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Scott Pitnick; Greg S. Spicer; Therese Markow

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PHYLOGENETIC EXAMINATION OF FEMALE INCORPORATION OF EJACULATE IN *DROSOPHILA*

SCOTT PITNICK,^{1,2} GREG S. SPICER,³ AND THERESE MARKOW^{4,5}

¹Department of Biology, Syracuse University, 108 College Place, Syracuse, New York 13244-1270

³Department of Biology, San Francisco State University, San Francisco, California 94132-1722

⁴Department of Zoology, Arizona State University, Tempe, Arizona 85287-1501

E-mail: attam@asuvm.asu.edu

Abstract.—Males of some invertebrate species transfer large ejaculates, and many of the substances contained therein are incorporated by females into their somatic and ovarian tissues. These incorporated substances are expected to be energetically costly for males to produce, but benefit males by enhancing their fertilization success and/or the viability of their offspring. A better understanding of the evolution and maintenance of this important reproductive strategy should come from phylogenetic examination. We therefore quantified the extent of ejaculate incorporation by females of 34 species of *Drosophila*. Substantive amounts of male-derived proteins were more frequently detected in female somatic tissue than in ovarian tissue. Substantive ejaculate incorporation by females was found to have arisen numerous times across the phylogeny and tended to be lineage specific in expression. The extent to which evolution of a nutritive function of the ejaculate may have been influenced by phylogenetic history in the genus *Drosophila* is discussed. Macroevolutionary relationships between the amount of ejaculate incorporated by females and other features of species' reproductive and life-history biology, including body size, sperm length, the formation of an insemination reaction in females, and sex-specific ages of reproductive maturity, also were examined after controlling for phylogenetic effects.

Key words.—Accessory gland secretions, comparative analysis, ejaculate, ejaculatory donation, paternal investment, phylogenetic constraint.

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Concepts of maleness and femaleness were originally based strictly on sex differences in the amount of investment in individual gametes (Bateman 1948). Sexual selection was perceived as typically more intense on males than on females because males invest relatively little energy in each gamete and relatively much energy into maximizing mating success. Trivers (1972) modified this purely gamete-based perspective of sex differences by incorporating levels of parental investment into the formula. Males of many species invest a considerable portion of their reproductive effort into parental care, and therefore less into mating effort (Low 1978). However, because females have retained much greater control than males over the fate of individual gametes (Alexander and Borgia 1979; Birkhead et al. 1993; Eberhard 1996), the nature of paternal investment is almost strictly postzygotic.

These theoretical issues are central to the debate over whether courtship feeding evolved because it functionally constitutes male parental effort (prezyotic investment) or mating effort (Wickler 1985, 1986; Gwynne 1986a,b; Quinn and Sakaluk 1986; Sakaluk 1986; Simmons and Parker 1989). In many species males provide their mates with a nuptial gift during courtship and copulation (Thornhill and Alcock 1983), either in the form of a food item (Thornhill 1976), regurgitated crop contents (Steele 1986), part or all of the male's body (Dodson et al. 1983; Elgar 1992), or in the form of seminal products that are either ingested by the female or, more typically, absorbed through her reproductive tract (Leopold 1976; Chen 1984). These seminal products have been termed "ejaculatory donations." Such donations have been demonstrated to enhance female reproductive output in some species, and hence

have been interpreted as a paternal investment (Sakaluk and Cade 1980; Gwynne 1984, 1988; Boucher and Huignard 1987; Butlin et al. 1987; Rutowski et al. 1987; Simmons 1988; Markow et al. 1990). In other species, large ejaculates have been found to enhance male fertilization success by various means but to have no effect on female productivity, and so have been interpreted as a form of mating effort (Greenfield 1982; Jones et al. 1986; Svård and Wicklund 1988, 1991; Wedell and Arak 1989; Wedell 1991, 1993; Reinhold and Heller 1993). Alternatively, these ejaculates may not be nutritive at all, but instead may represent materials that hormonally and/or neuronally influence the female's behavior (remating, oviposition, etc.) in ways that benefit the male (reviewed by Eberhard 1996). In this case, the copious amount of ejaculate transferred in some species may be a consequence of redundant and/or dosage-dependent mechanisms of physiological interaction with the female (Eberhard 1996).

Most of the research on ejaculatory donations has focused on various species of Orthoptera and Lepidoptera. Several species of fruitfly in the genus *Drosophila* similarly produce what have been interpreted as ejaculatory donations, as seminal proteins are incorporated into the somatic tissues and developing oocytes of their mates (Markow and Ankney 1984, 1988; Pitnick et al. 1991). Although the parental effort versus mating effort debate over the evolutionary origin and maintenance of ejaculatory donations cannot presently be resolved from studies of *Drosophila*, these ejaculatory substances have been suggested to contribute to female productivity in *D. mojavensis*, the only species that has been examined in detail (Markow et al. 1990). Moreover, a comparative study of 19 *Drosophila* species revealed a positive association between the size of ejaculatory donations and the formation in females of an insemination reaction plug (Mar-

² Corresponding author during review.

⁵ To whom correspondence should be addressed.

low and Ankney 1988), a swelling or other enlargement of the female's vaginal cavity that has been functionally interpreted as a mating plug (Patterson 1947; Parker 1970; Markow and Ankney 1988). The association between these two traits was presumed due to the secondary evolution of the insemination reaction as an adaptation to protect the male's paternal investment from being cuckolded by other males (Markow 1988).

The ejaculate production strategies of many *Drosophila* species are also unusual in that relatively few, very long sperm are produced. For example, sperm can be five to 20 times the total body length of males (Hihara and Kurokawa 1987; Joly et al. 1991; Pitnick and Markow 1994a,b; Pitnick et al. 1995a,b). In these species, the energetic investment of males in sperm, per se, may be substantial. In one species, *D. hydei*, which produces 23-mm long sperm, the costs of producing sperm have been suggested to contribute to body size-related variation in male reproductive success (Pitnick and Markow 1994a). Production of giant sperm may cause populations to be sperm limited (Pitnick 1993) and confer many costs upon males, including the production and transfer to females of relatively few gametes (Pitnick 1996), the need to develop and maintain huge testes (Pitnick 1996), and delayed male sexual maturation (Pitnick et al. 1995b). The relationship between sperm length and male ejaculatory donations has not been examined, nor has the relative cost to males of producing nutritive accessory gland secretions.

Our understanding of the evolutionary significance of ejaculatory donations can be furthered by examination of the evolutionary history of this trait, as revealed by phylogenetic analysis, within a taxon with diverse ecologies such as *Drosophila*. We therefore quantified the extent of female incorporation of male ejaculatory proteins into their ovarian and somatic tissues in 34 species of *Drosophila*, and then examined these data with respect to phylogenetic relatedness. This approach also facilitated examination of the correlated evolution of this trait relative to other reproductive and life-history traits, including sperm length, the formation of an insemination reaction in females, body size, and sex-specific ages of reproductive maturity. Relationships among these traits were examined, after controlling for phylogenetic effects, to test a priori predictions regarding the cost to males of producing presumptive ejaculatory donations.

It is worth noting that caution must be used when attempting to consider the evolution of ejaculatory donations based on empirical data. Although the term "donation" connotes the male component of the phenomenon, what is implied is the coordinated evolution of both male and female physiological processes. Males must synthesize or accrue sufficient quantities of substances in the ejaculate that are useful to the female, and females must have mechanisms to selectively transport these substances across the wall of their reproductive tracts, modify these molecules if necessary, and then concentrate the products in target tissues. Empirical evidence for both of these processes, however, is often lacking, as is evidence of a positive relationship between the expression of these processes and fitness of each participant. Moreover, it is possible for males to transfer relatively large ejaculates that females do not utilize, as well as for ejaculate quantity and composition to have evolved for reasons other than nu-

trition, yet for females to usurp these materials to serve their own needs and those of their offspring. Consequently, because we only quantified the female side of this evolutionary dynamic in the present study, we use the term "ejaculate incorporation" throughout the remainder of the paper when referring to our data, and "ejaculate donation" only when referring theoretically to evolved paternal investment.

MATERIALS AND METHODS

For 34 species of *Drosophila*, we present an analysis of the phylogenetic distribution in the amount of male-derived substance incorporated by females into their ovaries and somatic tissues as measured by corrected disintegrations per minute (see below). Data for 29 of these species were uniquely collected for these analyses. Data for the remaining five species (*D. melanogaster*, *D. wassermani*, *D. nanoptera*, *D. pachea*, and *D. acanthoptera*) were reported previously by Pitnick et al. (1991), but were reanalyzed as described below. In addition, evolutionary relationships between incorporation size and other traits were examined. Values for sperm length, thorax length, and sex-specific ages of reproductive maturity are those reported by Pitnick et al. (1995b). These analyses were conducted for 32 species, as thorax length, sperm length, and maturation data were not available for *D. putrida* and *D. repleta*. Values for degree of female insemination reaction were available for 22 species, as reported by Patterson and Stone (1952; table 67, p. 366), Markow and Ankney (1988), and Alonso-Pimentel et al. (1994).

Species and Culturing

Measurements were made on flies from laboratory cultures derived from multifemale collections. Species collection information is provided for *D. melanogaster*, *D. wassermani*, *D. nanoptera*, *D. pachea*, and *D. acanthoptera* by Pitnick et al. (1991), for *D. nigrospiracula* by Polak (1993), and for *D. subpalustris*, *D. recens*, *D. guttifera*, and *D. putrida* by Spicer and Jaenike (1996); *D. straubae* (ORV 22), *D. parisiensis* (902.9), and *D. mayaguana* (ORV 29A) were provided by William B. Heed; *D. busckii* (13000-0081.0), *D. navojoa* (15081-1361.0), *D. mettleri* (15081-1502.0), *D. repleta* (15084-1611.0), *D. eohydei* (15085-1631.0), *D. bifurca* (15085-1621.0), *D. melanica* (15030-1141.2), *D. micromelanica* (15030-1151.0), *D. americana* (15010-0951.0), *D. texana* (15010-1041.0), *D. novamexicana* (15010-1031.0), *D. lummei* (15010-1011.2), *D. virilis* (15010-1051.0), *D. kanekoi* (15010-1061.0), *D. ezoana* (15010-0971.0), *D. littoralis* (15010-1001.0), *D. borealis* (15010-0961.6), *D. lacicola* (15010-0991.0), and *D. montana* (15010-1021.19) were obtained from the National *Drosophila* Species Resource Center (Bowling Green State University, Ohio); *D. arizonae* was collected by T. A. Markow in San Carlos, Sonora, Mexico, in May 1988.

Every effort was made to culture all species under standardized conditions. All flies were reared under uncrowded conditions on medium in 200-mL bottles with live yeast at $24 \pm 1^\circ\text{C}$ at an approximate 12L:12D photoperiodic cycle and an approximate 1:1 sex ratio. However, some species had unique culturing requirements: *D. busckii* was reared on instant *Drosophila* medium (Formula 4-24, Carolina Biological

Supply Co.); *D. recens*, *D. subpalustris*, *D. guttifera*, and *D. putrida* were reared on instant medium to which dry powdered mushroom was added and a quarter of a grocery-store variety mushroom cap was placed in each culture; all remaining species were reared on standard banana medium; autoclaved, necrotic tissue of senita cactus, *Lophocereus schottii* (Englemann) Britton and Rose, was added to medium for *D. pachea*.

Males of each species were radiolabeled by transferring 50 first-instar larvae from culture bottles to an 8-dram vial containing 5 g of appropriate culture medium to which 50 μ Ci of an L-amino acid mixture of C^{14} (ICN 10147 or NEN NEC-445) had been added. Flies were collected on the day of eclosion and sorted by sex without anaesthetization. Radiolabeled males were then maintained in 8-dram vials containing medium, live yeast, and no more than nine other same-sex individuals; radiolabeled females were discarded.

Data Collection

Within two to four days of becoming reproductively mature (Pitnick et al. 1995b), single, virgin, radiolabeled males and virgin, nonradiolabeled females were gently aspirated into vials for mating. The duration of copulation was recorded to eliminate any "pseudocopulations"; in the rare event of a pseudocopulation, the female was substituted. Immediately following copulation, the sexes were separated by aspirating the male into a new vial. Flies were then undisturbed for six to eight hours, at which time they were processed for scintillation. Markow and Ankney (1988) have demonstrated, across a broad array of *Drosophila* species, that any measurable incorporation by females of radiolabeled ejaculate will occur within a six-hour period.

To determine the amount of male-derived protein incorporated by females into their ovarian and somatic tissue, respectively, each fly was processed for scintillation counting as follows. Thorax length of the ether-anaesthetized fly was measured using the ocular micrometer of a dissecting microscope. The fly was then washed by vigorously shaking it for 30 seconds in a 1.5-mL microcentrifuge tube containing phosphate-buffered saline (PBS) and 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 PBS, they were decapitated as some eye pigments are a natural quenching agent that could reduce counting efficiency during scintillation. Females were dissected in PBS; their reproductive tracts were removed intact and the remaining somatic tissues were collected into a separate dish to avoid contamination. The egg-bearing ovaries were then isolated from the distal end of the common oviduct using two fine probes. The ovarian and somatic tissues were then each vigorously rinsed twice in microcentrifuge tubes containing PBS as described above. For the ovary and somatic tissue samples, parts from three females were pooled for each experimental replicate. Males were scintillated individually. To control for background "noise" of the scintillation counters, ovaries and somatic tissue of females that had been mated to unlabeled males were prepared for scintillation counting by identical procedures. An equal number of control

and experimental replicates (see Fig. 2) were executed within each species to accommodate pairing for statistical analyses, as described below.

All samples were placed in a scintillation vial containing 100 μ L of Scintigest tissue solubilizer (Fisher Scientific), and crushed with glass rods prior to digestion for 24 hours at 50°C. Glacial acetic acid (17.5 μ L) was added to neutralize the solution, and 5 mL of ScintiVerse II scintillation fluid (Fisher Scientific) was added. Each vial was then vortexed and allowed to settle overnight at 24°C prior to counting in a Packard Tri-carb 1500 or 1600TR Liquid Scintillation Analyzer. Counts per minute were converted to disintegrations per minute (DPM) following a standard quench curve.

Standardization of Radiolabel Data for Interspecific Comparison

The number of DPM incorporated by females was expected to depend, in part, upon the extent of radiolabel incorporation by their mates. For example, comparing data for two conspecific females, one may be found to have twice as many DPM in her ovaries than the other, not because she incorporated twice as much male-derived protein as the other, per se, but because her mate's ejaculate contained twice the concentration of radiolabel. This phenomenon posed a particular problem for comparison of data among species, as species differed in both larval development time and adult body size, which were expected to affect the concentration of radiolabel incorporated by males.

We resolved this problem by standardizing all DPM data by the concentration of radiolabel in males as follows: (1) Using data we previously reported for thorax length and total dry body mass of males of 11 species (of which 10 are included in the present study; Pitnick 1996), we calculated the relationship between these traits by a least-squares (model I) linear regression of \log_{10} -transformed variables. The resulting power equation (\log_{10} body mass = 2.53 + [\log_{10} thorax length]^{1.86}) was then extrapolated to all 35 species examined in the present study to convert values of mean male thorax length to mean male dry body mass on a per replicate basis ($n = 3$ males per replicate). (2) Each value of mean male dry body mass was then divided by mean male DPM to calculate the mean number of DPM per μ g dry body mass for males of each replicate, a variable hereafter referred to as "male radiolabel concentration." Regression analyses using \log_{10} -transformed species mean values confirmed that variation among species in male radiolabel concentration was significantly contributing to interspecific variation in the number of DPM detected in female ovaries and somatic tissue, and therefore needed to be controlled statistically (ovaries: $F = 6.49$, $df = 1, 32$, $r^2 = 0.17$, $P = 0.016$; soma: $F = 6.60$, $df = 1, 32$, $r^2 = 0.17$, $P = 0.015$). (3) The number of DPM detected in female ovaries and soma were therefore corrected on a per replicate basis using the following formulae:

$$\begin{aligned} &\text{Corrected DPM ovaries} \\ &= [(\text{DPM ovaries} - \text{DPM paired control} \\ &\quad \text{ovaries})/\text{male radiolabel concentration}] * 100, \quad (1) \end{aligned}$$

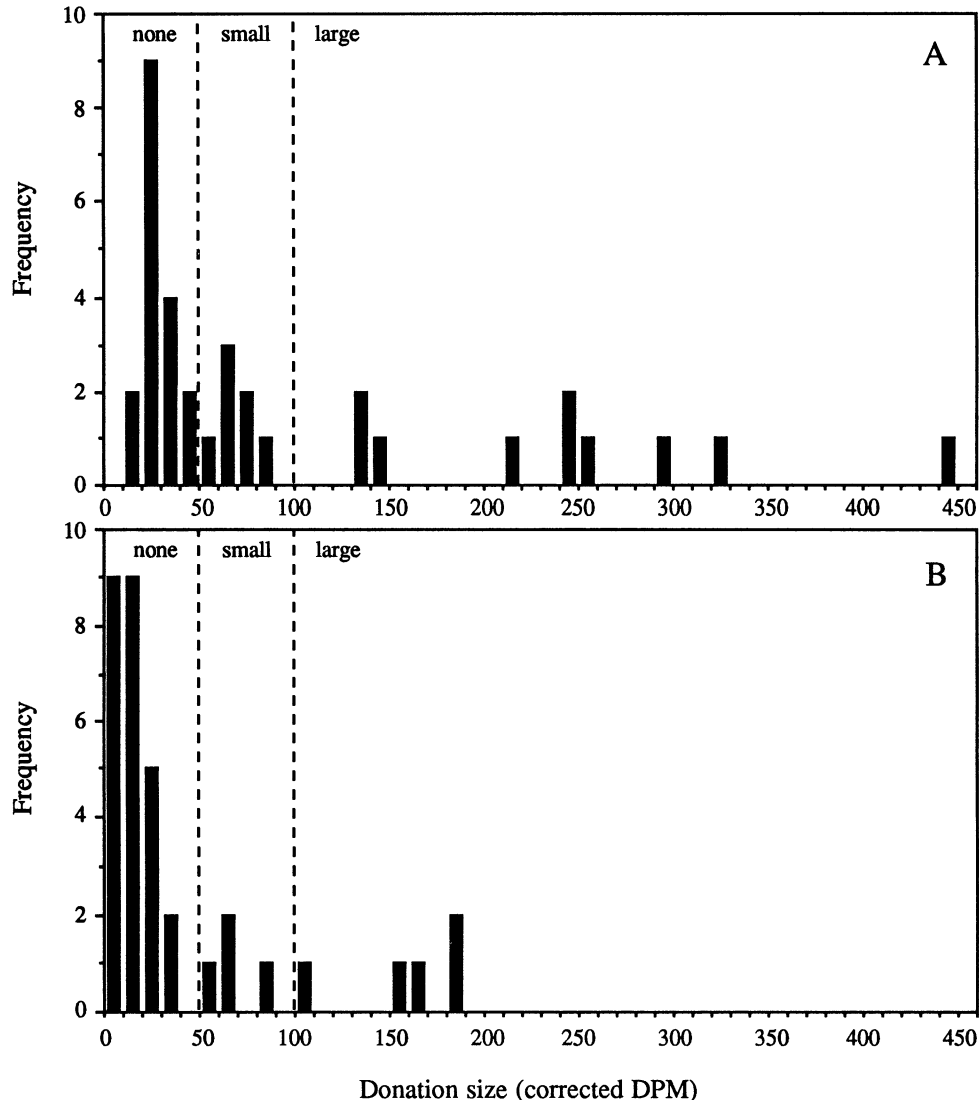


FIG. 1. Frequency distributions for mean size of female ejaculatory incorporation (corrected DPM) into (A) somatic tissue; and (B) ovaries of 34 species of *Drosophila*. Dotted lines distinguish donation size categories (see text for details).

and

$$\text{Corrected DPM soma} = \frac{[(\text{DPM soma} - \text{DPM paired control soma}) / \text{male radiolabel concentration}] * 100. \quad (2)$$

These formulae simultaneously remove the effects of background noise of the scintillation counters and differential male radiolabel concentrations.

Statistical Analyses

For the purpose of mapping somatic and ovarian incorporation size onto a preexisting phylogeny, it was necessary to categorize the data. As fewer than three replicate experiments were performed for many species, statistical methods of categorization were not applicable. We therefore examined frequency plots for corrected somatic and ovarian incorporations and conservatively categorized the data along natural breaks in the distributions (Fig. 1). For both datasets, cor-

rected DPM values from 0 to 50 were categorized as “no incorporation,” values from 51 to 100 were categorized as “small incorporation,” and values greater than 100 were categorized as “large incorporation.” Consistent with our categorization here, male *D. melanogaster* have been reported to provide “no incorporation” to female somatic and ovarian tissues, and male *D. mojavensis* have been reported to provide a “large incorporation” to both female somatic and ovarian tissues (Markow and Ankney 1984; Pitnick et al. 1991). The tree and character mapping manipulations were accomplished by using the program MacClade (Maddison and Maddison 1992).

To comparatively examine evolutionary relationships between characters, it was first necessary to control for phylogenetic effects (Felsenstein 1985; Harvey and Pagel 1991). We used Felsenstein’s (1985) method of phylogenetically independent contrasts, which provides statistical independence of data points. Independent contrasts were computed (using

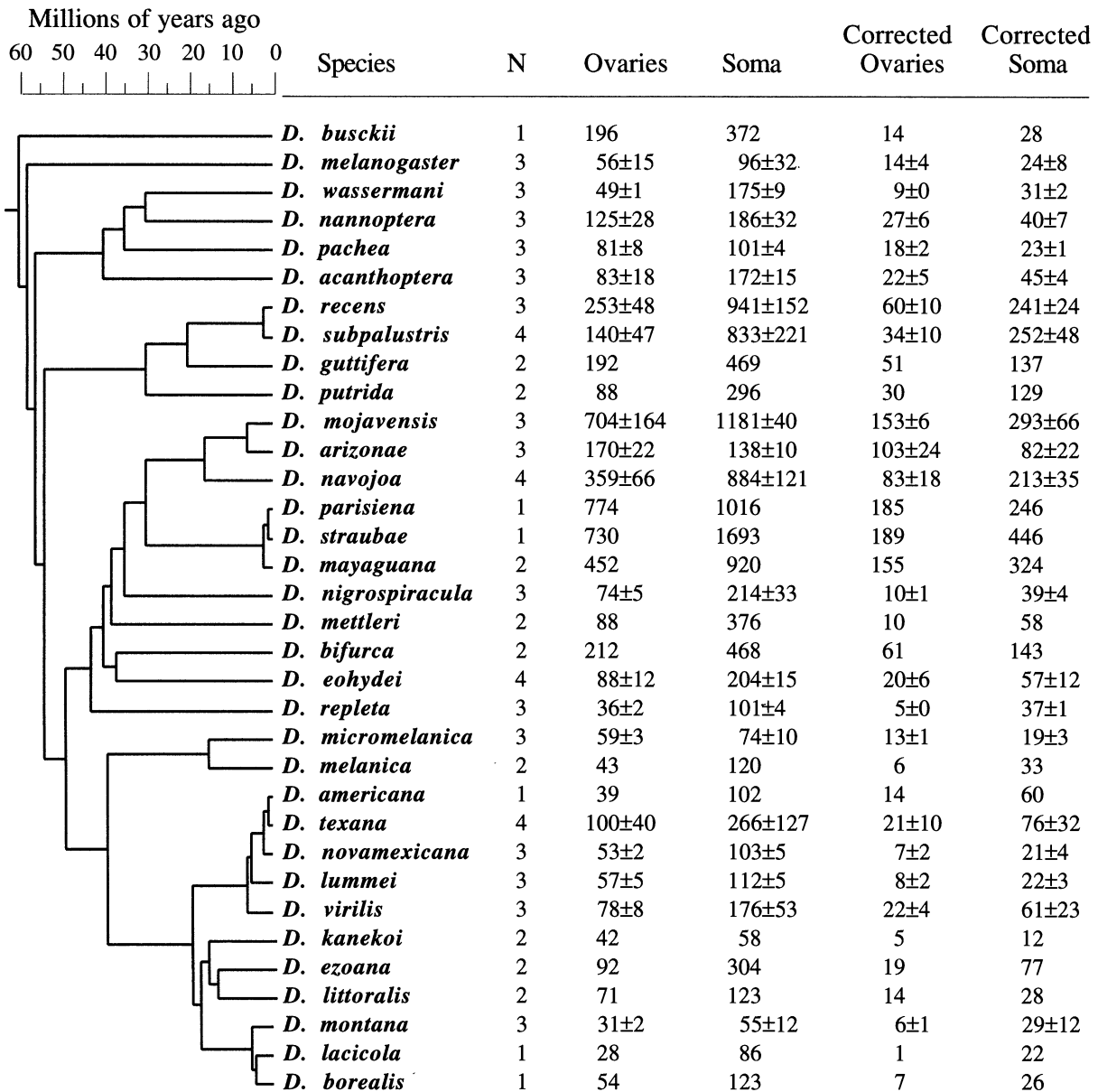


FIG. 2. The phylogeny and amount of male-derived radiolabeled substance (mean disintegrations per minute, DPM, \pm SE) incorporated by females into soma and ovaries for 34 species of *Drosophila*. Both absolute and corrected DPM values (see text for details) are presented. The scale bar represent times since divergence.

the phylogenetic topology and branch lengths presented in Fig. 2) using the Phenotypic Diversity Analysis Program of Garland et al. (1993) and the CMSINGLE program of Martins and Garland (1991). Each variable, except the degree of insemination reaction, was \log_{10} transformed prior to computation of contrasts. Standardization was accomplished by dividing each contrast by its standard deviation (the square root of the sum of its branch lengths; Garland et al. 1992). Adequacy of this procedure was verified by a lack of significant linear or nonlinear trends in plots of the absolute value of each standardized independent contrast versus its standard deviation (Garland et al. 1992, 1993). The analyses presented employ a model that assumes gradual evolutionary change in variables, with branch lengths equal to estimated times of

divergence (Felsenstein 1985; Martins and Garland 1991). Conclusions did not change qualitatively when a punctuational model of evolutionary change was assumed (i.e., all branch lengths equal; Martins and Garland 1991) or when "minimum evolution" methods were used (Martins and Garland 1991).

The phylogeny was compiled from a number of sources. The higher level relationships were inferred from several morphological (Throckmorton 1975; Grimaldi 1992) and molecular (Beverley and Wilson 1982, 1984; Spicer 1988; Sullivan et al. 1990; Caccone et al. 1992; DeSalle 1992; Pelandakis and Solignac 1993; Kwiatowski et al. 1994; Powell and DeSalle 1995) datasets, some of which were reanalyzed to construct the figure. The lower-level relationships were de-

terminated both by published sources and by our unpublished DNA sequence comprising about 1.5 Kb of the mitochondrial cytochrome oxidase subunits (G. S. Spicer, unpubl. data). Phylogenetic relationships for *melanogaster* (Ashburner 1989) and *quinaria* (Spicer and Jaenike, 1996) species groups were inferred entirely from the literature. The *virilis* (Spicer 1991, 1992) and *repleta* (Wasserman 1992; Spicer and Pitnick 1996) species groups were determined by using a combination of published phylogenies and by our sequencing studies. Relationships for the *nannoptera* and *melanica* species groups were inferred entirely from our sequencing studies. Details of the phylogeny will be published elsewhere.

RESULTS

Phylogenetic Distribution of Ejaculatory Incorporations and Insemination Reactions

Substantial interspecific variation was observed in the size of ejaculatory incorporations into both female somatic and ovarian tissues (Figs. 1,2). With respect to somatic tissues, 50% of the species were found to have no incorporation ($n = 17$), 21% showed a small incorporation ($n = 7$), and 29% had a large incorporation ($n = 10$) (Fig. 1a). Fewer species were found to have ovarian incorporations: 74% of species had no incorporation ($n = 25$), 12% showed a small incorporation ($n = 4$), and 15% had a large incorporation ($n = 5$) (Fig. 1b).

It is clear from examination of the somatic and ovarian incorporation data distributed across a phylogeny that these traits have independently arisen (and/or been lost) numerous times and are therefore somewhat evolutionarily labile (Fig. 3). These results are consistent irrespective of the optimization algorithm employed for reconstructing the character state distributions (Swofford and Maddison 1987); the Deltran optimization is figured. Both the presence and size of incorporations, however, were not randomly distributed across the phylogeny (Fig. 3). Somatic incorporations were fairly widespread, being found in some species from most lineages. However, nine of the 10 species found to have large somatic incorporations were confined to just two narrowly defined lineages. All three examined members of the *quinaria* species group (*D. subpalustris*, *D. recens*, *D. guttifera*), and *D. putrida* of the closely related *testacea* group had large somatic incorporations. Similarly, five of the six examined members of the *mojavensis* cluster of the *repleta* species group (*D. mojavensis*, *D. navajoa*, *D. straubae*, *D. parisiensis*, and *D. mayaguana*) had large somatic incorporations. The remaining species from this lineage (*D. arizonae*) had a small incorporation. The only remaining species found to have a large somatic incorporation was *D. bifurca* of the *hydei* subgroup of the *repleta* species group.

Phylogenetic distribution of the ovarian incorporation data was even more localized, with species making either a small or large incorporation being found in only three lineages (Fig. 3). Large ovarian incorporations were found only in species (five of six) belonging to the *mojavensis* cluster of the *repleta* species group. The sixth species of this lineage had a small incorporation. Conversely, there were three lineages for which multiple species were examined in which no species was found to have an ovarian incorporation: the *nannoptera*

species group, the *melanica* species subgroup, and the *virilis* species group.

We also examined the phylogenetic distribution of insemination reactions among the 22 species examined in this study for which these data were available (Fig. 4). This character also revealed a distinct pattern of nonrandom phylogenetic distribution. No two species related at the subgroup level or below differed in their insemination reaction classification.

Relationships between Ejaculatory Incorporations and Other Traits

We examined relationships between the size of somatic and ovarian incorporations and body size, sperm length, sex-specific ages of reproductive maturity, and formation of the insemination reaction in females, after controlling for phylogenetic effects. There was a significant positive relationship between the size of ovarian and somatic incorporations across species (Fig. 5; reduced major axis slope = 1.11; $F = 19.90$; $r^2 = 0.40$; d.f. = 30; $P = 0.0001$).

Due to preexisting knowledge of significant relationships between male thorax length, sperm length, and male age at maturity (Pitnick et al. 1995b), the relationships between these variables and incorporation size were examined in a multiple regression model. These analyses revealed that the size of both somatic and ovarian incorporations were unrelated to male thorax length and sperm length. However, significant positive relationships were found between male age at maturity and the size of both types of incorporation (Table 1). No relationship was found between female age at maturity and size of either type of incorporation, as determined by least-squares (model I) linear regression through the origin (soma: reduced major axis slope = 2.14; $F = 1.59$; $r^2 = 0.05$; df = 30; $P = 0.217$; ovaries: reduced major axis slope = 2.38; $F = 0.543$; $r^2 = 0.02$; df = 30; $P = 0.467$). The degree to which an insemination reaction forms in females following mating did not explain a significant amount of the interspecific variation in either form of ejaculatory incorporation (Table 2).

DISCUSSION

Interpreting Nutritive Function of Ejaculates

The male ejaculate tends to be complex in composition, which is not surprising given the many diverse functions of its constituents (reviewed by Leopold 1976; Chen 1984; Eberhard 1996). In addition to facilitating sperm transport to the female during insemination, the ejaculate may influence movement of the male's sperm to the female's sperm-storage organs, the maintenance of sperm viability, the fate of other males' sperm already present within the female, egg maturation, ovulation, oviposition, and receptivity of the female to further mating, as well as provide a source of nourishment to the female and/or her progeny.

Demonstrating that ejaculatory substances contribute nutrients to egg production is complicated by the difficulty in experimentally discriminating nutritive from non-nutritive effects of male ejaculatory substances on female productivity. Support for a nutritive role of the ejaculate has primarily come from experiments in which egg production (number

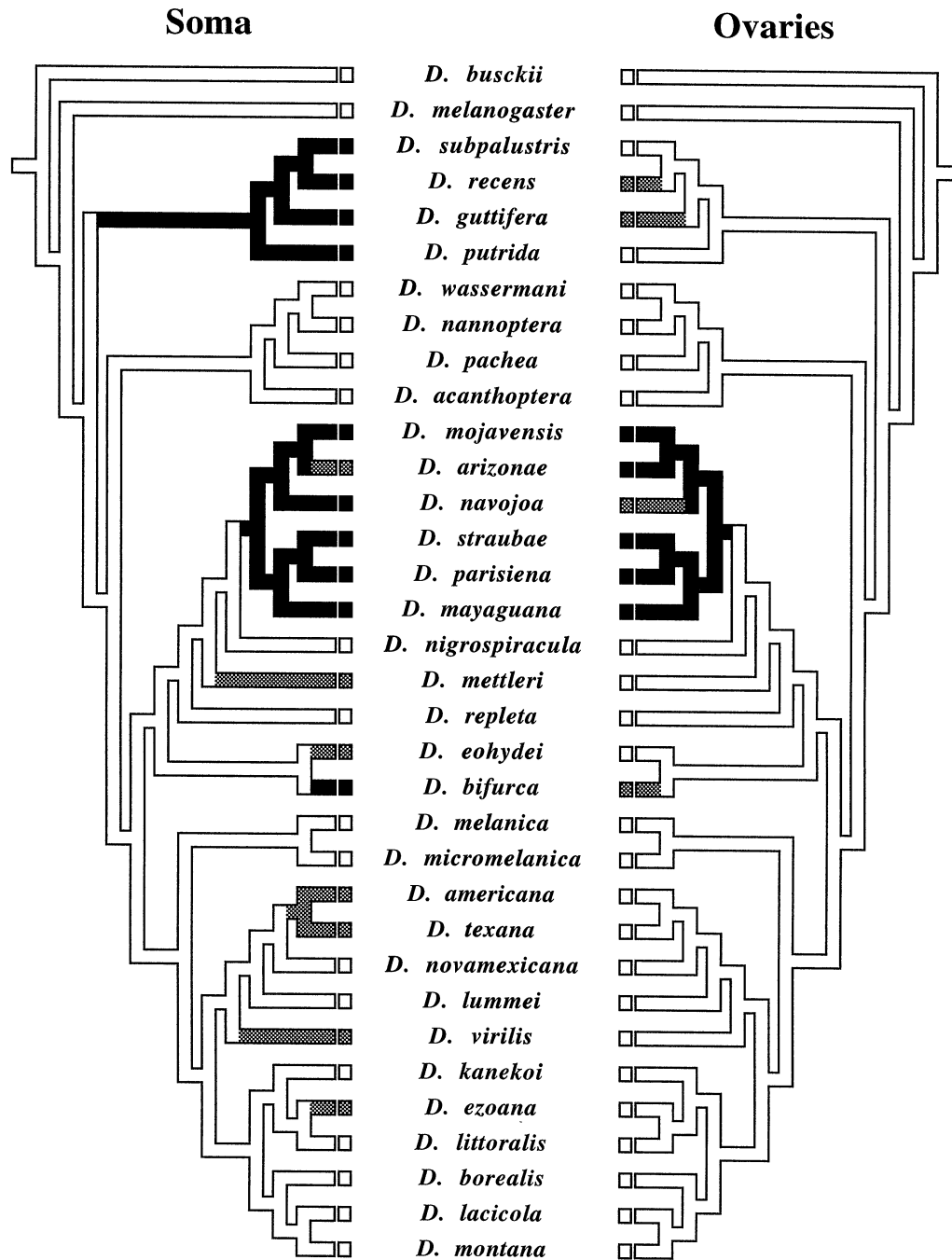


FIG. 3. Phylogeny for 34 species of *Drosophila* illustrating distribution of ejaculatory incorporations to female soma and ovaries. The Deltran optimization is presented. White bars indicate no incorporation (0–50 corrected DPM); gray bars indicate a small incorporation (51–100 corrected DPM); black bars indicate a large incorporation (> 100 corrected DPM).

and/or size) is compared among single-versus multiple-mated females (Sakaluk and Cade 1980; Simmons 1988), among females mated to virgin versus recently mated males that are ejaculate, but not sperm, depleted (Rutowski et al. 1987; Markow et al. 1990), or among females that have been permitted to feed for different lengths of time on the spermatophylax, or nonsperm containing portion of the spermatophore (Gwynne et al. 1984). Each of these experimental protocols likely varies the amount of male accessory gland se-

cretions that presumptively stimulate female egg production and oviposition in addition to the presumptively nutritive secretions. Productivity differences among experimental treatment groups, therefore, cannot be confidently interpreted.

Support for a nutritive role of ejaculatory substances is strengthened by demonstrations that male-derived substances or their derivatives are incorporated into developing oocytes (Boggs and Gilbert 1979; Boggs and Watt 1981; Bowen et

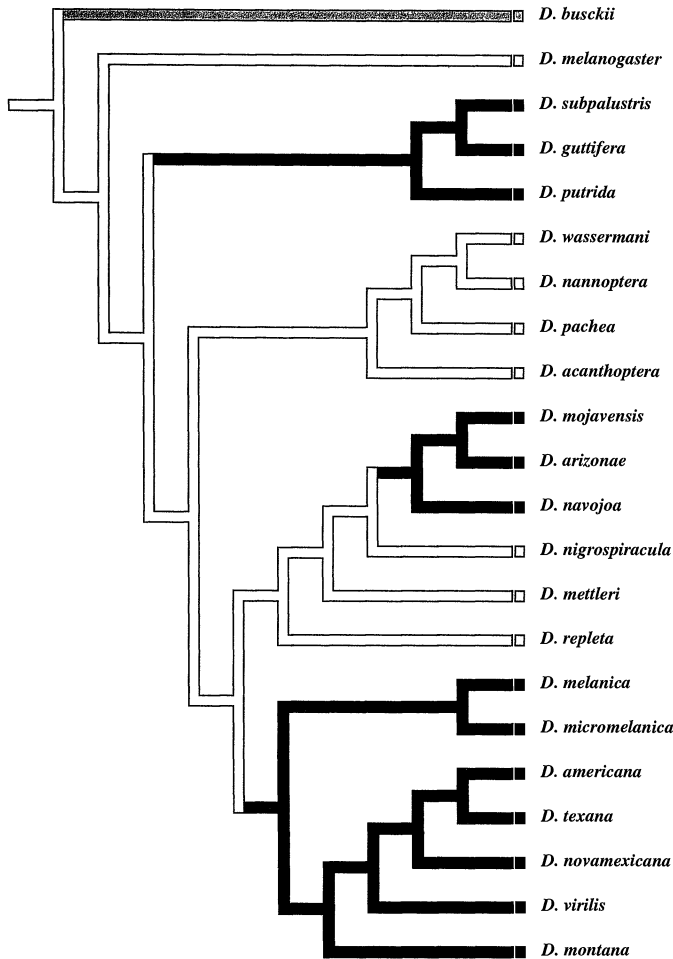


FIG. 4. Phylogeny for 22 species of *Drosophila* illustrating distribution of the formation of insemination reactions in females. White bars indicate no reaction; gray bars indicate a moderate reaction; black bars indicate a strong reaction.

al. 1984; Markow and Ankney 1984, 1988; Boucher and Huignard 1987; Bownes and Partridge 1987; Butlin et al. 1987; Pitnick et al. 1991; Simmons and Gwynne 1993). Data of this sort must also be cautiously interpreted, however, as the techniques used to detect male secretions in eggs are so highly sensitive that quantities too small to be of likely nutritional value can be found (e.g., Bownes and Partridge 1987; Cheeseman and Gillott 1989). In the present study, for example, radiolabel from the male ejaculate above background

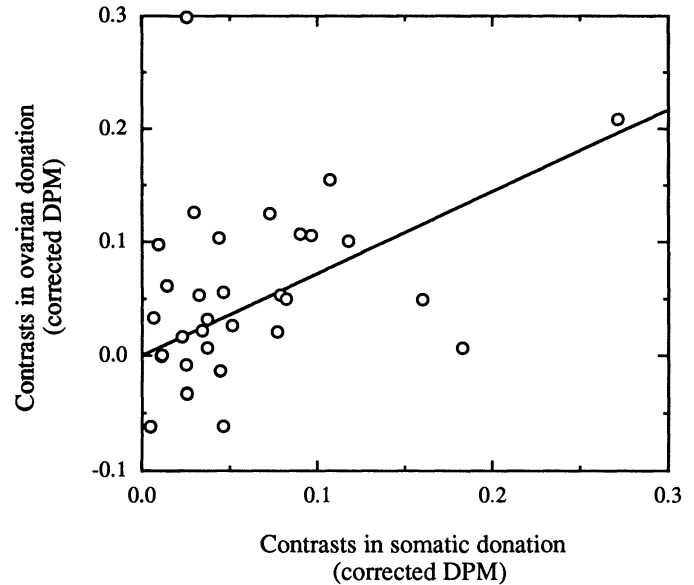


FIG. 5. The interspecific relationship between mean size of ejaculatory incorporation into female ovaries and female soma. Each point is a standardized independent contrast; linear regression line was forced through the origin.

(control) levels was detectable in nearly all species examined (Fig. 2).

That some male accessory gland products find their way into developing oocytes in nearly all species is not surprising. Very few seminal products affect the female from within her reproductive tract; typically they enter the female hemolymph unaltered, often with great rapidity, and thereby arrive at the target organs. Most of these substances are believed to interact with the female's nervous system (see reviews by Chen 1984; Gillott 1988; Eberhard 1996). Moreover, two selection pressures associated with sexual selection by cryptic female choice (Thornhill 1983) are expected to favor the production of large and complex ejaculates. First, there is selection on males to provide redundant signals to females, that is, to produce multiple substances that function in triggering each female behavioral or physiological response (reviewed by Eberhard 1996). Second, because many female responses are dosage-dependent, selection favors males who increase the intensity of their signals by transferring greater quantities of substance (reviewed by Eberhard 1996). Given the relatively high metabolic activity of the ovaries, it is not surprising that

TABLE 1. Results of multiple regression analyses (forced through the origin) of standardized independent contrasts examining relationships between dependent variables: somatic and ovarian incorporation size (corrected DPM), and the independent variables: thorax length, sperm length, and male age at maturity.

Dependent variable	Independent variable	Regression coeff. (SE)	<i>t</i>	<i>P</i>
Somatic incorporation	Thorax length	-1.38 (1.32)	-1.044	0.306
	Sperm length	-0.50 (0.35)	-1.409	0.170
	Male age at maturity	0.96 (0.47)	2.056	0.049
Ovarian incorporation	Thorax length	-1.60 (1.41)	-1.132	0.267
	Sperm length	-0.37 (0.38)	-0.971	0.340
	Male age at maturity	1.21 (0.58)	2.424	0.022

TABLE 2. Results from least-squares (Model I) linear regressions (forced through the origin) of standardized independent contrasts examining relationships between ejaculate incorporation size (corrected DPM) and the independent variable: insemination reaction.

Dependent variable	Reduced major axis slope	F	r ²	P
Somatic incorporation	0.879	0.808	0.045	0.719
Ovarian incorporation	0.807	0.911	0.051	0.691

some of these male-derived substances would be used to build eggs.

In the absence of information regarding the molecular identification and activity of presumptively nutritive ejaculatory substances, therefore, interpretation of their role must be subjectively based on their quantity. We have identified several species in which a very large quantity of seminal material is incorporated into oocytes (Figs. 1,2), wherein the interpretation of nutritive function is intuitive. Our assessment that no nutritive ejaculatory incorporation has evolved in those species for which very little seminal material was found in developing oocytes, however, must be viewed with greater caution, as nutritive donations/incorporations may be qualitative rather than quantitative in nature, with very small amounts being both costly for males to produce and limiting for female egg production (Marshall 1982; Gwynne 1988; Marshall and McNeil 1989).

Relationships between Ejaculatory Incorporations and Other Traits

Sex-Specific Ages of Reproductive Maturity.—Among *Drosophila* species there is striking variation in rates of sexual maturation. Whereas in some species flies are reproductively competent on the day of the final moult (eclosion), in other species many days posteclosion are required for sexual maturation. A comparative study of 42 species of *Drosophila* (Pitnick et al. 1995b) revealed that the rate of male maturation was much more variable, and typically more protracted, than the rate of female maturation (ranges: males, 0 to 19 d; females, 1 to 8 d).

Life-history theory contends that the advantages of rapid reproduction and short generation time must be balanced by trade-offs with other fitness components to explain the evolution of delayed sexual maturity (Roff 1992; Stearns 1992). Pitnick et al. (1995b) therefore assumed that delayed maturity was costly and hypothesized that delayed male maturity in *Drosophila* represented a constraint on the need to produce or accrue costly materials necessary for successful reproduction. This hypothesis was examined with respect to sperm length, as the production of long sperm has been shown to bear significant costs (Pitnick and Markow 1994a; Pitnick 1996). A significant amount of the variation in male age at maturity was explained by variation in sperm length, thereby supporting the hypothesis.

Here we examine the hypothesis that some interspecific variation in sex-specific ages of sexual maturity is due to the time and energy requirements of ejaculatory donations. As such costs are expected to be associated with the production of nutritive ejaculatory secretions, but not with their incor-

poration, we predicted there to be a positive relationship between donation size and male age at first reproduction, yet no relationship with female age at first reproduction. As predicted, significant positive relationships were found between the size of both ovarian and somatic incorporations and male maturation time. These relationships suggest that female ejaculate incorporation (as male ejaculate production was not measured) is correlated with male production of ejaculatory donations and that such production is costly to males. Also as predicted, there was no significant relationship between the amount of ejaculate incorporated and female age of maturity.

Sperm Length.—There is tremendous variation among *Drosophila* species in the length of their sperm (e.g., Pitnick et al. 1995b). While the functional significance of longer sperm tails has not been determined for *Drosophila* (see Karr 1991; Pitnick and Markow 1994a), they have not evolved to serve some postfertilization function (Bressac et al. 1994; Karr and Pitnick 1995; Pitnick et al. 1995a). Consequently, long sperm and nutritive ejaculatory incorporations are not alternative means by which males provision offspring.

Nevertheless, there is reason to expect a relationship between the length of sperm and the size of ejaculatory incorporations. As both longer sperm (Pitnick and Markow 1994b; Pitnick et al. 1995b; Pitnick 1996) and ejaculatory incorporations are costly to produce, and because males have limited energy to invest in the production of ejaculates, we predicted that males of any given species might be likely to produce either a sizeable ejaculate incorporation or long sperm, but not both. The expectation of a negative relationship was not met; there was no significant relationship between the size of ejaculate incorporations and sperm length (Table 1). It is however notable that male *D. bifurca*, which produce the longest known sperm (Pitnick et al. 1995a) in addition to a moderate incorporation, exhibit extremely delayed reproductive maturation (17 d; Pitnick et al. 1995b), which may reflect the additive cost of this dual investment.

Insemination Reactions.—In certain *Drosophila* species, the anteroventral portion of the female's vaginal cavity becomes distended to three or four times its normal size during or shortly following copulation. This insemination reaction, as first described by Patterson (1946), appears as an opaque mass that persists for approximately eight hours, although the actual timing of events varies among species (Patterson and Stone 1952). The reaction results from swelling of the epithelial cells of the vaginal wall, either due to increased secretion by these cells or by the uptake of fluid, such as that contained in the semen. Examination of females mated to males transferring sperm-free semen (Patterson 1947) and of females artificially inseminated with extracts of various male organs (Lee 1950) indicates that live sperm are not necessary for the induction of the insemination reaction, but that the active component is likely derived from the testes. The extent to which the insemination reaction occurs varies greatly among species (Wheeler 1947; Patterson and Stone 1952).

The insemination reaction has been speculated to be analogous to a mating plug (Patterson 1947; Parker 1970), and therefore to function in delaying female remating. Consistent with this hypothesis, Markow and Ankney (1988) found a positive correlation between formation of the insemination reaction and the size of male ejaculatory incorporations

among 18 species of *Drosophila*. A positive relationship between these traits is concordant with the theoretical expectation that in species with nuptial gifts, males are expected to evolve mechanisms to manipulate females to ensure rapid use of the gift and paternity of offspring receiving the gift (Parker and Simmons 1989; Simmons and Parker 1989).

Despite using identical experimental protocol, the present study found no relationship between formation of the insemination reaction and the size of ejaculatory incorporations among 22 species. Several factors may have contributed to the contrasting conclusions of the two studies. First, the two studies shared only eight species in common. Second, Markow and Ankney (1988) did not control for body size and phylogenetic effects in their data analyses. Body size did contribute to interspecific variation in male radiolabel concentration in the present study, and the evolution of body size (Pitnick et al. 1995b), ejaculate incorporations (Fig. 3), and insemination reactions (Fig. 4) have all been influenced by phylogenetic history.

Determining the evolutionary significance of the insemination reaction is further complicated by a lack of detailed understanding of the physiological basis of this postmating vaginal response. Recent ultrastructural and biochemical analyses of vaginas of five species of *Drosophila* following mating suggest that the insemination reaction, as defined by Patterson (1946), is unlikely to be a single phenomenon (Alonso-Pimentel et al. 1994). Alonso-Pimentel et al. (1994) suggest three terms to describe postmating vaginal features in *Drosophila*: the true insemination reaction, the mating plug, and the sperm sac. Distinguishing between mating plugs and true insemination reactions is difficult without careful examination, as demonstrated by Alonso-Pimentel et al. (1994) for *D. hexastigma*, which has a mating plug but was described by Patterson (1946) as exhibiting a strong insemination reaction. Only to the extent that mating plugs and true insemination reactions evolved to serve the same function, therefore, does the present study reveal there to be no evolved relationship between these traits and ejaculatory incorporations.

Phylogenetic Distribution of Ejaculatory Incorporations

A distinct phylogenetic pattern in ejaculatory incorporations was detected; closely related species were more likely to exhibit incorporations of similar size than were more distantly related species (Fig. 3). Because many ejaculatory components function by interacting with the female outside of her reproductive tract, the detection of substantive levels of ejaculate-derived substances in female somatic tissue of many species is to be expected. Distinguishing for which of these species the data can be interpreted as a donation of nutrients by the male versus some ulterior function is difficult. In contrast, the accumulation of male-derived substances in developing oocytes can more confidently be interpreted as a nutrient donation by males. To the extent that the macroevolutionary pattern in ejaculatory donations has been shaped by history, therefore, a more discernible pattern for ovarian than somatic incorporations would be expected, and was observed (Fig. 3).

The observed phylogenetic patterns in ejaculatory incor-

porations may be largely attributable to phylogenetic niche conservatism (Harvey and Pagel 1991). When a niche becomes vacant, it is most likely to be invaded and maintained by a species occupying a similar niche in an adjacent environment, because of its superior competitiveness. As a consequence of this adaptive process, species within lineages are more likely to occupy similar environments and to have similar ecologies (and therefore to share similar adaptations) than are species from different lineages. This pattern is clear throughout the genus *Drosophila*. For example, of the species examined here, two of the three *quinaria* group species (*D. recens* and *D. guttifera*) and the closely related *D. putrida* breed on decaying mushrooms (Spicer and Jaenike 1996); the four *nannoptera* group species (*D. wassermani*, *D. nannoptera*, *D. pachea*, and *D. acanthoptera*) all breed on the necrotic tissue of columnar cacti (Heed 1982); the six *mulleri* subgroup species (*D. mojaviensis*, *D. arizonae*, *D. navojoa*, *D. straubae*, *D. parisiana*, and *D. mayaguana*) all breed on necrotic cacti (Heed 1982; Heed and Grimaldi 1991); and the 11 *virilis* group species (*D. americana*, *D. texana*, *D. novamexicana*, *D. lummei*, *D. virilis*, *D. kanekoi*, *D. ezoana*, *D. littoralis*, *D. borealis*, *D. laticola*, and *D. montana*), despite being widely distributed throughout the northern hemisphere, all probably breed on tree fluxes (Throckmorton 1982). Subsequent to invasion of new host plant niches, further specialization, primarily biochemical, likely has contributed to the speciation process in these flies (Fogleman and Heed 1989). Because more similar host plants have more similar chemical compositions, closely related *Drosophila* species will be subject to similar conditions of nutrient limitations to female egg production, and similar selection pressures on males to provide nutritive ejaculatory donations. In addition, similar host plants may be more similar in resource distribution and their effects on fruitfly population structure, and may therefore favor more similar mating system features (Emlen and Oring 1977), including the presence or absence of ejaculatory donations/incorporations. The process of phylogenetic niche conservatism may therefore explain why, for instance, all of the cactus-breeding *mulleri* subgroup species have substantive somatic and ovarian incorporations, as do all of the closely related mushroom-breeding flies.

This process, however, cannot explain why *D. subpalustris*, the one *quinaria* group species examined that has made a radical ecological shift from breeding on mushrooms to breeding on decaying water plants, also exhibits substantial ejaculate incorporation. Niche conservatism also cannot explain why there has been a lack of convergent evolution, such that other cactus-breeding species (*D. nigrospiracula*, *D. mettleri*, and the four *nannoptera* group species) do not exhibit ejaculatory incorporations. This lack of convergence in ejaculatory strategies would seem unlikely due to phylogenetic constraint on male cellular physiology, as evidence suggests that ejaculate proteins in *Drosophila* are evolutionarily highly labile. For example, a comparison of eight tissues from four species (*D. melanogaster*, *D. simulans*, *D. mauritiana*, and *D. sechellia*) found the greatest genetic divergence in the testis and accessory gland tissue. Moreover, there was a correlation between the levels of reproductive tract-protein divergence and the degree of reproductive isolation among species (Thomas and Singh 1992). Other comparative studies of the major

accessory gland proteins among eight *Drosophila* species revealed the rapidly divergent and species-specific nature of male accessory gland proteins (Chen et al. 1985; Stumm-Zollinger and Chen 1988), illustrating the rapidity with which ejaculate chemistry can evolve. Consequently, with all other things being equal, similar selection pressures acting on phylogenetically disparate species occupying similar niches might be expected to result in convergent evolution of ejaculatory donations/incorporations.

The possibility, therefore, that related *Drosophila* species express similar ejaculatory incorporation phenotypes simply due to common ancestry cannot be dispelled; this trait may not be of adaptive significance in all extant species. Non-adaptive traits may be retained as long as suitable genetic variance is absent or if they are pleiotropically involved with other traits that are selectively favored (Harvey and Pagel 1991). Determining the evolutionary significance of ejaculatory incorporations will require intraspecific studies of the contribution of donations/incorporations to male and female fitness in a variety of species. A complete understanding of interspecific variation in ejaculatory incorporation awaits a cohesive biochemical approach to the examination of ejaculate constitution, resource substrate chemistry, and identification of those factors limiting female productivity. If resource ecology is also phylogenetically conservative, then defining the relative contributions of ancestry and diet-based selection to ejaculate evolution will be difficult.

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