

# How *Drosophila* males make eggs: it is elemental

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Oogenesis in *Drosophila* requires a significant amount of phosphorus. Oocytes mature in follicles, each of which contains 15 highly polyploid, transcriptionally active chromosomes. We show that the demand for phosphorus is met in part from the male's ejaculate following mating. Females incorporate phosphorus-32 from radiolabelled males into their ovaries, specifically into their nucleic acids. Male-derived phosphorus is also present in significant amounts in mature oocytes. The mechanism by which phosphorus uptake from the female reproductive tract occurs must differ from that previously reported for radiolabelled carbon and hydrogen derived from ejaculatory proteins, as phosphorus uptake is observed in species not showing female incorporation of radiolabel derived from ejaculate proteins.

**Keywords:** *Drosophila*; ejaculate; phosphorus; oogenesis; donation

## 1. INTRODUCTION

The hosts of phytophagous insects vary widely in their nitrogen content and this variability directly affects growth and reproduction (Mattson 1980). Recently, phosphorus and, in particular, its abundance relative to nitrogen has been shown to be an important determinant of the growth and reproduction of aquatic invertebrates (Elser *et al.* 1996). An investigation of the nitrogen and phosphorus contents of the hosts of frugivorous and cactophilic *Drosophila* species (Markow *et al.* 1999) revealed striking differences in the levels of these two elements: both exist in quantities several-fold higher in fruits than in columnar cacti. When the resident *Drosophila* species were examined for body nitrogen and phosphorus, the relative values mirrored those found in their respective hosts: the bodies of cactophilic species were found to contain less nitrogen and less phosphorus than those of frugivorous species. As this pattern was unalterable by changing the elemental composition of their food (Markow *et al.* 1999) it is likely that these species differences have an evolutionary explanation reflecting long-term associations with their hosts.

Another striking finding was that, in all species examined, regardless of resource type, females had significantly higher body phosphorus than conspecific males. The hypothesis offered for explaining this sexual dimorphism in phosphorus was based upon the nature of oogenesis. *Drosophila* eggs develop in follicles containing 15 nurse cells, the chromosomes of which replicate 1024 times. These polytene chromosomes are transcriptionally active, with the nascent RNA passed to the developing oocytes in order to support embryogenesis (King 1968). Because each of a female's paired ovaries contains hundreds of developing follicles, the high requirement for phosphorus in nucleic acid biosynthesis in oogenesis was proposed to explain the phosphorus bias in females (Markow *et al.* 1999). However, this hypothesis has never been tested.

Markow *et al.* (1999) further suggested that, under certain conditions, female phosphorus limitation could be

associated with female acquisition of phosphorus from sources other than their typical hosts. For example, in a number of *Drosophila* species, males pass large quantities of ejaculatory proteins to females who then incorporate these male-derived substances into their ovarian oocytes (Markow & Ankney 1984, 1988; Markow 1988*b*; Pitnick *et al.* 1991, 1997). While such 'seminal feeding' of *Drosophila* females has never been investigated for phosphorus or any other element, the sequestering and copulatory transfer of phosphorus by males in Lepidoptera (Lai-Fook 1991) and mosquitoes (Quraishi *et al.* 1966; Patterson *et al.* 1968; Quraishi 1968) and of sodium in the Lepidoptera (Pivnick & McNeil 1987; Boggs & Jackson 1991) suggests that this route of acquisition is possible in fruit flies.

In the present study, we test the hypotheses that (i) the female bias in phosphorus reflects a relatively higher need for phosphorus in ovarian versus testicular tissue, and (ii) that females obtain phosphorus from seminal fluid for use during oogenesis, specifically egg production. We used phosphorus-32 (<sup>32</sup>P) for radiolabelling males and females and examined the amount of radiolabel in the gonads of sexually mature flies. We also mated <sup>32</sup>P males to unlabelled females in order to determine whether phosphorus is transferred to females and, if so, if it is detectable in ovarian tissue and mature eggs. We examined two species of *Drosophila*. One, the cosmopolitan *Drosophila melanogaster* is largely frugivorous, breeding in comparatively phosphorus-rich hosts, while the other, *Drosophila nigrospiracula*, feeds and breeds in either saguaro (*Carnegiea gigantea*) or cardón (*Pachycereus pringlei*) cacti which have extremely low relative phosphorus levels (Markow *et al.* 1999). In addition to the ecological difference between these two species, they were selected because of their similarity in two reproductive features. Their ovariole number is virtually identical. Female *D. melanogaster* have *ca.* 42 ovarioles (Robertson 1957) and *D. nigrospiracula* have 40 (Heed & Mangan 1986), so that a similar requirement for phosphorus in the ovaries would be expected. Second, neither species has been found to show significant incorporation of male seminal proteins into somatic or ovarian tissue. Thus, the detection of male-derived phosphorus use would represent a previously unknown male contribution to oogenesis.

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## 2. MATERIAL AND METHODS

### (a) *Drosophila stocks and culture techniques*

Two species of *Drosophila* were used. One was *D. melanogaster* of the standard laboratory strain Oregon-R. The strain of *D. nigrospiracula* was derived from a multifemale collection made at Mulege, Baja California Sur, in March 1998. Unless specified otherwise, *D. melanogaster* were reared on standard cornmeal–molasses–agar medium, whereas *D. nigrospiracula* were reared on potato–cactus medium in all experiments (Castrezana 1997). In order to examine the effect of culture medium type on  $^{32}\text{P}$  incorporation and distribution, we cultured *D. melanogaster* on both cornmeal medium, its standard laboratory food, as well as on potato medium, the only laboratory food on which *D. nigrospiracula* is reliably reared. *Drosophila nigrospiracula* cannot be reared on cornmeal medium.

### (b) $^{32}\text{P}$ -labelling of flies

Fifty microcuries of  $^{32}\text{P}$  (ICN no. 64014) (carrier-free phosphoric acid) were added to shell vials containing 5 g wet weight of culture medium and mixed with a glass rod. Thirty second-instar larvae were gently transferred to the radioactive food. Once pupae were observed, the vials were closely monitored and newly emerging males and females were separated without anaesthesia. The sexes were stored separately until sexually mature, when they were used either to assess the distribution of label in tissues or to measure transfer to females and incorporation into their tissues.

### (c) *Distribution of $^{32}\text{P}$ in sexually mature adults*

One of our predictions was that the higher amount of phosphorus relative to nitrogen observed in female *Drosophila* was due to the high demand for phosphorus in oogenesis compared to spermatogenesis. We examined this prediction by removing the gonads of sexually mature males and females reared on  $^{32}\text{P}$ -labelled culture medium and determining the amount of radioactivity in these organs and the remaining carcasses. In the case of males, their accessory glands were also examined as these are a primary source of ejaculatory proteins transferred to females. In order to test the hypothesis that female nitrogen:phosphorus ratios are phosphorus biased due to an excess demand for phosphorus in oogenesis, ovaries were dissected from sexually mature  $^{32}\text{P}$ -labelled females and compared to the testes and accessory glands of males. Carcasses and dissected tissues were washed in phosphate-buffered saline (PBS) and digested for 24 h in Scintigest (Fisher Scientific, Pittsburg, PA, USA) tissue solubilizer. Scintiverse BD (Fisher Scientific) scintillation fluid was added and the samples were counted in a Packard Tricarb scintillation counter for 10–60 min. Counts per minute (CPMs) were converted to decompositions per minute (DPMs) following a standard quench curve.

### (d) *Copulatory transfer of male $^{32}\text{P}$ to mated females*

In order to assess male transfer of  $^{32}\text{P}$  during copulation, we mated radiolabelled males to unlabelled virgin females. *Drosophila melanogaster* females were 2–3 days old and *D. nigrospiracula* females were 5–7 days old. All pairs were observed until copulation occurred. Mated males and females were immediately prepared for scintillation counting as described above, with the exception that digestions were performed on washed bodies of whole flies rather than dissected parts. Conversions to DPMs used a standard quench curve.

Total male body counts were determined by adding the total counts remaining in the mated male to those found in the female immediately after mating.

### (e) *Determination of $^{32}\text{P}$ in mated female nucleic acids*

Virgin females of both species were mated to radioactive males when sexually mature, as described above. All pairs were observed until copulation occurred and sacrificed 30–72 h after copulation in order to allow enough time for male-derived phosphorus to become incorporated into female nucleic acids. Ovaries from ten females were washed in PBS, pooled and RNA or DNA was extracted. RNA was extracted and purified from ovaries using RNazol B (Teltest Corporation, Friendswood, TX). Pelleted RNA was washed in ethanol, resuspended in Tris/EDTA buffer and counted in Scintiverse BD (Fisher Scientific) cocktail in a Beckman LS 7500 scintillation counter. Controls were RNA samples from females mated to unlabelled males treated in the same manner. Subsamples of labelled, purified RNA were treated with RNase A (Sigma, St Louis, MO), reprecipitated, washed, resuspended and counted. RNase A treatment caused a reduction of ca. 96% in recoverable counts. DNA was extracted and purified from ovaries using DNazol (Molecular Research Center Inc., Cincinnati, OH). DNA pellets were resuspended in Tris/EDTA buffer and counted as above. Controls were DNA samples from the ovaries of females mated to unlabelled males and treated in the same manner. CPMs were converted to DPMs by direct spiking of samples with a known amount of label in order to determine counting efficiency. Subsamples of labelled, purified DNA were treated with DNase I, reprecipitated, washed, resuspended and counted. DNase I treatment resulted in a complete loss of recoverable counts from the samples.

### (f) *Determination of male-derived phosphorus in mature eggs*

Virgin female flies were mated at either 3 days of age in the case of *D. melanogaster* or 5 days of age in the case of *D. nigrospiracula*. Mature, stage-14 eggs were collected 24 h later in the case of *D. melanogaster* and 72 h later in the case of *D. nigrospiracula*. These times were predetermined to be those at which male-derived label began to appear in eggs. The ovaries of *D. nigrospiracula* females are less mature than those of *D. melanogaster* at the time of eclosion, so completion of oogenesis requires longer. Eggs were individually collected and thoroughly washed in PBS. Groups of 100 eggs were digested in 200  $\mu\text{l}$  of Scintigest for 24 h at 60 °C and the digested material was counted in Scintiverse BD in the Beckman LS7500 scintillation counter. Controls were the eggs from unlabelled females mated to unlabelled males treated in the same manner. CPMs were converted to DPMs by direct spiking of samples with a known amount of label in order to determine counting efficiency.

## 3. RESULTS

### (a) *Distribution of $^{32}\text{P}$ in sexually mature adults*

Males'  $^{32}\text{P}$  levels and distributions are presented in table 1. The higher total DPMs for *D. nigrospiracula* most probably reflect their larger size (Markow 1996). The dry weight for *D. melanogaster* males was  $230 \pm 6 \mu\text{g}$  ( $n = 10$ ), as compared to  $346 \pm 14 \mu\text{g}$  ( $n = 10$ ) for *D. nigrospiracula*. The greater amount of label detected per microgram of dry body weight in *D. nigrospiracula* (26 DPMs  $\mu\text{g}^{-1}$ ) compared

Table 1. Distribution of  $^{32}\text{P}$  in tissues of adult virgin males reared on radiolabelled medium.

(The unlabelled control fly DPMs were  $21.1 \pm 1.0$  for *D. melanogaster* and  $25.6 \pm 1.2$  for *D. nigrospiracula*. The percentages of labelled phosphorus in testes and accessory glands were determined by first subtracting the control DPMs from the total and tissue DPMs. \* $p < 0.01$  above control DPMs.)

species	food	<i>n</i>	total body DPMs $\pm$ s.e.	testes DPMs $\pm$ s.e.	% testes $\pm$ s.e.	accessory gland DPMs $\pm$ s.e.	% glands $\pm$ s.e.
<i>D. melanogaster</i>	cornmeal	18	1925 $\pm$ 108*	122 $\pm$ 7*	5.2 $\pm$ 0.2	195 $\pm$ 17*	9.2 $\pm$ 0.2
	potato	17	5133 $\pm$ 359*	298 $\pm$ 23*	5.4 $\pm$ 0.2	513 $\pm$ 45*	9.5 $\pm$ 0.4
<i>D. nigrospiracula</i>	potato	18	13 785 $\pm$ 5032*	960 $\pm$ 315*	6.8 $\pm$ 1.6	1103 $\pm$ 433*	7.8 $\pm$ 1.0

Table 2. Distribution of  $^{32}\text{P}$  in the tissues of adult virgin females reared on radiolabelled medium.

(The unlabelled control fly DPMs were  $22.8 \pm 0.9$  for *D. melanogaster* and  $21.8 \pm 1.1$  for *D. nigrospiracula*. The percentages of labelled phosphorus found in ovaries were determined by first subtracting the control DPMs from the total and tissue DPMs. \* $p < 0.01$  compared to control DPMs.)

species	food	<i>n</i>	total DPMs $\pm$ s.e.	ovaries DPMs $\pm$ s.e.	% ovaries $\pm$ s.e.
<i>D. melanogaster</i>	cornmeal	23	3993 $\pm$ 577*	1049 $\pm$ 223*	25.8 $\pm$ 2.1
	potato	8	6538 $\pm$ 834*	1610 $\pm$ 280*	24.3 $\pm$ 2.3
<i>D. nigrospiracula</i>	potato	5	19 985 $\pm$ 3190	6087 $\pm$ 1162	30.1 $\pm$ 3.9

Table 3. Proportion of male  $^{32}\text{P}$  transferred to females during copulation.

(The unlabelled control ovarian DPMs were  $21.0 \pm 1.5$  for *D. melanogaster* and  $20.3 \pm 1.3$  for *D. nigrospiracula*. The percentages of labelled phosphorus transferred were determined by first subtracting the control DPMs from the male and female DPMs. \* $p < 0.001$  compared to control DPMs.)

species	culture medium		<i>n</i>	male DPMs	female DPMs	% transferred
	female	male				
<i>D. melanogaster</i>	cornmeal	cornmeal	7	3097 $\pm$ 254*	158 $\pm$ 30*	4.5 $\pm$ 0.5
	potato	cornmeal	9	4127 $\pm$ 319*	285 $\pm$ 47*	6.2 $\pm$ 0.7
	potato	potato	5	14 704 $\pm$ 3818*	755 $\pm$ 251*	5.1 $\pm$ 0.7
	cornmeal	potato	6	7427 $\pm$ 1298*	411 $\pm$ 63*	5.2 $\pm$ 0.9
<i>D. nigrospiracula</i>	potato	potato	7	21 996 $\pm$ 6504*	263 $\pm$ 46*	1.2 $\pm$ 0.2

to *D. melanogaster* (8 DPMs  $\mu\text{g}^{-1}$ ) may reflect the longer development time of *D. nigrospiracula*. It may also reflect a greater uptake of  $^{32}\text{P}$  in potato medium, as *D. melanogaster* grown on potato food had higher total DPMs compared to cornmeal-reared flies. This was the case for total body DPMs ( $t = 12.78$  and  $p < 0.001$ ), testes DPMs ( $t = 11.00$  and  $p < 0.001$ ) and accessory gland DPMs ( $t = 11.36$  and  $p < 0.001$ ). While the species differed in overall body label and label found in specific tissues, these differences have no influence on our primary question of whether significant levels of  $^{32}\text{P}$  are detected in reproductive tissues. A significant amount of label was found in the testes and accessory glands of males of both species, regardless of food type (see table 1). Food type in *D. melanogaster* did not influence the actual proportion of DPMs found in either their testes or accessory glands, nor was the difference between species in the proportions in these tissues significant ( $t = 2.01$  and n.s.).

Females of both species are larger than males (Markow 1996), which is consistent with the higher total amounts

of  $^{32}\text{P}$  they contained (table 2). Whereas higher amounts of  $^{32}\text{P}$  were again found in potato-reared compared to cornmeal-reared *D. melanogaster* (total DPMs,  $t = 9.90$  and  $p < 0.001$  and ovarian DPMs,  $t = 9.84$  and  $p < 0.001$ ), the proportion of DPMs in the ovaries (24–30%) was virtually identical for both species, regardless of food type. Furthermore, the proportion of radioactivity was several times higher in ovaries relative to testes for both species. These results support the hypothesis that oogenesis creates an excess requirement for phosphorus in females compared to males.

#### (b) Copulatory transfer and fate of male $^{32}\text{P}$

Despite the lack of an effect of food type on the proportion of  $^{32}\text{P}$  in the ovaries in *D. melanogaster*, we felt it necessary to rule out the possibility that food type did not influence transfer or incorporation of male-derived label. The total DPMs observed in this experiment (table 3) were higher for both species than those reported in tables 1 and 2. These differences reflect the different batches of

Table 4.  $^{32}\text{P}$  in RNA and DNA of ovaries after mating with radiolabelled males.

(The means and standard errors for individual replications are presented for three, 5-min counting periods in the scintillation counter, while the overall means are those of the four- and three-replicate experiments. One-tailed paired *t*-tests were used for all comparisons.)

species	replicate	RNA		DNA	
		control	$^{32}\text{P}$ mated	control	$^{32}\text{P}$ mated
<i>D. melanogaster</i>	1	42.7 ± 2.0	87.6 ± 0.2	47.7 ± 0.9	104.9 ± 2.5
	2	34.7 ± 3.2	62.5 ± 3.2	36.7 ± 2.6	105.0 ± 3.2
	3	36.2 ± 1.0	76.0 ± 0.5	36.3 ± 3.4	91.6 ± 2.4
	4	36.2 ± 1.0	49.2 ± 1.2	—	—
	mean	37.5 ± 1.8	68.8 ± 8.3	40.4 ± 3.7	100.5 ± 4.4
	paired <i>t</i>	<i>t</i> = 4.42	<i>p</i> = 0.01	<i>t</i> = 14.62	<i>p</i> = 0.002
<i>D. nigrospiracula</i>	1	50.9 ± 1.5	81.7 ± 2.0	43.2 ± 0.9	93.0 ± 2.8
	2	42.2 ± 2.2	61.7 ± 3.8	36.2 ± 1.2	105.8 ± 4.5
	3	37.0 ± 0.2	55.9 ± 3.2	34.0 ± 2.5	152.8 ± 8.1
	4	37.4 ± 2.1	56.9 ± 1.6	—	—
	mean	41.9 ± 3.2	64.0 ± 6.0	37.8 ± 2.8	117.2 ± 18.2
	paired <i>t</i>	<i>t</i> = 7.67	<i>p</i> = 0.002	<i>t</i> = 3.87	<i>p</i> = 0.03

Table 5.  $^{32}\text{P}$  detected in mature eggs of females mated to  $^{32}\text{P}$ -labelled males.

(The means and standard errors for individual replications are presented for three, 5-min counting periods in the scintillation counter, while the overall means are those of the three-replicate experiments. One-tailed paired *t*-tests were used for all comparisons.)

species	replication	DPMs ± s.e. (number of eggs)			
		control	$^{32}\text{P}$ mated	<i>t</i>	<i>p</i>
<i>D. melanogaster</i>	1	40.1 ± 1.6 (100)	109.8 ± 1.5 (120)	—	—
	2	39.2 ± 6.8 (100)	169.5 ± 7.6 (113)	—	—
	3	38.2 ± 1.8 (100)	131.9 ± 1.5 (101)	—	—
	mean	39.2 ± 0.05	137.1 ± 17.4	5.56	0.02
<i>D. nigrospiracula</i>	1	37.4 ± 1.6 (102)	125.4 ± 3.3 (93)	—	—
	2	36.7 ± 1.2 (102)	132.7 ± 2.4 (103)	—	—
	3	40.7 ± 1.4 (102)	183.3 ± 4.7 (108)	—	—
	mean	38.2 ± 1.2	147.1 ± 18.2	6.38	0.01

$^{32}\text{P}$  used and have no bearing on the conclusions. All combinations of *D. melanogaster* matings were tested (potato × ♀ $^{32}\text{P}$  potato ♂, potato × ♀ $^{32}\text{P}$  cornmeal ♂, cornmeal × ♀ $^{32}\text{P}$  potato ♂ and cornmeal × ♀ $^{32}\text{P}$  cornmeal ♂) and no significant food effect was observed in a two-way ANOVA ( $F = 0.724$  and  $p = 0.39$ ). Therefore these treatments could be pooled for comparison with *D. nigrospiracula* (table 3). Male *D. melanogaster* transferred ca. 5% of their body  $^{32}\text{P}$  to females on a given mating, compared to 1.6% in *D. nigrospiracula*. This species difference could stem from several sources. One could be male age. While males of both species were sexually mature by standard laboratory criteria, optimal ages with respect to the phosphorus content of the seminal fluid might be somewhat different. It is also known that, in *D. melanogaster* (Gromko *et al.* 1984), males pass thousands more sperm to females during copulation than do males of *D. nigrospiracula* (Pitnick 1996) and the  $^{32}\text{P}$  in sperm heads may have, when considering the large number passed in *D. melanogaster*, contributed to the difference. While this species difference may be interesting in itself, the intent of

the present study is to ask whether females in two different species receive phosphorus from males during copulation. Our data clearly show that significant  $^{32}\text{P}$  is transferred during mating in both species.

### (c) Incorporation of male-derived phosphorus into ovarian nucleic acids

Our second hypothesis, that females use male phosphorus for synthesizing the high levels of nucleic acids necessary for making eggs, was tested by examining the incorporation of male  $^{32}\text{P}$  into ovarian RNA and DNA. The values of  $^{32}\text{P}$  incorporated into ovarian RNA and DNA are given in table 4. RNA determination was performed in four separate experiments for *D. melanogaster* and three for *D. nigrospiracula*. The higher control DPMs in this compared to the previous tables reflects the change to a new scintillation counter following our move to the University of Arizona, but have no influence on the interpretation of the results. For both species the amount of  $^{32}\text{P}$  in ovarian RNA and DNA of females mated to labelled males in each experiment was

significantly greater than observed in the unlabelled control samples.

**(d) Male-derived phosphorus in mature, stage-14 eggs**

Late in oogenesis, DNA-rich ovarian nurse cells dump their degraded nuclear contents into the developing oocytes where these breakdown products subsequently serve as a pool of resources for supporting early embryonic development. If male-derived phosphorus, regardless of its form, becomes part of this pool, it should be detectable in mature oocytes.  $^{32}\text{P}$  is clearly present in significant amounts (table 5), which is consistent with the prediction.

#### 4. DISCUSSION

Females of *D. melanogaster* and *D. nigrospiracula* contain approximately three times as much phosphorus in their gonads as males. In both species, females clearly obtain phosphorus from the ejaculate and incorporate it into their ovaries, where it is detectable in RNA and DNA. Male-derived phosphorus is also found in mature eggs. All previous studies of *Drosophila* non-sperm ejaculatory components and their functions have focused on the seminal proteins produced in the male accessory glands, the accessory gland proteins (ACPs) (Wolfner 1997; Pitnick *et al.* 1997). ACPs are attractive study molecules because their direct relationships to the coding regions of the DNA and their demonstrated roles in stimulating oviposition and delaying remating facilitate examination of their evolution and function. Whereas radiolabelled carbon and hydrogen derived from some of these seminal proteins are, in certain species of *Drosophila*, also incorporated into ovarian eggs (Markow & Ankney 1984; Pitnick *et al.* 1997), in contrast the transfer to and use of phosphorus by females has the potential to link mating system evolution with resource availability in nature more directly. For example, the natural cactus host of *D. nigrospiracula* is poor in phosphorus relative to the fruits used by *D. melanogaster* (Markow *et al.* 1999) and females of *D. nigrospiracula* remate much more frequently than do *D. melanogaster* females (Markow 1988a).

The uptake and incorporation of seminal fluid components into the tissues of mated female insects as a form of 'nuptial gift' was reviewed by Boggs (1998). Although examples of seminal protein incorporation are now well-known among Lepidoptera and *Drosophila*, only one element, sodium, which is found to pass from the seminal fluid of mud puddling butterflies into ovarian eggs (Pivnick & McNeil 1987; Boggs & Jackson 1991), appears associated with a fitness benefit. In this case, the sodium is thought to prevent the desiccation of oviposited eggs. To the authors' knowledge, our study is the first to show the incorporation of a male-derived element into a specific class of molecules in females, the nucleic acids. Fitness benefits, particularly under conditions of severe phosphorus limitation, of the ability to use non-dietary phosphorus for manufacturing oocytes are easily envisioned. Unpublished observations from our laboratory indicate that reduced phosphorus in adult *Drosophila* female diets retards oogenesis. Additional studies designed for testing the importance of male-derived phosphorus for female

fitness more directly across *Drosophila* species of contrasting ecologies will be highly informative.

Larval phosphorus limitation may have forced *D. nigrospiracula* larvae to allocate their dietary phosphorus to somatic growth rather than to gonadal development. Females of *D. melanogaster* eclose with ovarian follicles that have reached stage 7 (Kambysellis 1968; King 1968), the most advanced pre-vitellogenic stage in a classification that ranks follicles in 14 stages (King 1968). The most mature follicle seen in eclosing female *D. nigrospiracula* is stage 2, although most are at stage 1 (Markow, unpublished data). This species difference appears to be hard wired, as rearing flies on enriched or deprived diets has no effect on follicular stage at eclosion. Both males and females of *D. nigrospiracula* exhibit delayed reproductive maturity compared to *D. melanogaster*, but in *D. nigrospiracula* males require substantially longer relative to females than those of *D. melanogaster*. The delay in females compared to *D. melanogaster* may reflect the need for developing mature follicles prior to mating. In male *Drosophila* of several other species, delays in reproductive maturity relative to females has been strongly associated with sperm gigantism (Pitnick *et al.* 1995) and, to a lesser degree, with the production of protein ejaculate donations to females (Pitnick *et al.* 1997). The degree of delay in adult male sexual maturity in *D. nigrospiracula* could in fact reflect the time required by males for accumulating adequate phosphorus in their reproductive tracts for transfer to females. Future studies focusing specifically on the allocation of larval versus adult dietary phosphorus to reproduction are suggested by the results of the present study.

At present, we are uncertain of the form of phosphorus found in the ejaculate and incorporated by females. However, we feel that it is unlikely that a substantial proportion of the incorporated phosphorus comes from the haploid nuclei in the heads of sperm. First, there is no evidence that *Drosophila* females digest sperm for nutritive uptake (Snook & Markow 1997). Second, there is clear evidence that, although sperm may be lost immediately after copulation, those sperm remaining in storage are efficiently used for fertilization (Pitnick 1996). Moreover, sperm themselves do not enter the ovaries where the radiolabel is subsequently detected. Pyrophosphate has been reported to exist in seminal fluid (Heller *et al.* 1950; Piechowska 1956), but other possibilities include ATP, phospholipids or phosphoproteins. In those insects in which phosphorus has been documented to be transferred to females in the ejaculate, such as butterflies (Lai-Fook 1991) and mosquitoes (Quraishi *et al.* 1966; Patterson *et al.* 1968; Quraishi 1968), the form of the transferred phosphorus was not documented. However, the mechanism of phosphorus transport from the uterus or seminal receptacle into female somatic tissues and ovaries is likely to differ from that responsible for the uptake of ACPs, as neither *D. melanogaster* nor *D. nigrospiracula* females exhibit the significant degree of incorporation of ACPs into somatic tissue or ovaries seen in many other *Drosophila* species (Pitnick *et al.* 1997). Of the ca. 100 ACPs (Wolfner 1997), the possibility that some may be involved in the transport of phosphorus and perhaps other elements should not be overlooked.

The scenario under which such uptake and use evolved is speculative. Seminal fluid is rich in protein and other

molecules, both organic and inorganic, that serve a variety of functions, from maintaining sperm viability and motility to stimulating oviposition and delaying female remating (Wolfner 1997). Depending upon the ecological factors important to a specific species, female reproductive tracts that are able to co-opt these substances for functions that are beneficial to female interests would be favoured by selection. For example, if a species were phosphorus limited and the ejaculate contained significant ATP for another purpose, such as maintaining sperm motility, females capable of diverting the ATP from the males and using it to make eggs would have a fecundity advantage over females that could not. Male responses could include altering their ejaculates in order to make them less co-optable, modifying the ratios of the costly versus inexpensive ejaculate components transferred to females and being selective, prior to mating with a given female, with respect to her condition and the likelihood that his gametes would fertilize eggs into which he has invested substances.

Markow (1996) proposed that, in some species, the ejaculate is 'expensive' because of trade-offs involving sperm length or the quantity of ejaculate proteins required for 'feeding' females. Under that scheme, *D. melanogaster* and *D. nigrospiracula* were considered to be 'cheap' ejaculate species because no seminal proteins for incorporation into female tissues were donated by males. Our new findings suggest that such species classifications may have been inexact. The issue of whether phosphorus controls male reproductive maturation time in the same way that, for example, sperm gigantism does (Pitnick *et al.* 1995) requires investigation. Clearly, interactions between the ejaculate and female reproductive tract are more complex than previously believed, raising new questions and highlighting the need for relating these interactions to the greater ecological picture.

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