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# Large-male advantages associated with costs of sperm production in *Drosophila hydei*, a species with giant sperm

(sexual selection/body size/testis size/age at maturity)

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ABSTRACT **Males of the fruit fly *Drosophila hydei* were found to produce 23.47 ± 0.46-mm-long spermatozoa, the longest ever described. No relationship was found between male body size and sperm length. We predicted that if these giant gametes are costly for males to produce, then correlations should exist between male body size, rates of sperm production, and fitness attributes associated with the production of sperm. Smaller males were found to make a greater relative investment in testicular tissue growth, even though they have shorter and thinner testes. Smaller males were also found to (*i*) be maturing fewer sperm bundles within the testes at any point in time than larger males, (*ii*) require a longer period of time post-eclosion to become reproductively mature, (*iii*) mate with fewer females, (*iv*) transfer fewer sperm per copulation, and (*v*) produce fewer progeny. The significance of these findings for body size-related fitness and the question of sperm size evolution are discussed.**

Disparity between the sexes in the size and number of gametes produced is the keystone of sexual selection theory (1–3). The conventional perspective is that, relative to sperm, eggs are costly to produce, and thus females make relatively few of them. One consequence of this cost is that a positive relationship frequently exists between female body size and egg production measures (4–6). Conversely, only a trivial amount of energy is invested in each gamete by males (2), so that production of adequate numbers of sperm is expected not to limit male reproductive success (4, 5). This perspective has been modified by the recognition that production of ejaculates may limit male reproductive success, due to the large numbers of sperm and potentially costly secretions they may contain (for reviews, see refs. 7 and 8). Intraspecific, body size-related variation in males' ability to produce and transfer costly accessory-gland secretions that are nutritive to females has been demonstrated in some spermatophore-producing insects (e.g., refs. 9–12), but not in others (e.g., refs. 13 and 14). Whether variation among males in their ability to produce sperm contributes to differential male reproductive success in any species is unknown.

If sperm production is costly, then males with more energy to invest (i.e., larger males) should produce greater numbers of sperm. They should therefore also have larger testes, as daily sperm production is often positively correlated with testis dimension (e.g., refs. 15–17). A positive correlation between male body size and testis size has been reported for field-collected rats, *Rattus rattus* (18); swamp buffalo, *Bubalus bubalis* (19); Egyptian buffalo, *Bos bubalis* (20); bonnet macaques, *Macaca radiata* (21); rams (22); and goats (23). Unfortunately, all of these studies examined immature as well as young and old adult males, yet none controlled for male age in their statistical analyses. Because both body and testis mass are highly correlated with male age (e.g., refs. 15–17 and 24), conclusions regarding body size and testis size relationships from these studies are suspect. Among studies which have controlled for male age, a significant positive relationship between body size and testis size has been found only in savanna baboons (25). Lack of any relationship between body size and testis size has been reported for European bulls, *Bos taurus* (26); stallions (17); dusky leaf monkeys, *Presbytis obscura* (27); stumptail macaques, *Macaca arctoides* (28); chimpanzees, *Pan troglodytes* (29); humans (30, 31); capybaras, *Hydrochaeris hydrochaeris* (24); and 21 of 25 subspecies (16 species) of voles (32).

The general lack of unequivocal intraspecific relationships between body size and testis size suggests that sperm (and testicular tissue) are not so costly to produce or that selection to produce a species-specific optimal number of sperm is so strong that even small males make the requisite investment. Furthermore, due to the large numbers of sperm ejaculated in most species and limitations on the opportunity for males to copulate with multiple females, production of relatively greater numbers of sperm will not necessarily confer a fitness advantage on males. In fact, Bercovitch (25) convincingly argues for savanna baboons that, despite a significant positive relationship between adult male body weight and testicular volume, variation in neither of these characters contributes to variation in male reproductive success.

A new challenge to our understanding of male reproductive strategy evolution is being posed by species in which males make relatively few, large gametes (8, 33, 34), characteristic of the typical female reproductive strategy. Here we report on the sperm of *Drosophila hydei*, the longest sperm described. In *Drosophila* species with giant sperm, males are known to produce and transfer few sperm per copulation (8). We therefore test the prediction that a positive relationship between body size and testes size will exist in *D. hydei*, and illustrate mechanisms by which increased ability to invest in sperm production may confer a fitness advantage on larger males.

## MATERIALS AND METHODS

**Fruit Flies.** All experiments were conducted using *D. hydei* reared from a laboratory culture derived from a multifemale collection taken from fallen citrus in Tempe, AZ, in 1989. Additional sperm measurements were recorded for a "Hess and Meyer" strain of *D. hydei* obtained from Thomas Gregg (Miami University, Oxford, OH), and for *D. hydei* strains from Australia (stock no. 15085-1641.30); I-Lan, Taiwan (15085-1641.32); Zurich, Switzerland (15085-1641.0); and Pisco, Peru (15085-1641.31), obtained from the National Drosophila Species Resource Center, Bowling Green, OH. The *Drosophila melanogaster* males examined were from a

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laboratory culture derived from flies collected from fallen citrus in Tempe, AZ, in 1990.

All flies were reared on banana medium with live yeast at 24 ± 1°C at an approximate 12 hr light/12 hr dark photoperiod and an approximate 1:1 sex ratio. Virgin flies used in experiments were collected on the day of eclosion, anesthetized with ether to facilitate sorting of sexes, and maintained from that time in 8-dram vials containing medium, live yeast, and no more than 10 other same-sex individuals.

The body size of males varied with the age of the culture bottle from which they eclosed. As somewhat smaller males came from older bottles, a strong environmental contribution to body size variation is suggested. Size of individuals was determined by measuring thorax length, a standard measure of body size in studies of *Drosophila* because it strongly correlates with the size of other characters such as wing length (35). In the present study, thorax length was found to be a highly reliable indicator of the total dry body weight of *D. hydei* males (regression analysis: $F = 418.91$; df = 1, 18; $P = 0.0001$; $r^2 = 0.96$).

**Sperm Production.** Measurement of sperm length using the ocular micrometer of a dissecting microscope was facilitated by a technique that releases all sperm bundles intact from the testis (8). We defined sperm length of each male as the mean length of the four most mature sperm cysts in his testis, those just beginning to enter the seminal vesicle. The number of sperm bundles simultaneously undergoing development within a single testis of each male was determined at the mid-testis cross-section.

To acquire testicular dimensions, testes were dissected from anesthetized, mature flies into white paraffin oil and measured with an ocular micrometer. Testis width of each male was determined by the mean value of 10 equidistant width measures spanning the entire length of his testis. Because testis shape approximates that of a long cylinder, we were able to use the length ($l$) and width ($w$) data to calculate testis volume = $\pi(0.5w)^2 l$.

To determine the dry weight of testes relative to body size, testes were dissected from anesthetized, mature males into double-distilled water and then transferred to a preweighed piece of aluminum foil. All remaining tissue was placed on another preweighed piece of foil, and the head and thorax were ruptured to facilitate desiccation. Samples were then dried at 55°C for 1 hr before weighing on a Cahn C-31 microbalance accurate to the nearest 1.0 μg.

**Mating and Sperm Use.** Two replicates of an experiment were conducted in which 15- to 16-day-old virgin males were placed in individual 8-dram food vials and presented with an *ad libitum* series of virgin receptive females for 3 hr on each of two successive days. In addition to the number of times each male mated, we recorded the number of progeny (pupae and/or adults) produced by the first, third, and fifth female mated to each male on both days. In a separate experiment, females were dissected immediately following copulation, to determine the number of sperm transferred by males to their first and third mates on the first day only. Sperm numbers were quantified by epifluorescence microscopy (8).

**Reproductive Maturity.** Two replicates of an experiment were conducted in which, beginning at 6 days of age, each male was paired in the morning with two mature virgin females in 8-dram food vials. At the end of each day, females were removed from vials and dissected to assay for the presence of sperm in storage. Age of reproductive maturity was determined as the number of days between eclosion and the transfer of sperm to a female. Sperm transfer was a necessary criterion, as *D. hydei* males in the laboratory will copulate before any mature sperm have been produced (S.P., unpublished observation). All males were observed to copulate on all days.

## RESULTS

Mean total sperm length for *D. hydei* from the Arizona strain was 23.47 ± 0.46 mm (mean ± SE, $n = 7$). Because a much shorter sperm length was originally reported in the literature by Hess and Meyer (36), and because the strain they utilized was still available, we measured sperm length in the "Hess and Meyer" strain, as well as for *D. hydei* collected around the world. Males of all strains produced sperm of approximately equivalent length ("Hess and Meyer," 23.02 ± 0.45 mm, $n = 4$; Australia, 25.42 mm, $n = 1$; Taiwan, 25.35 mm, $n = 1$; Switzerland, 24.97 mm, $n = 1$; Peru, 25.91 mm, $n = 1$). Mean total sperm length (± SE) for *D. melanogaster* was 1.91 ± 0.01 mm ($n = 5$), a measure consistent with other reports (37, 38).

Across a wide range of body sizes among males of the Arizona strain of *D. hydei* (male thorax length, 1.000–1.308 mm), no statistically significant relationship was found between body size and sperm length (regression analysis: $F = 0.003$, df = 1, 5; $P = 0.96$; $r^2 = 0.001$).

We examined the dimensions of testes and the number of sperm bundles simultaneously undergoing development in *D.*

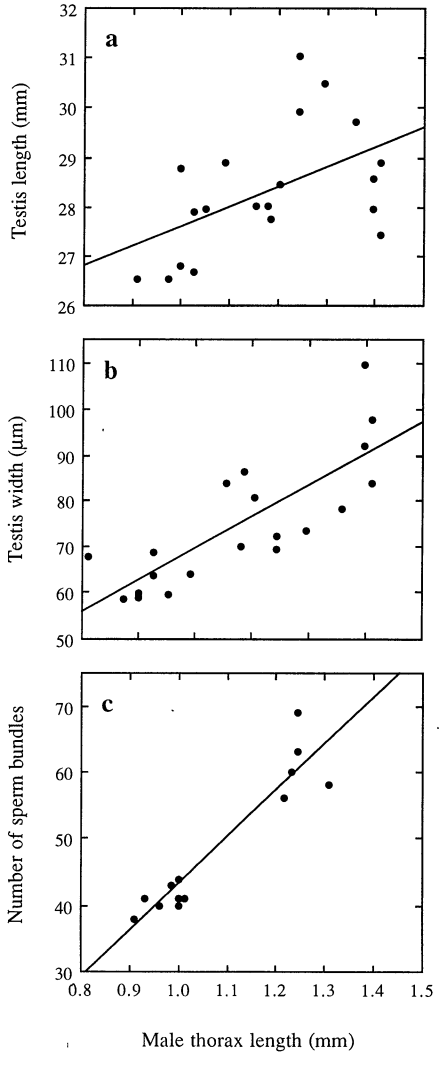


FIG. 1. (*a*) Relationship between male body size measured as thorax length, and testis length (the line is described by the equation $y = 23.562 + 4.038x$). (*b*) Relationship between male body size and testis width (the line is described by the equation $y = -6.600 + 69.212x$). (*c*) Relationship between male body size and the number of sperm being produced (the line is described by the equation $y = -27.190 + 70.298x$).

*hydei* males representative of the continuum in body size. Significant positive relationships were found between the thorax length of males and the length of their testes (Fig. 1*a*; regression analysis; $F = 6.64$, df = 1, 18; $P = 0.02$, $r^2 = 0.27$), the width of their testes (Fig. 1*b*; regression analysis: $F = 32.71$; df = 1, 18; $P = 0.0001$; $r^2 = 0.64$), and the number of sperm bundles simultaneously undergoing development (Fig. 1*c*; regression analysis: $F = 78.34$; df = 1, 11; $P = 0.0001$; $r^2 = 0.88$). In contrast, the relationship between thorax length (range, 0.731–0.897 mm) and testis length (range, 1.782–1.987 mm) among *D. melanogaster* males was not significant (regression analysis: $F = 1.65$; df = 1, 8; $P = 0.24$; $r^2 = 0.17$).

To determine the relative cost to *D. hydei* males of testis production, we first estimated the amount of testicular tissue produced by individuals of different sizes. Not surprisingly, there was a significant positive relationship between male thorax length and testis volume (regression analysis: $F = 36.519$; df = 1, 18; $P = 0.0001$; $r^2 = 0.67$). Larger males also produced heavier testes (Fig. 2; regression analysis: $F = 64.498$; df = 1, 28; $P = 0.0001$; $r^2 = 0.70$). Despite this, smaller males were found to make a significantly greater relative investment in testes than larger males, as measured by the ratio of dry testes weight to dry body weight (Fig. 2; regression analysis: $F = 7.515$; df = 1, 28; $P = 0.01$; $r^2 = 0.21$). In contrast, among *D. melanogaster* males there were no significant relationships between dry body weight and either dry testes weight (regression analysis: $F = 0.912$; df = 1, 8; $P = 0.37$; $r^2 = 0.37$) or the relative dry weight of testes (regression analysis; $F = 0.288$; df = 1, 8; $P = 0.61$, $r^2 = 0.04$) (ranges: dry body weight, 127–229 μg; dry testes weight, 5–13 μg; relative dry testes weight, 2.96–6.67%).

To investigate whether larger male *D. hydei* have greater reproductive success than do smaller males, and whether body size-related variation in sperm production might contribute to any such advantage, males representative of the continuum of body sizes were each provided with an *ad libitum* series of virgin females on two successive mornings. We recorded the number of times males mated, in addition to the number of sperm transferred and the productivity of specific matings, measured by the number of progeny produced. Significant positive relationships were found between male body size and the number of copulations achieved on each day (regression analysis for day 1: $F = 149.80$; df = 1, 40; $P = 0.0001$; $r^2 = 0.79$; for day 2: $F = 113.22$; df = 1, 40; $P = 0.0001$; $r^2 = 0.74$), as well as on the two days combined (Fig. 3*a*; regression analysis: $F = 196.89$; df = 1, 40; $P = 0.0001$; $r^2 = 0.83$). Male body size was also positively related to the number of sperm transferred per copulation (Fig. 3*b*; regression analysis for first copulation: $F = 28.77$; df = 1, 13; $P = 0.0001$; $r^2 = 0.69$; third copulation: $F = 55.04$; df = 1, 13; $P = 0.0001$; $r^2 = 0.81$). Finally, significant ($P = 0.0001$) positive relationships were found between male size and the productivity of all three matings measured on both of the experimental days. Fig. 3*c* illustrates these data for the first and third mating of the first day (regression analysis for first mating: $F = 20.63$; df = 1, 38; $P = 0.0001$; $r^2 = 0.35$; third mating: $F = 60.08$; df = 1, 39; $P = 0.0001$; $r^2 = 0.61$). Female body size was unrelated to productivity in these experiments (regression analysis: $F = 1.488$; df = 1, 233; $P = 0.22$; $r^2 = 0.006$), reinforcing the conclusion that the observed variation in productivity was attributable to body size-related variation in male fertility. Data from the two replicates of this experiment were statistically similar and so were pooled for analysis and illustration in Fig. 3.

There was also a significant negative relationship between male body size and the time required for males to become

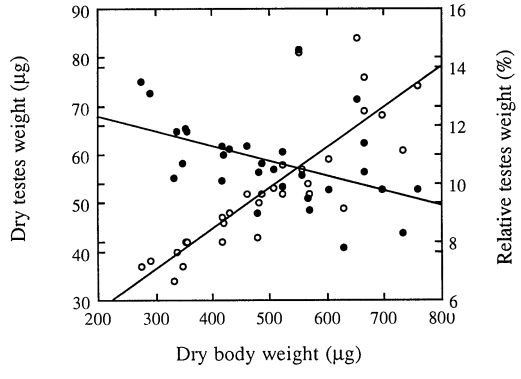


FIG. 2. Relationships between the dry body weight of males (excluding testes) and the dry weight of their testes [open circles; the line is described by the equation $y = 11.380 + (8.322 \times 10^{-2})x$] and between dry body weight and the relative weight of testes, measured as the ratio of dry testes weight to dry body weight [filled circles; the line is described by the equation $y = 13.337 - (5.099 \times 10^{-3})\ x$].

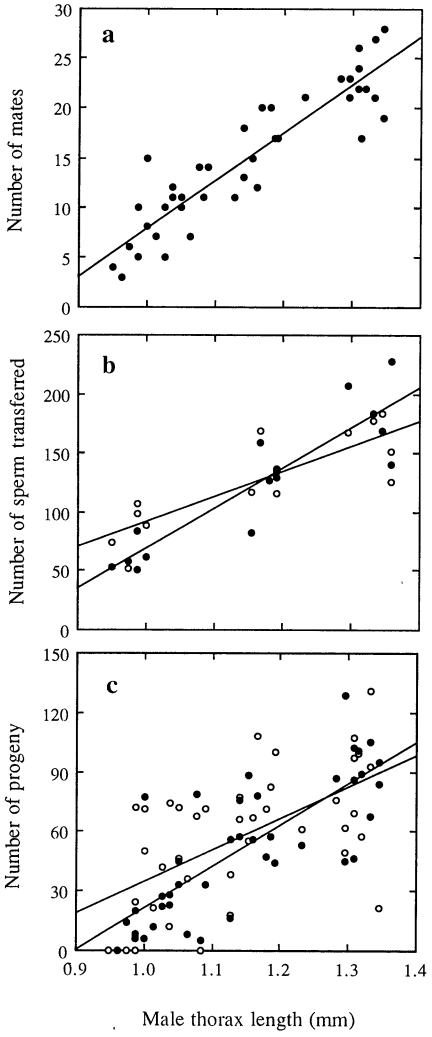


FIG. 3. (*a*) Relationship between male body size and the total number of matings achieved (the line is described by the equation $y = -40.471 + 48.246x$). (*b*) Relationship between male body size and the number of sperm transferred in the first (open circles) and third (filled circles) copulation by each male (first copulation, the line is described by the equation $y = -119.19 + 210.47x$; third copulation, the line is described by the equation $y = -271.03 + 339.62x$). (*c*) Relationship between male body size and the number of progeny produced by the first (open circles) and third (filled circles) mating by each male on day 1 (first mating, the line is described by the equation $y = -123.56 + 157.92x$; third mating, the line is described by the equation $y = -187.86 + 208.57x$).

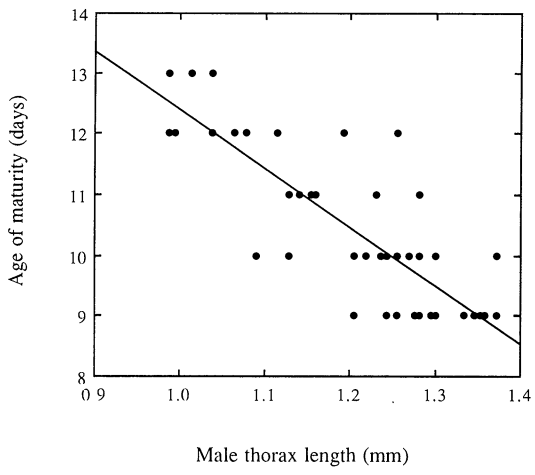


FIG. 4. Relationship between male body size and the time required for males to become reproductively mature (the line is described by the equation $y = 22.063 - 9.668x$).

reproductively mature (regression analysis: $F = 167.39$; df = 1, 58; $P = 0.0001$; $r^2 = 0.74$). Data from the two replicates of this experiment were statistically similar and so were pooled for analysis and illustration in Fig. 4.

## DISCUSSION

Previously reported measures of spermatozoan length for *D. hydei* are inconsistent. An initial measurement of 6.6 mm for total sperm length was reported by Hess and Meyer (36). Subsequent reports of "greater than 10 mm" were minimum estimates based on examination of sperm fragments (36, 39). We were able to accurately measure intact sperm bundles in *D. hydei*, including the strain originally used by Hess and Meyer (36) and strains collected around the world, and found them to consistently be over 23 mm long, or ≈10 times the total body length of this species. Most of this length is comprised of flagellum, as the sperm head is only 75 μm long in this species (39).

The nature of spermatogenesis in *Drosophila* requires the testes to be longer than the sperm they manufacture (40, 41). In *D. hydei*, the numerous coils of the ≈30-mm-long testes largely fill the abdominal haemocoel, even in the largest males. We therefore questioned whether small male *D. hydei* would be able to produce the giant sperm. However, all males were found to produce sperm of equivalent size.

If sperm production is costly, then we expected to find correlations between body size and sperm production values (42), similar to correlations often found between female body size and clutch volume in insects (4–6). Testis size measurements for *D. hydei* males of varying body size revealed that smaller males have shorter and thinner testes, perhaps because, relative to larger males, they have less energy available for testicular tissue growth or less space available within the abdominal cavity. Variation in testis width was congruent with a difference among males in the number of sperm produced; the largest males were manufacturing roughly 50% more sperm bundles than were the smallest males. [There are 32 sperm per bundle in *D. hydei* (43).] Larger males also have heavier testes. Moreover, measures of the dry weight of the testes as a percentage of the dry body weight suggest that as body size decreases in a continuous fashion, males produce fewer gametes despite a greater relative investment.

As is true for some other species of *Drosophila* (refs. 44 and 45; but see refs. 45 and 46), larger male *D. hydei* are at an advantage when competing to mate with females (47). The mating experiment described here excluded competition among males, as it was designed to determine whether differences observed among males in sperm production may contribute to size-related variation in other potential fitness attributes, such as the number of times males can mate, the number of sperm transferred in matings, and the number of progeny sired. Significant positive relationships between each of these characters and male size were observed.

The significant difference among males in mating frequency (Fig. 3*a*) is difficult to interpret, first, because the opportunity for males to acquire multiple mates in nature is unknown for this species, and second, because the mechanisms contributing to the observed pattern are also unknown. For example, smaller males may have mated fewer times than larger males because of more rapidly diminishing assets (i.e., stamina, sperm reserves) or because females somehow discrimated against them. Variation in the productivity of matings, however, may be influenced by body size-related variation in the number of sperm produced (Fig. 1*c*), as variation in sperm production most likely affects variation in the number of sperm contained in ejaculates (Fig. 3*b*). Moreover, because females of this species mate multiply, and ejaculates of males randomly mix within females (47), these results suggest that smaller males will be at a disadvantage in numerical sperm competition (48). They are therefore likely to suffer lower fertilization success than larger males. Insofar as fertilization success influences the number of adult progeny produced, our measurements of the reproductive advantage of large males may therefore reflect their observed superiority in sperm production.

Finally, in some *Drosophila* species, males take longer to become reproductively mature than females (33, 49). This is true for *D. hydei*, as females mature within ≈3 days of eclosion, whereas males require 9–11 days (47). Because growth of the long testes has been implicated as the causal agent of this delayed male maturity (33), and given the greater relative investment in testicular tissue made by smaller male *D. hydei* (Fig. 2), we predicted that smaller males should require more time to become reproductively mature than larger males. This prediction was confirmed (Fig. 4).

To summarize our findings, smaller males invest relatively more energy in testicular tissue than do larger males yet have smaller testes which manufacture fewer numbers of sperm at any given time. Smaller males require more time to become reproductively mature, do not mate with as many females, transfer fewer numbers of sperm per copulation, and sire fewer progeny. These patterns illustrate that sperm or the machinery required for their production is not so cheap in this species as to be lacking measurable costs.

While it is clear that *D. hydei* represents a phenotypic extreme with respect to sperm length, it is unclear whether or not the costs of sperm production identified here may also apply to species with less exceptional sperm morphologies, as comparative data are scant. For *D. melanogaster*, which produces relatively small sperm, we found no statistically significant relationships between male body size and testis length or between male dry body weight and the absolute or relative dry weight of testes. Moreover, although the underlying mechanism has not been identified, female *D. melanogaster* singly mated to small males have been shown to produce more progeny than females mated to larger males (50), a pattern opposite to that identified for *D. hydei*. In the cockroach *Diploptera punctata*, male body size did not significantly affect the number of sperm transferred per copulation (13). As in *D. hydei*, the numbers of sperm that male flesh flies, *Neobellieria* (= *Sarcophaga*) *bullata*, produce and ejaculate are positively correlated with their body size (42), although neither testis nor sperm size was examined. More comparative data are therefore required before the costs of sperm production relative to sperm length can be fully assessed.

Given the apparent costs associated with their manufacture, why has such extreme sperm gigantism evolved? Several selective forces have been suggested to explain the evolution of sperm morphology. First, interspecific variation in sperm dimensions may correspond to variation in conditions faced within the female reproductive tract (for review, see ref. 51). It is clear, however, that sperm size can differ tremendously among closely related species in the genus *Drosophila*, despite negligible variation in gross measures (e.g., length) of female reproductive-tract morphology (8). Second, sperm competition (48) has been invoked to explain increased sperm length in rodents and primates (52) and in birds (53). Longer sperm in these taxa may have a motility advantage in their race to fertilize ova or occupy limited female sperm-storage organ space (52, 53). A simple motility advantage of longer sperm is unlikely to explain sperm length evolution in *D. hydei* and many other invertebrates, however, as the sperm are many times longer than any distance they may travel within the female tract (8, 54–56). Moreover, males of *Drosophila* species with giant sperm tend to transfer relatively few sperm per copulation, only partially filling the sperm storage capacity of females (8). Any mechanism by which longer sperm may provide an advantage in sperm competition in these species, such as resisting displacement by females' subsequent mates or their sperm (e.g., ref. 57), is a mystery. Finally, although ejaculates of *D. hydei* males do not include nutritive accessory-gland secretions that are incorporated into females' somatic tissue or into developing oocytes (47), the giant sperm of *Drosophila* may represent provisioning of gametes by males as a paternal investment, as the entire sperm cell, including the intact flagellum, does enter the egg (58). The sperm tail is composed of a variety of substances that may be of functional significance to the developing embryo (34, 59, 60).

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