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Male Accessory Gland Secretory Proteins in *nasuta* Subgroup of *Drosophila*: Synthetic Activity of *Acp*

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ABSTRACT—The quantity of male accessory gland secretory proteins in relation to the number of cells in the gland, size of the gland and the duration of copulation has been studied in seven members of the *nasuta* subgroup of *Drosophila*. The study revealed that the difference in the quantity of secretions is independent of the number of secretory cells in the gland. However, a positive correlation exists between the quantity of secretions and size of the gland; while there is no correlation between the copulation duration and the quantity of secretions. Further, there is an increase in the values of all the parameters studied, with increasing distance of the species from the ancestor.

Key Words: *Drosophila*, accessory gland proteins, copulation duration, phylogeny

INTRODUCTION

There are two accessory glands in the male reproductive system of *Drosophila* adults. They develop from a special set of cells in the genital imaginal disk, upon instruction by genes that determine the sexual phenotype of the animal (Nothiger *et al*., 1977). These glands synthesize and secrete a complex mixture of proteins, carbohydrates, lipids and amino acids (Chen, 1984) that are developmental stage specific, tissue specific, sex specific (Chapman and Wolfner, 1988) and are transferred to the female during copulation (Monsma *et al.,* 1990). Ultrastructural studies on these glands in *D. melanogaster* and *D. funebris* have provided information on the types of secretory cells and the nature of secretions (Bairati, 1968; Bertram *et al*., 1992). Studies on age dependent qualitative and quantitative variations in the accessory gland secretions in different species of *Drosophila* have shown that there is variation in the quantity of proteins synthesized (see Chen, 1984, Wolfner, 1997). Qualitative analysis of these proteins (Ravi Ram and Ramesh, 1999, 2001) have revealed that unlike in *D. melanogaster*, the SDS-PAGE patterns in various members of the *nasuta* subgroup are simple and some of the protein fractions show X-linked pattern of inheritance. However, the extent of quantitative variation and the significance of variation if any, among different members of *D. nasuta* subgroup has not been analyzed so far. Hence, present investigations were undertaken by employing 7 members of *D. nasuta* subgroup to (a) find out whether differences exist, in the extent of accessory gland proteins synthesized (b) investigate whether the difference if any, in the quantity of accessory gland proteins is a consequence of variation in number of cells in the glands or variation in synthetic activity and (c) establish the relationship if any, between copulation duration and the extent of secretory proteins synthesized.

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MATERIALS AND METHODS

Fly stocks

Seven members that belong to *Drosophila nasuta* subgroup namely *D. nasuta nasuta* (Coorg, India; Stock Number 201.001), *D. n. albomicans* (Okinawa, Japan; Stock Number 202.001), *D. n. kepulauana* (Sarawak, Stock Number 203.001), *D. kohkoa* (Thailand, Stock Number 204.001), *D. sulfurigaster sulfurigaster* (Queensland, Australia; Stock Number 205.001), *D. s. albostrigata* (Cambodia; Stock Number 207.001) and *D. s. neonasuta* (Mysore, India; Stock Number 206.001) were employed for the present study. These stocks were obtained from Drosophila Stock Centre, University of Mysore, Mysore, India. 50 synchronized eggs collected from the stock cultures by following the modified method of Delcour (Ramachandra and Ranganath, 1988) were placed in each vial (8 X 2.5 cms) containing wheat cream agar medium seeded with yeast. Care was taken to maintain the constancy of temperature, moisture and quantity of food in these cultures which otherwise would influence the larval development and ultimately the size of the adults. Unmated males isolated within 3hr of their eclosion from the pupal case were kept in fresh media vials. All the stocks and experimental cultures were maintained at 22±1°C.

Quantitative estimation of accessory gland secretory proteins

In a separate study we have found that during development of the adult, there is progressive and rapid accumulation of accessory gland secretions reaching almost maximum levels by 7th day in all the members under study. Hence, for the present investigations seven days old unmated males were utilized and the samples were prepared by dissolving accessory gland secretions of a pair of glands (from single individual) in 25µl of sample buffer (see Ravi Ram and Ramesh, 2001). The protein quantity in these samples was determined by micromethod (Neuhoff, 1985) in which, the samples were blotted onto small pieces of cellulose acetate strip (SM11200, Sartorious, Germany) and were allowed to dry. After staining these strips in 0.5% Amido Black 10B for 10 min, they were destained in 90:10 Methanol Acetic acid mixture with three changes of 10 min each. The strips were dried again, the stained portions were excised and dissolved in 4 ml of Dimethyl sulfoxide. Similarly the samples were prepared with known quantity of Bovine serum albumin. The O.D. of the sample was read against blank at 630nm. The protein quantity in the unknown samples was determined by extrapolation with calibration curve of Bovine serum albumin. Twenty five samples were prepared from each strain to determine the average quantity of secretions.

Determination of number of cells per lobe

The accessory glands dissected out from 7 days old unmated male flies in Medium A (Ashburner, 1970) were fixed in 1N HCl for 5 min. and later transferred to 2% lactoaceto-orcein. After 20 min, the glands were gently opened up with the help of the fine entomological needles and squashed between a slide and cover glass in 45% acetic acid so as to spread the cells in a single layer. Under low magnification, the number of main cells in one lobe of a pair of glands were counted by using a tally counter. Twenty five such preparations were used to determine the average number of main cells.

Determination of the size of the gland

The male reproductive system isolated carefully from 7 days old flies was mounted on a slide. Diagrams of only the accessory glands were drawn with a magnification of 40X with the help of camera lucida. The diagram of the gland was considered as a ribbon with edges in polygonal shape approximately. For the sake of convenience we divided the whole area into smaller areas consisting triangles, trapeziums and rectangles and accordingly the areas were marked. The areas of these geometrical forms were calculated individually through well known mathematical formulae. The sum of these areas was considered as the size of the gland (cm^2). Actual area of the gland in the fly was calculated by dividing these values with the magnification. Twenty five such replicates were used from each member to determine the average size of the gland.

Observation of copulation duration

Virgin females and unmated males from each culture were collected within 3hrs. after eclosion from pupal case and aged for 7 days in vials (8×2.5 cms) containing fresh medium seeded with yeast. These flies were maintained at 22±1°C under normal laboratory light conditions (12:12). With the help of an aspirator, a male and a female were introduced into a fresh culture vial and the duration of copulation that included the period from mounting of the male to parting was recorded in each case. Twenty five replicates were observed for each member and the average copulation duration was determined. All the pair matings were conducted during morning hrs. (7–9AM) when the temperature ranged between 22–24°C. The copulation duration was tested in all the members concurrently.

The data obtained from these analyses were individually subjected to ANOVA followed by DMRT (see Broota, 1989) to analyze the significance of differences. Further, correlation coefficients were calculated for the comparisons between the number of cells per gland and the quantity of secretions; size of the gland and the quantity of secretions, so also for quantity of secretions and the duration of copulation. Student t-test was applied to test the significance of correlation coefficient.

RESULTS

Quantity of accessory gland secretory proteins

Among the members analyzed, the amount of secretions on 7th day in case of *D. n. albomicans* was found to be maximum, while lowest quantity was a characteristic feature of *D. kohkoa*. The differences in the quantity of secretions are found to be non-significant only among *D. n. nasuta*, *D. s. albostrigata* and *D. s. sulfurigaster*. While in all other comparisons, the differences observed were found to be significant. (Table 1)

Number of cells per lobe

Perusal of Table 1 which shows the data on number of cells present in the accessory glands and the quantity of secretions synthesized in different members of *nasuta* subgroup, reveals that the glands of *D. s. neonasuta* are composed of maximum number of cells that is significantly different when compared with the number in all other members

Table 1. Number of cells, size of the gland, the secretory protein quantities and copulation duration in different members of *nasuta* subgroup of *Drosophila*. (The values are Mean ± SE)

	Number of Cells per lobe	Size of the gland[1] (cm^2)	Quantity of secretions per pair of glands (µg)	Copulation duration (min)
D. n. nasuta	1902 ± 6.74[a]	0.273 ± 0.001[a]	13.00 ± 0.03[a]	17.00 ± 0.48[a]
D. n. albomicans	1942 ± 6.15[a]	0.301 ± 0.001[b]	20.00 ± 0.05[b]	25.20 ± 0.52[b]
D. n. kepulauana	2219 ± 7.63[b]	0.224 ± 0.001[c]	10.50 ± 0.05[c]	17.60 ± 0.37[a]
D. kohkoa	1544 ± 8.12[c]	0.215 ± 0.001[d]	9.50 ± 0.04[d]	15.00 ± 0.55[a]
D. s. sulfurigaster	2214 ± 8.35[b]	0.268 ± 0.002[a]	13.00 ± 0.09[a]	17.80 ± 0.15[a]
D. s. albostrigata	2225 ± 11.32[b]	0.230 ± 0.001[e]	13.50 ± 0.06[a]	9.60 ± 0.35[c]
D. s. neonasuta	2905 ± 4.52[d]	0.257 ± 0.001[f]	15.20 ± 0.01[e]	16.40 ± 0.24a
F value	2887.23	1421.4	1297	124.19

[1]Average size of the single lobe of accessory gland
The strains with the same alphabet in superscript are not significantly different at 5% level according to DMRT.
Note: df = *(6, 168);

under study. In contrast, the cell number in the glands of *D. kohkoa* was found to be least and is significantly different when compared with the number of cells in the glands of rest of the members analyzed. The cell number in *D. n. nasuta* and *D. n. albomicans* was found to be non-significant among themselves but significant with others. The differences in cell number among *D. n. kepulauana, D. s. sulfurigaster* and *D. s. albostrigata* was found to be non-significant while in comparison with others, the differences were found to be significant.

Size of the gland

The average gland size of a 7 days old unmated male varied from 0.215±0.001 cm^2 (in *D. kohkoa*) to 0.301±0.001 cm^2 (in *D. n. albomicans*). The differences in the size of the gland were found to be non-significant only between *D. n. nasuta* and *D. s. sulfurigaster*, while all other comparisons were found to be significant (Table 1).

Copulation duration

Copulation duration was found to be minimum in *D. s. albostrigata* and maximum in *D. n. albomicans*, which were found to significantly differ with all the members understudy. In all other comparisons, the differences were found to be statistically non-significant (Table 1).

Correlations

The correlation coefficient for the comparison between number of cells per gland and the quantity of secretions was found to be 0.31; while the correlation coefficient for the size of the gland versus quantity of secretions was found to be 0.86. The correlation coefficient for the comparison of quantity of secretions and the duration of copulation was found to be 0.62. Student t-test showed significance of correlation coefficient only for the comparison between gland size and quantity of secretions (Table 2).

Table 2. Correlation co-efficients for different comparisons and their significance

Comparison	Correlation coefficient (r)	t	P
Number of cells/gland *Vs* quantity of secretions	0.31	0.73	>0.05
Size of the gland *Vs* quantity of secretions	0.86	3.76	<0.05
Quantity of secretion *Vs* copulation duration	0.62	1.67	>0.05

Note: df = 5

DISCUSSION

Different *Drosophila* species, in contrast to other insects (Ramalingam and Craig, 1978; Happ, 1984), show virtually the same basic features of the accessory glands; wherein each gland is an elongate sac, the wall of which consists of a single layer of secretory epithelium resting on a thin layer of muscle cells. Bertram *et al*., (1992) have shown that in *D. melanogaster*, the predominant type are the binucleate, hexagonal main cells which constitute 96% of the secretory cells of the gland (1004±84 cells per lobe). The remaining 4% (43±9 cells per lobe) are the secondary cells that are binucleate and spherical, containing large vacuoles.

Employing two species of *nasuta* subgroup, namely *D. n. nasuta* and *D. s. neonasuta*, Shivanna and Ramesh (1995a) have documented that the pattern of synthesis and accumulation of the accessory gland secretions is very much similar to that of *D. melanogaster* though there are differences in total quantities. The quantity of proteins synthesized differs significantly in at least four out of seven members presently analyzed. We presumed that these differences might be due to differences in size of the gland and/or number of cells in the gland and hence those components were analyzed. Such an analysis revealed that except in case of *D. kohkoa*, the number of cells that constitute the glands is nearly double when compared with that of *D. melanogaster*. Further, it is interesting to note that even the glands with less number of cells produced secretions, the quantity of which is much more than that of the glands with large number of cells. For instance, the glands in *D. s. neonasuta* with 2905 cells per lobe produce only 15.2 μg of secretory protein while the glands in *D. n. albomicans* having nearly 1000 cells less (1942 cells per lobe) produce 20 μg of secretory protein. These differences in the number of cells and quantity of secretions are statistically significant. Further, the difference in the number of cells per lobe in *D. n. nasuta* and *D. n. albomicans* was found to be non-significant while the differences in secretory protein quantities synthesized in these two were found to be significant. Similarly, the differences in the number of cells among *D. n. kepulauana*, *D. s. sulfurigaster* and *D. s. albostrigata* were found to be non-significant while the differences in protein quantity was found to be significant. Analysis of coefficient of correlation ($r = 0.31$; $P>0.05$) revealed that the number of cells in the gland has no correspondence with the quantity of secretory protein production. Absence of correlation between the number of cells and the quantity of secretions as well as size of the gland and the quantity of secretions has been reported in case of larval salivary glands of *Drosophila* (Shivanna and Ramesh, 1995b). In contrast to this situation, in the present study, when the quantity of secretions were analyzed in relation to the size of the gland, a positive correlation between the size of the gland and the quantity of secretions ($r=0.86$; $P<0.05$) was found to exist. The increase in the size of the organ may occur either due to hyperplasia and/or due to hypertrophy (Lofts, 1978). When the gland size is compared with the number of cells (see Table 1), two situations exist; in one, the differences in gland size were found to be non-significant though there is a significant difference in the cell number and in the other situation, the gland size was found to be significantly different though the cell number is same. The first case is exemplified by *D. n. nasuta* and *D. s. sulfurigaster* and the second situation prevails among *D. n. kepulauana*, *D. s. sulfurigaster* and *D. s. albostrigata*. This clearly indicates that these differences are a consequence of variation in the extent of cellular hypertrophy. Further, this also supports the findings of Cunnigham *et al.*, (1978) that the increase in the size of the glands is associated with increased secretions of the respective hormones and/or proteins. Present study has revealed that in the accessory glands of members of *D. nasuta* subgroup, it is the secretory activity of the cells which decides the quantity of secretions, but not the number of cells in the glands. The differences in the quantity of secretions observed in different members are a consequence of differential secretory activity of the *Acp* genes. In *D. s. neonasuta*, these genes are hypoactive as they produce lesser quantity with maximum number of cells in the glands; while those of *D. n. albomicans* are hyperactive as they synthesize more protein with significantly less number of cells. Investigations by Monsma *et al.,* (1990) in *D. melanogaster* have revealed that the synthesis of two specific accessory gland proteins namely msP355a (Acp26Aa) and msP355b (Acp26Ab) is developmentally regulated. Further, they have shown that these proteins are also synthesized following copulation. In the present study as we have analyzed the accessory gland proteins in the unmated males, the differential accessory gland protein quantities observed thus is due to developmental regulation, which is species and/or subspecies specific. Whether this regulation occurs at the transcriptional or translational level needs to be evaluated.

The duration of copulation is species specific but considerable individual variation also exists. The shortest time recorded is that of *D. enigma* with a mean of 5 sec (Grossfield and Rockwell, 1979) and the longest is that of *D. acanthoptera* with a mean of 62 min (Spieth, 1952). Majority of the species have copulatory time of less than 10 min and closely related species almost invariably have similar mean duration of copulation (Wheeler, 1947; Patterson, 1947).

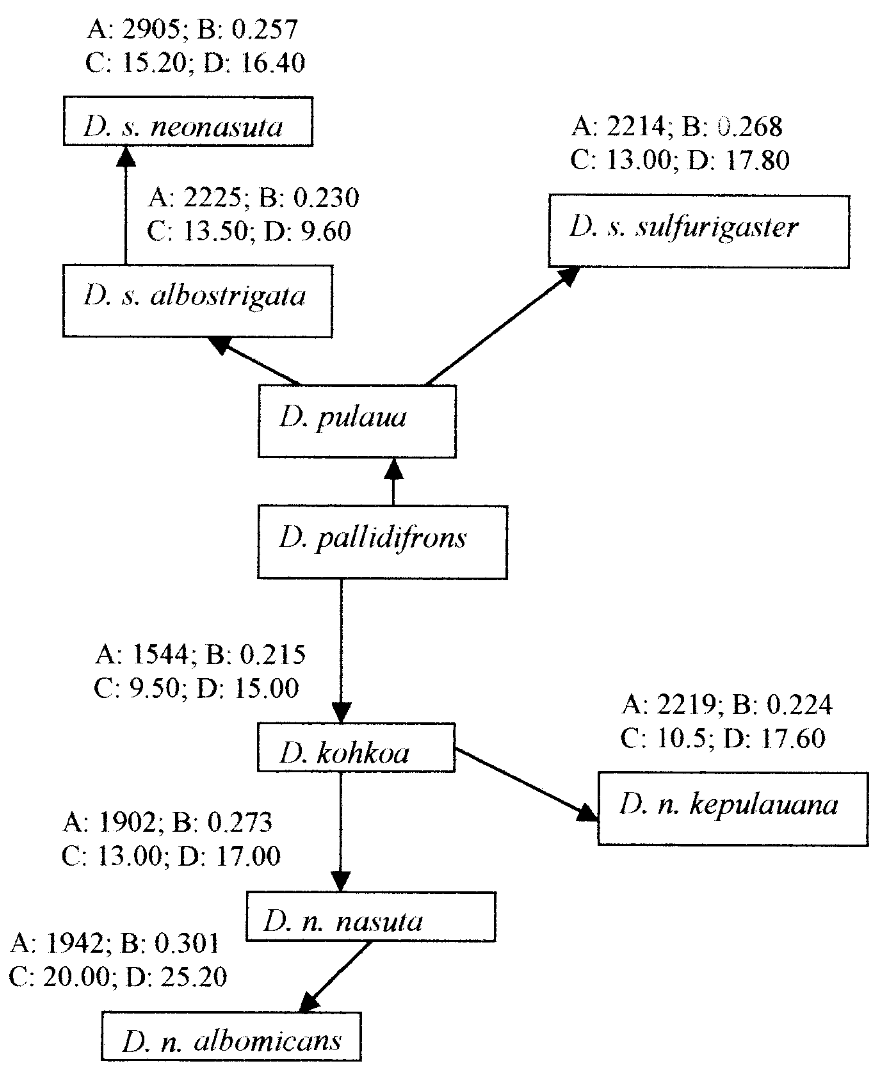

Fig. 1. Phylogenetic tree of *D. nasuta* subgroup showing the trends of different parameters under study. A- Number of cells/gland. B- Size of the gland. C- Quantity of secretions. D- Copulation duration

Working with four members of *D. nasuta* subgroup, Spieth (1952) has demonstrated that the duration of copulation is relatively long when compared to many other species of *Drosophila*. Even in the present study, the mean copulation duration was found to vary from 9.6 min (in *D. s. albostrigata*) to 25.2 min (in *D. n. albomicans*). The duration of copulation is determined by male in various species of *Drosophila* and is an expression of rate of sperm transfer (Mac Bean and Parsons, 1967). Male accessory gland secretory proteins form a component of the ejaculate and are transferred to the female during copulation (Monsma *et al.,* 1990). These secretions are essential for transfer, storage and utilization of the sperm (Fowler, 1973; Neubaum and Wolfner, 1999; Lung and Wolfner, 1999; Chapman *et al*., 2000). Further, Krebs (1991) has suggested that longer copulations stimulate earlier oviposition, possibly by increasing accessory gland secretions that are passed by males during copulation. Taking these aspects into consideration, an attempt was made to check whether there is any correlation between quantity of secretions and the duration of copulation. A comparative analysis of the data reveals that *D. s. albostrigata* with 13.5 µg of secretions has a copulation duration of only 9.6 min., while *D. n. nasuta* and *D. s. sulfurigaster* with similar quantities have a copulation duration of 17 min. Further, *D. kohkoa* with least quantity of secretions has copulation duration that was found to be non-significant with all the members except *D. n. albomicans* and *D. s. albostrigata*. Analysis of the data shows the absence of correlation between these two parameters ($r=0.62$; $P>0.05$). Spiess (1968) recorded mating latency and time of copulation in different karyotypes of *D. pseudoobscura* and the results clearly indicate that the duration of copulation was the most uniform part of male mating activity and is quite species-specific.

David *et al.,* (1994) by comparing the reaction norms of size traits of a natural population of *D. simulans* and *D. melanogaster* have demonstrated that the reaction norms have diverged within an evolutionary time of about 2 million years. The species differences with regard to several parameters found in present investigation might reflect the phylogenetic changes that have occurred during the course of evolution of *D. nasuta* subgroup. Perusal of the Fig. 1 which shows the divergence of various members of *D. nasuta* subgroup with *D. pallidifrons* as the ancestral species (Kitagawa, 1991), reveals that though the genetic basis of each one of the parameters analyzed in the present study is different from one another, with the increased distance from *D. pallidifrons* there is an increase in the values, be it the number of cells, quantity of secretions, size of the gland or copulation duration. On the contrary, Harini and Ramachandra (1999, 2000) by their analyses on *D. n. nasuta*, *D. n. albomicans* and the progenies of their hybridization, namely the cytoraces have documented that the newly evolved cytoraces show reduced body size and abdominal bristle number during the course of their differentiation under laboratory conditions. Further, they have also shown that these cytoraces have better fitness. However, the differences observed in the study may also be strain specific.

Therefore, present study on one hand has revealed a lack of correlation when the quantity of accessory gland proteins synthesized is compared with the number of cells and duration of copulation; while on the other, it has shown a positive correlation between the quantity of male accessory gland secretions and size of the gland indicating that there is a differential synthetic activity among different members.

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