obtain young male) This procedure helps us to culture all young, middle aged and old male in the same environment and also conduct the experiment at the same time.

Effect of male age on the quantity of Acps

To quantify Acps young, middle aged, or old males were separately etherized using insect saline using entomological needles to obtain accessory glands. Males of each age class were either unmated or had recently copulated (<5 min before they were sacrificed). Accessory glands were fixed in 95% ethanol obtained as above then these fixed glands were placed individually on a glass slide, and the membrane was removed using a fine needle and a stereomicroscope. The isolated secretions were washed in methanol/chloroform (1:1) and dried at 37°C for 15 min. Approximately 100 µL sample buffer (0.625 M Tris-HCl, pH 6.8, 1% SDS, 1% β-mercaptoethanol, and 10% glycerol) was added to each sample to dissolve the glands and secretions. Twenty pairs of accessory glands from each age class (10 mated and 10 unmated males for each trial) were collected and total Acps (Accessory Gland Proteins / secretions) was estimated using the Bradford method (Bradford, 1976). Fifty trials were run for each male age class (young, middle aged and old).

Bradford method

Approximately 50 µL of Acps (obtained as described above) were mixed with 5 ml Bradford reagent, which was generated by adding 100 mg Coomassie Brilliant Blue G-250 (in 50 ml 95% ethanol) to 100 ml 85% phosphoric acid and then diluting the mixture to 1 L with distilled water. The solution was allowed to stand for 5 min to develop color. The quantity of proteins in each sample was determined by measuring optical density at 595 nm using a spectrophotometer. Bovine serum albumin was used as the standard. Fifty trials were run for each male age class (young, intermediate aged, and old).

Effect of male age on copulation duration, quantity of Acps, and sperm count

To understand interaction between male age and quantity of Acps and sperms a young, middle-aged, or old unmated male was placed in an Elens-Wattiaux mating chamber (Elens-Wattiaux, 1964) [8] with a virgin female (5–6 days old). The pair was observed for 1 h. Pairs that did not mate were discarded. If mating occurred, the copulation duration was recorded. Soon after mating, the reproductive organ of the female was dissected in 20 µL Beadle-Ephrussi Saline (128.3 mM NaCl, 4.7 mM KCl, 23 mM $CaCl_2$) (Ephrussi-Beadle, 1936) [9]. Because sperm could dissociate into the solution, 20 µL lacto-aceto orcein was added to the slide without draining the saline. The number of sperm was then counted using an Olympus CX21 microscope. Total sperm counts includes sperms in the spermatheca, seminal vesicle and sperms in the genital tract of female mated. The quantity of Acps was measured for the mated males as described above. Fifty trials were run for each male age class. Repeatability index was also calculated to understand the consistency of an individual (repeatability, often symbolized as r, ranges from 0 to 1, and expresses the proportion of variations in a trait that is due to differences among individuals not due to differences within and individual).

Eggs and progeny produced by females that mated with males of different ages

A virgin female (5–6 days old) and a male (young, intermediate aged, or old) were placed in an Elens-Wattiaux mating chamber (Elens-Wattiaux, 1964) [8] and observed for 1 h. Pairs that did not mated were discarded. If mating occurred, the duration of copulation was recorded. Individual mated females were collected and placed into a vial. Every 24 h, a female was placed into a new vial, and this procedure was repeated until the death of the fly. The total number of eggs and emerged progeny were counted from the time of its eclosion until death of each mated female was considered as female longevity.

Results and Discussion

Table 1 shows one -way ANOVA followed by Tukey's post hoc test. According to the table the size of accessory glands and quantity of protein of unmated middle-aged males was found to be significantly greater compared to young or old males, as shown by Tukey's post hoc test. Furthermore, the size of the accessory glands was significantly greater in middle-aged males than in old males, as shown by Tukey's post hoc test. The quantity of protein showed insignificant variation between unmated middle-aged males and old males. Further the interaction between copulation duration and quantity Acps and sperms transferred to mated females in *D. malerkotliana*. except quantity of Acps of mated males transferred to mated female fecundity and fertility of female mated with middle aged male was greater than female mated with young or old male. Acps and sperms transferred to Mated females in *D. malerkotliana.* One -way ANOVA followed by Tukey's post hoc test showed that females mated with middle aged males had transferred significantly greater quantity of Acps and sperms and greater number of eggs and progeny productions than females either with young or old males. Design of the experiment used in the study allow us to study interaction between copulation duration, quantity of Acps and sperms transferred to the mated female as we used the same pair of flies involved in copulation were allowed to complete copulation, then they were used to as the quantity of transferred protein and sperm to the female to understand the relationship between male age, copulation duration, and the amount of Acps and sperm transferred (Tables.1). In all the three age classes of *D. malerkotliana* middle males copulated longer than young or old-aged males but the difference was non-significant. Although the reason why middle-aged males copulated longer is unknown but this result could also be explained by three other hypotheses. First, middle aged males may be unable to rapidly transfer sperm and hence require longer copulations. Second, middle aged males may transfer larger quantities of sperm, therefore requiring more time (Table.1) third, middle aged males might transfer more accessory fluid in their ejaculates during extended copulations. The

first explanation suggests that middle aged males are worse at transferring sperm than young males. The second and third explanations suggest that middle aged males invest more resources per mating. Table 1 show that male age was positively related with duration of copulation and the quantity of Acps and sperm transferred to mated females, suggesting that in *D. malerkotliana*, middle aged males which would fit the second and third theoretical explanation. Old males, with their shorter durations of copulation, transferred significantly less Acps and sperm to mated females.

Conclusion

In the present study the same pairs of flies used in copulation were also used to record copulation duration, fecundity, and fertility to understand interrelation between male age copulation duration, fecundity and fertility (Table 2). Male age significantly effects on duration of copulation, the quantity of Acps and sperms transferred (Tables 1& 2) to the mated females. Table.1 shows that in *D. malerkotliana*, middle aged males with longer copulation duration transferred a greater quantity of protein, and as a result, females who mated with middle aged males had significantly greater fecundity than females who mated with old or young males. Our study supports the idea of the role of accessory gland proteins in egg production (Wolfner 1997) [18, 19]. The greater the quantity of protein transferred to the females, the greater the egg production. The results also confirm the role of accessory gland secretion in post-mating physiological changes in the females, i.e., receptivity of females, fecundity, and fertility (Chen *et al.* 1996; Wolfner 1997) [18, 19]. In the present study on *D. malerkotliana*, females who mated with middle aged males received more Acps and more sperms, allowing them to have a higher fecundity and produce more progeny than females who mated with young and old aged males. In *D. bipectinata* (Santhosh and Krishna.) showed that female mated with old male had copulated longer transferred greater quantity of Acps and sperms and produced greater number of eggs progenies than female mated either with young or middle-aged males. Three age classes were used in our present study because the female preference for middle aged males was found in *D. malerkotliana* in other studies, the results of the present study in relation to accessory gland variation in young, middle, and old males and its effect on fecundity and fertility could also be extended to other strains. Females who mated with middle aged males obtained fitness benefits.

Table 1: Female mating with middle aged males obtains greater sperms and accessory gland proteins in *D. malerkotliana* [N=50; df=2,147]

Parameter	Male age classes			
	Young male	Middle aged males	Old males	F-value
Number of sperms in seminal vesicle (In no)	155.72 ±0.75^{a}	171.49±0.38^{b}	118.28±0.65^{c}	5848.92**
Total number of sperms transferred to mated female (In no)	4380.8±17.31^{a}	4807.8±11.28^{b}	4117.6±10.18^{c}	1853.74**
Quantity of Acps in unmated males (in µg)	13.39 ± 0.03^{b}	16.17 ± 0.07^{b}	15.03 ± 0.06^{b}	834.784**
Copulation duration (min)	11.9 ±1.791	13.2±0.529	10.21±0.139	1.125^{NS}
Quantity of Acps in mated males (in µg)	10.07 ± 0.07^{a}	9.22 ± 0.08^{b}	11.21 ± 0.05^{c}	384.784**
Transferred quantity of Acps to mated female (in µg)	3.32 ± 0.08^{b}	6.95 ± 0.11^{b}	3.82 ± 0.08^{c}	773.624**

****Significant at *P* < 0.001**

Different letters in superscript indicate significant at 0.05 level by Tukey's post hoc test

[Transferred quantity of protein was calculated by subtracting the quantity of mated males from unmated males]

Table 2: Female mating with middle aged males obtained greater fecundity and fertility in *D. malerkotliana* [N=50; df =2,147]

Parameter	Male age classes			
	Young male	Middle aged males	Old males	F-value
Fecundity (in no)	325.34±17.31^{a}	359.42±11..28^{b}	211.12±10.18^{c}	18.29.**
Fertility (in no)	265.34 ± 9.07^{a}	301.56 ± 8.08^{b}	186.23 ± 12.05^{c}	92.24.**

NS insignificant;**Significant at *P* < 0.001

Different letters in superscript indicate significant at 0.05 level by Tukey's post hoc test

Acknowledgements

The authors are grateful to the Chairman, Department of Studies in Zoology, University of Mysore, for providing facilities to carry out this work

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