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TRANSFER OF EJACULATE AND INCORPORATION OF MALE-DERIVED SUBSTANCES BY FEMALES IN THE NANNOPTERA SPECIES GROUP (DIPTERA: DROSOPHILIDAE)

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Among insect mating systems, the amount of paternal investment varies from the typical case in which males provide females with sperm alone to extreme situations in which the male himself is consumed by his mate. Unique parental investment strategies are presumed to reflect a maximization of male fitness and to have evolved in the context of varying resource ecologies (Thornhill and Alcock, 1983). The degree to which paternal investment strategies are constrained by phylogeny, however, has never been examined.

One mating system feature that appears to vary widely is ejaculate function. Various components of insect seminal fluid may influence sperm activation, motility, and viability, as well as female receptivity, egg production, ovulation, and oviposition (see reviews by Leopold, 1976; Chen, 1984; Gromko et al., 1984). The ejaculate may also contain nutrients that are quickly absorbed across the female reproductive tract (Boggs and Watt, 1981; Markow and Ankney, 1984). Because they reduce the male's probability of acquiring additional mates (e.g., Rutowski, 1979), these nutrients likely represent a nonpromiscuous mating effort (Alexander and Borgia, 1979; Gwynne, 1984). Additionally, male secretions may be paternal investment that directly benefits the male's mate, offspring, or both. In several species, male-derived nutrients have been demonstrated to enhance egg-production by recipient females (Boggs and Gilbert, 1979; Gwynne, 1981; Steele, 1986; Butlin et al., 1987; Markow et al., 1990; but see Jones et al., 1986; Wedell and Arak, 1989).

Several factors will determine whether males will be selected to employ this kind of reproductive effort. First, a male must be able to effect significant reproductive gain by virtue of his investment. Therefore, the nutritive component is expected to be a substance that limits the rate of reproduction by females. Substances that limit reproductive success are likely to vary among species and habitats. Consequently, the kinds of nutritive secretions contained within the ejaculate are expected to vary among species according to their unique ecology (Marshall, 1982). Second, reinforcement of male nutritive investment requires adequate certainty of paternity. Markow (1988) has demonstrated the potential for multiple mates of a female to cuckold each other's nutritive contribution in *Drosophila mojavensis.* It is also true, however, that throughout the genus *Drosophila* the presence of ejaculatory donations is strongly associated with the formation of a copulatory plug (Markow and Ankney, 1988), which presumably enhances a male's paternity assurance. Third, the reproductive costs, in terms of lost mating opportunities, must be outweighed by the reproductive

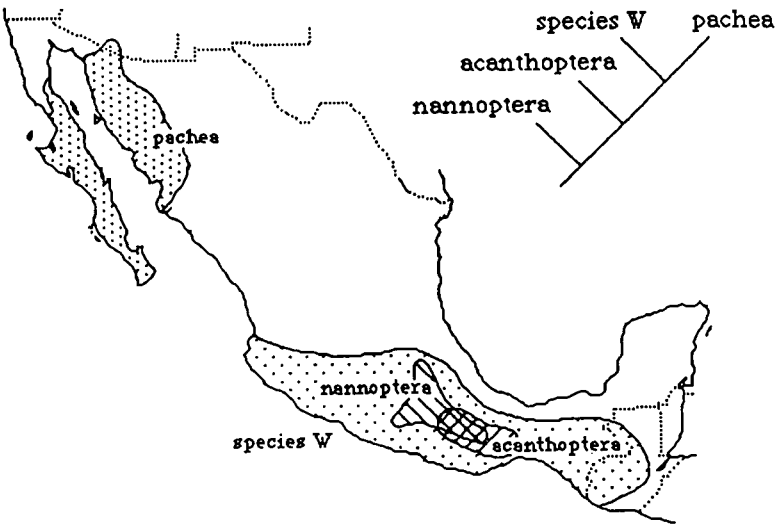

FIG. 1. Distribution and phylogeny of the nannoptera species group. Distributions are based on collections by author and localities listed in Heed (1982).

gains. This variable may differ among populations, primarily due to varying resource distribution and abundance (Thornhill and Alcock, 1983).

Species under similar ecological constraints, especially with respect to larval nutrient limitation, are expected to display similar patterns of male reproductive investment. However, no pattern is revealed by comparison among the various *Drosophila* studied, at least with respect to broad ecological categorization of feeding ecology. For instance, among the cactophilic species endemic to the Sonoran Desert, males of *D. mojavensis* and *D. mettleri* make large ejaculatory contributions, while *D. nigrospiracula* males pass no nutritive substance to females. Further, among six species with highly generalized diets, males of three species (*D. melanogaster, D. simulans, and D. hydei*) provide no ejaculatory contribution (but see Bownes and Partridge, 1987), one species (*D. pseudoobscura*) shows a moderate contribution, and males of two species (*D. arizonensis* and *D. immigrans*) provide a very large contribution (Markow and Ankney, 1988).

On the other hand, data presently available suggest that production of nutritive accessory gland secretions by males is a phylogenetically conservative trait. Within the subgenera *Sophophora* and *Drosophila,* the amount of male-derived material incorporated into female somatic tissue and ovaries remains consistent among the members of each monophyletic "species group." Although this observation appears to apply to the many species groups that have been examined (Markow and Ankney, 1988), no single group has been exhaustively sampled. One goal of the present study was therefore to quantify the amount of male-derived substance incorporated into female tissue by all members of a species group. The "nannoptera" group was chosen because it consists of only four species: *D. nannoptera, D. acanthoptera, D. pachea,* and an undescribed "species W" (Ward and Heed, 1970), and because the presumed phylogenetic relationships have been established for this group (Fig. 1; Ward and Heed, 1970; Heed, 1982).

MATERIALS AND METHODS

Cultures of each species were initiated from flies collected on their natural hosts in the wild. *D. nannoptera, D. acanthoptera,* and "species W" were collected in Chiapas, Mexico in August 1988. *D. pachea* and *D. mojavensis* were collected in San Carlos, Sonora, Mexico in March 1989 and May 1987, respectively. *D. melanogaster* were collected in Tempe, AZ in November 1988 and *D. mulleri* were collected in the Parajito Mountains, 20 miles west of Nogales, AZ in May 1988. Fly strains were cultured in uncrowded conditions on banana medium with yeast at 24° ± 1°C on an approximate 12L:12D photoperiodic cycle. Medium for *D. pachea* additionally contained necrotic, autoclaved senita cactus tissue.

Males were radiolabeled by collecting 50 early first instar larvae from culture bottles of each species. All 50 larvae were placed in a vial containing 5 g of culture medium prepared with 50 μCi of a mixture of ^{14}C labeled amino acids (ICN 10147). Virgin males were separated upon eclosion and stored under low densities (10 per vial) until they were mated to virgin unlabeled females.

In all of the species studied, females become reproductively mature within a few days following eclosion. All females were mated at five days of age. Time required for males to attain sexual maturity, however, varies greatly among species. With the exception of *D. melanogaster,* all species studied are protogynous, the males requiring 7–13 days after eclosion to copulate successfully (Pitnick, unpubl. data; Markow, 1982). *D. nannoptera* and *D. acanthoptera* males were mated approximately seven days after achieving reproductive maturity. The remaining species were mated within two days of maturity. Labeled males were aspirated individually into shell vials containing single females.

TABLE 1. Copulation duration and ^{14}C radiolabel (disintegrations per minute + SE) in whole females immediately after copulation and in body parts and ovaries six hours later in various *Drosophila* species. Differences between species were examined with a Tukey's studentized range test at $\alpha = 0.05$ for each variable. Subset membership by this test is indicated by the letter following the mean radioactivity for that variable.

Species	Copulation duration	Whole flies (0 hr)	Body parts (6 hr)	Subset	Ovaries (6 hr)	Subset
D. mojavensis	2.5 min	3,102 + 571	2,543 + 89**	a	1,785 + 351**	a
D. mulleri	0.37 min	1,416 + 167	555 + 221**	b	416 + 136**	b
D. nannoptera	7.4 min	431 + 43	186 + 32*	c	125 + 28	c
"Species W"	16.1 min	220 + 18	175 + 9**	c, d	49 + 1	d
D. acanthoptera	139.8 min	552 + 60	172 + 15**	c, d, e	83 + 18	c, d
D. pachea	41.3 min	177 + 11	101 + 4*	d, e, f	70 + 4	c, d
D. melanogaster	15.4 min	2,220 + 312	96 + 32	e, f	56 + 14	d
Cold control (*D. pachea*)		80 + 5	80 + 5	f	80 + 5	c, d

Copulating pairs were undisturbed. After males had dismounted, they were removed, and females were processed according to the experiment being conducted.

Two experiments were performed. The first measured the amount of ejaculate (radiolabel) transferred during copulation. To this end, females were processed for scintillation counting immediately following copulation (0 hr). The second experiment measured the amount of male-derived substance incorporated by females into their body tissue and oocytes. These females were left undisturbed to await dissection six hours after copulation. Markow and Ankney (1988) have demonstrated, across a broad array of *Drosophila* species, that any measurable incorporation of radiolabeled ejaculate will occur within a six-hour period.

All flies were processed for scintillation counting as follows. First, the thorax length of the ether-anesthetized fly was measured. Second, the fly was washed by vigorously shaking it for 30 sec in a 1.5 ml micro centrifuge tube containing Schen's solution with a drop of the detergent Triton X to remove any radiolabeled material from the surface of males or that which was transferred to females by physical contact with males. After similarly rinsing the flies in pure Schen's solution, they were decapitated to eliminate the natural quenching effect of eye pteridines. At this point, "0 h" females were placed in scintillation vials and "6 h" females were dissected in Schen's solution. Reproductive tracts were carefully removed intact from females, and the remaining somatic tissues were collected into a separate dish. Ovaries were then isolated from the distal end of the common oviduct using two fine probes. The ovary and somatic tissues were then each rinsed twice in micro centrifuge tubes as described above. The remaining reproductive tracts were slide mounted and examined to confirm that sperm had been transferred during the copulations. For the ovary and somatic tissue samples, parts from three females were pooled. As a control, females that had been mated to unlabeled males were obtained and prepared for counting by identical procedures.

All samples were placed in a scintillation vial containing 100 μl of Scintigest tissue solubilizer (Fisher Scientific), and crushed with glass rods prior to digestion for 24 hr at 50°C. Glacial acetic acid (17.5 μl) was added to neutralize the solutions, and then 5 ml of ScintiVerse II scintillation fluid (Fisher Scientific) was added. Each vial was then vortexed and allowed to settle for an additional 24 hr at 24°C prior to counting in a Beckman LS 7000 Liquid Scintillation System. Counts per minute were converted to distingegrations per minute (DPM) following a standard quench curve.

RESULTS

The levels of radioactivity found in whole, recently mated females and in their body parts are shown in Table 1. Unlabeled control samples for each species showed virtually identical amounts of background radioactivity. Table 1 presents the control values for *D. pachea*. Among the species examined there is great variability in the duration of copulation and in the amount of ejaculate transferred per copulation, as measured by female DPM. However, no relationship was found to exist between these two variables, either among species ($r = -0.37$, $P = 0.413$) or within any of the species ($r = -0.06$, $P = 0.794$ for *D. nannoptera*; $r = -0.44$, $P = 0.101$ for *D. acanthoptera*; $r = 0.22$, $P = 0.274$ for *D. pachea*; $r = -0.17$, $P = 0.438$ for species W).

Variability in ejaculate size is further illustrated in Table 2, which shows the average relative amount of ejaculate transferred by males of each species, expressed as the proportion of the male's total premating DPMs contained in his ejaculate. Relative to body size, estimated by thorax length (Robertson and Reeve, 1952), *D. mojavensis* and *D. melanogaster* males transfer many times more ejaculate per copulation than do all members of the nannoptera species group.

A significant amount of radiolabel, relative to controls, was incorporated into somatic tissue by females of all species except *D. melanogaster*. However, only *D. mojavensis* and *D. mulleri* females incorporated a significant amount of radiolabel into ovarian tissue (Table 1). Both the mean disintegrations per minute for body parts and the mean disintegrations for ovaries (oocytes) from different species were compared by the Tukey's studentized range test at $\alpha = 0.05$. Species assigned to different subsets show significantly different amounts of label (Table 1). A striking amount of label was present in tissues of female *D. mojavensis*, and somewhat less in tissues of female *D. mulleri*, resulting in their placement into discrete subsets. Relatively low amounts of label detected in tissues of female *D. melanogaster* and all the nannoptera species caused them

TABLE 2. Male thorax length, male total premating DPM, and size of ejaculate (mean ± SE) calculated as the percent of a male's total premating ^{14}C radiolabel that is transferrred during copulation.

Species	Male thorax length (mm)	Premating male DPM*	Ejaculate size	N
D. mojavensis	1.03 ± 0.01	201,430 ± 20,413	1.51 ± 0.15	3
D. melanogaster	0.88 ± 0.01	128,419 ± 6,003	1.73 ± 0.19	3
Nannoptera species group:				
D. nannoptera	1.03 ± 0.00	159,622 ± 3,788	0.27 ± 0.03	23
D. acanthoptera	1.10 ± 0.01	143,785 ± 6,803	0.38 ± 0.04	19
"Species W"	1.11 ± 0.01	228,440 ± 10,665	0.10 ± 0.01	24
D. pachea	1.03 ± 0.01	162,334 ± 7,251	0.11 ± 0.01	26

* Male premating DPM calculated by adding each male's postmating DPM value with the DPM value of his mate.

to be grouped into a series of subsets that overlap with cold controls.

Caution is required when making intra- and interspecific comparisons based on data from radiolabel experimentation. For instance, because we are not measuring ejaculate volume per se, the 0-hour DPM values can only represent relative measures. A male that has incorporated a higher concentration of radiolabel into body tissue may be falsely interpreted as transferring a larger volume of ejaculate, because his sperm and accessory gland secretions are "hotter" than those of other males. Although every attempt was made to be precise and consistent in establishing each radiolabel culture, variation in radiolabel concentration is inevitable, primarily due to variation in developmental time and adult body size. This was not a problem in the present study as whole body counts for males were highly consistent within cultures (Table 2), and there was a strong positive relationship between body size and DPM for males of all species used in this study ($R^2 = 0.249$, $P < 0.0001$). Therefore, intra- and interspecific comparisons were possible based on DPM for recently mated females relative to whole body counts of their respective mates (Table 2). These values were consistent with the absolute values presented in Table 1.

DISCUSSION

The most striking observation of this study is the diminutive ejaculates of all members of the nannoptera species group. For instance, although both species are endemic to the Sonoran desert, have similar cactophilic lifestyles, and are equal in body size, *D. mojavensis* males transfer 13.7 times more ejaculate per copulation than do *D. pachea* males (Table 2). Understanding the functional significance of this variation requires detailed knowledge of the materials an ejaculate comprises and of the fate of each component within the female. Although such information in not currently available for *Drosophila,* the adaptive significance of ejaculate size can be investigated by searching for consistencies between ejaculate size and phylogeny, as well as other aspects of the reproductive biology of these species, such as copulation duration, the presence of nutritive ejaculatory contributions, formation of an insemination reaction, and fertility.

Copulation duration varies enormously among species in the present study, but is unrelated to ejaculate size. The evolutionary significance of copulation duration is still not well understood. Females can determine the length of copulation in many insect species (Thornhill and Alcock, 1983), and premature termination of copulation can reduce male fitness by limiting the number of sperm transferred (Thornhill, 1976). Sperm competition has been postulated to select for longer copulations due to its effect on female behavior (Jackson, 1980; Eberhard, 1985) or on the level of sperm displacement (e.g., Siva-Jothy, 1987). Copulation duration has been shown to be primarily male-determined in various *Drosophila* species (MacBean and Parsons, 1967; Kaul and Parsons, 1965; Krebs, 1989), and to be positively correlated with fertility in *D. melanogaster* (Gromko, 1987, 1989; but see Pitnick, 1990). However, attempts to attach evolutionary significance to the interspecific variation in copulation duration have been unrevealing (e.g., Grant, 1983). The present study found no relationship between copulation duration and ejaculate size in either interspecific or intraspecific analyses. *D. mulleri,* which performs the briefest copulation (29 sec) of all *Drosophila* species (Spieth, 1952), was included in this study specifically to test this relationship. *D. mulleri* males transferred an average of 2.6 times more label than did male *D. acanthoptera,* which exhibit the longest copulation (2.2 hr) of all *Drosphila* (Table 1).

In a comparative study of 19 *Drosophila* species, Markow and Ankney (1988) established a correlation between incorporation of male-derived substances by females and the formation of an insemination reaction (swelling of the uterine wall interpreted as the functional equivalent of a copulatory plug; Patterson, 1946). They interpret the incorporated substances as representing a paternal investment, and a later study by Markow et al. (1990) supports this hypothesis. The swelling is presumably an evolved mechanism by which males (and females?) protect their fitness-limiting investment. When males invest in offspring, they are expected to evolve the "feminine" trait of higher confidence of parentage (Gwynne, 1984). This trend is further strengthened by the present study, for female *D. mulleri,* which form a large insemination reaction, were found to incorporate a significant amount of label into body tissue and oocytes (Table 1). No label was incorporated into ovarian tissue by any of the nannoptera group species, albeit a small yet significant amount was found in female somatic tissue, and these females exhibit no insemination reaction.

The hypothesis that male accessory gland secretions, which can be utilized by females, represents an evolved paternal investment is intuitively acceptable for species

such as *D. mojavensis* and *D. mulleri,* where a large amount of ejaculate is provided and most of it is sequestered by female-derived tissues. The pattern of incorporation of label by nannoptera group females observed here is far less easily interpreted. Because so little ejaculate is transferred with each copulation, even less incorporated into female tissue, and then only into somatic tissue, this phenomenon is unlikely to represent a nutritive parental investment. Although contributing solely to a mate's soma could increase her longevity, fecundity, or both, thereby enhancing the male's own reproductive success, remating by females would minimize the profitability of this strategy (Markow, 1988). Female *D. pachea* are known to mate several times daily (Pitnick, unpubl. data), which suggests that the pattern of label incorporation observed in this species does not represent a nutritive investment by males.

Evidence from two of the species examined here suggests that ejaculate size is not correlated with fertility. In *D. melanogaster,* males whose accessory glands have become depleted from serially mating continue to transfer a normal complement of sperm (Hihara, 1981). Likewise, the amount of label transferred by serially mating *D. mojavensis* males declines significantly after two or three copulations. Still, male fertility remains constant across six copulations (Markow et al., 1990), suggesting that sperm number remains more constant. It is worth noting, however, that *D. pachea,* which transfers the smallest known ejaculate of any *Drosophila* species (Table 1; Markow and Ankney, 1988) also transfers the fewest number of sperm (Pitnick, unpubl. data).

The results of this study reinforce the phylogenetically conservative nature of ejaculatory contributions suggested by the Markow and Ankney (1988) study. The amount of label incorporated and its fate within the female were similar for all four nannoptera group species (Table 1). Also, the large contribution made by *D. mulleri* (Table 1) is similar to that provided by the two other members of the mulleri species subgroup that have been examined previously (Markow and Ankney, 1988). There is an inherent difficulty, however, in trying to distinguish the roles of phylogenetic inertia and ecological convergence in creating such patterns, because closely related species are often ecologically similar.

All members of the nannoptera species group are ecologically similar in that each utilizes necrotic tissue of columnar cacti as feeding and breeding substrate (Heed, 1982). There appears to be, however, important ecological differences that would generate interspecific variation in the selection pressures affecting the composition of male accessory gland secretions. For instance, variation exists in the range of host plants utilized by each species, with host plant specificity becoming increasingly specialized through evolutionary time. The ancestral species, *D. nannoptera,* has been reared from a variety of cacti from four genera, while the most derived species, *D. pachea,* is monophagous on senita cactus (Heed, 1982; Fogelman et al., 1986). *D. pachea* is restricted in range within the Sonoran Desert while its relatives occupy more tropical regions of southern Mexico and Guatemala (Fig. 1). The flies feed primarily on the variety of yeasts and bacteria growing on the rotting cactus, and yeast communities are known to vary seasonally, geographically, and with host identity (Starmer et al., 1987, 1990; Heed et al., 1976). Finally, *D. pachea* is more likely to suffer cyclical periods of nutritional stress during the dry desert summers, when habitable cactus necroses become extremely scarce (Pitnick, pers. obs.). Each of these ecological differences could theoretically contribute to variation in the quantity or quality of paternal investment among species, but the trait remains conservative.

The quantity and composition of a male's ejaculate may determine his mate's willingness to remate with a competitor, the fate of his sperm within her reproductive tract, the rate at which she manufactures and lays eggs, and the viability of his offspring. Provided there is sufficient additive genetic variation, traits with such profound fitness consequences should respond rapidly to selection, and therefore be evolutionarily labile. The pattern of phylogenetic conservativism observed for incorporation of male-derived substances by female *Drosophila* is therefore surprising. It raises the possibility that this character may have been adaptive only in the context of an ancestral ecology, and persists simply because superior alternatives have not arisen in descendant taxa (Dobson, 1985). Understanding the evolution of ejaculatory contributions awaits a molecular characterization of male accessory gland secretions and their fate within females of ecologically diverse taxa, combined with a detailed knowledge of specific dietary stresses encountered by each species.

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A TEST OF THE LOW MARGINAL VARIANCE (LMV) THEORY, IN *LEPTOSPERMUM SCOPARIUM* (MYRTACEAE)

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Population geneticists have long theorized that for a particular species, within-population genetic variance will be lower in sites geographically peripheral to the species' range (da Cuhna et al., 1950; White, 1951; Carson, 1956). Mayr (1959) suggested that one reason would be that at the geographical margin the environment would be marginal too, so selection would be more severe. Agnew (1968) applied this theory to plants, making it clear that he was envisaging greater stabilizing selection near the margin of a species' ecological range, where its environment is more critical. We term this the Low Marginal Variance (LMV) theory. Many authors have considered the theory (e.g., Avise and Selander, 1972; Grant and Antonovics, 1978; Silander, 1985), without a consensus emerging on its validity.

We tested the theory using *Leptospermum scoparium* J. R. et G. Forst. (Myrtaceae), a shrub/small-tree indigenous to New Zealand and widespread through the three main islands.

To test the theory we examined ecological marginality directly, rather than assume it from geographical peripherality. We must therefore define what conditions we regard as ecologically marginal. *L. scoparium* occurs over a broad ecological range in New Zealand, particularly of altitude, temperature, and moisture conditions (Yin et al., 1984). The latter two variates were the best correlates of morphological variation. In its temperature range, *L. scoparium* occurs up to the warmest areas in the north of New Zealand (Yin et al., 1984; Enright, 1989), so we assume it is not limited by high temperatures in the field. The species does not occur above c 900 m altitude (Yin et al., 1984), which suggests the expected limitation by cold temperatures, and this is confirmed by its restriction, at its upper altitudinal limit, to northerly (sunny) aspects (Wilson et al., 1989). As for moisture range, it can grow for several years in standing water (Cook et al., 1981), and none of the sites examined here are really wet sites. However, in the driest areas it is absent at low to mid altitudes, being ecologically replaced by the related *Kunzea ericoides*. We therefore assume that within the current range of sites the wetter ones are the more favorable.

METHODS

Leptospermum scoparium was sampled at 17 sites throughout New Zealand—from the north of North Island to Stewart Island, from near sea level to 700 m elevation, and covering a sevenfold difference in rainfall.

Seed was collected from each population, and sown in Petri dishes. Ten seedlings from each population, selected at random, were grown, in potting compost (pots 8 cm × 8 cm × 7 cm deep), in the greenhouse, in a randomized block design.

After 16 months' growth, several measurements were made on each plant. Most simple plant characters are affected by overall plant size, and straight analyses of them all would not have been independent. Therefore, most characters were expressed as ratios, reflecting shape rather than absolute size. The characters analyzed were:

leaf length: measured with a microscope eyepiece micrometer;
leaf length/width (L:W) ratio;
real height: measured with the main stem extended vertically;
erectness: natural height/real height. Natural height was measured from the ground to the highest point of the unsupported plant;
apical dominance: real height/length of lowest branch (the latter measured from its attachment on the primary trunk, to the branch apex);
plant shape: natural height/plant width;
branch angle: angle of the lowest branch with respect to the horizontal.

The purpose of this reexpression was to reduce necessary correlations, and indeed within-population cor-