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Sperm size evolution in *Drosophila*: inter- and intraspecific analysis

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Abstract

Numerous reports were devoted to the variation of sperm length in relation to sperm competition amongst species. However, studies on intraspecific variations of sperm size are very scarce and the number of sperm measured, very limited. This paper investigates within-individual, between-individual and between-population variation of sperm length in the two cosmopolitan species, *D. simulans* and *D. melanogaster*. Sperm length distributions are completely discriminated against with these two species, with the mean values equal to 1.121 ± 0.002 and 1.989 ± 0.008 mm, respectively. Results of intraspecific variation show a contrasting pattern between the two species. The mode of sperm length distributions is much less variable in *D. simulans* than in *D. melanogaster*. The sperm size divergence is unaffected whenever the two species are in sympatry (tested at 'Evolution Canyon', Mount Carmel, Israel) or in allopatry, but the two species react differentially to abiotic local factors. *D. melanogaster*, in contrast to *D. simulans*, shows a clinal pattern in sperm size associated with drought. We discussed this pattern in relation to the potential role of sperm length in the ongoing process of non-random mating and incipient sympatric speciation observed in this locality in *D. melanogaster.*

Introduction

In recent years much attention has been paid to the relative roles of sperm quality versus sperm quantity in sperm competition, one of the major components of sexual selection. Sperm size is one aspect of sperm quality that is supposed to play a role in fruitfly evolution because of the amazing variation among species. A striking diversity of species-specific anisogamous strategies has evolved within the genus *Drosophila*, including all-short, all-giant or heteromorphic sperm strategies (i.e., concomitant production of two sperm length classes by each male). Comparative studies have examined the adaptive significance of sperm length in a variety of taxa, including *Drosophila* (Pitnick & Markow, 1994b; Karr & Pitnick, 1996). They concluded that relatively long sperm provide an advantage in sperm competition (Gomendio & Roldan, 1991; Briskie & Montgomerie, 1992; Roldan, Gomendio & Vitullo, 1992; Briskie, Montgomerie & Birkhead, 1997; Hosken, 1997; Ward, 1998; LaMunyon & Ward, 1999). However, very few studies analyzed the extent, causes, and consequences of intraspecific variation in sperm morphology, and only some of them were devoted to insects (Otronen, Reguera & Ward, 1997; Ward, 1998; Morrow & Gage, 2000).

Here we investigated intraspecific variation in sperm size in two cosmopolitan sibling species *D. melanogaster* and *D. simulans.* In our results, we considered the potential role of sperm size in assortative fertilization, according to sperm competition theory and life history features. First, background on sperm morphology and diversity in the family is given, followed by an investigation of the intra- and interspecific sperm size variation in the sibling species. Special attention is given to the variation in sperm length distributions in relation to the variation in sperm

length means. The discussion points out future ways of investigation that could account for a better understanding of sperm size variation in the context of sexual selection and reproductive systems.

Material and methods

Sperm and nucleus length measurements

Flies were reared in uncrowded conditions on cornmeal medium at $21 \pm 1°C$ with a natural photoperiodic cycle, and a sex ratio of approximately 1:1, and maintained until sexual maturity was reached. Generally, for species with testes longer than 2 mm the measurements of mature cysts were preferred to that of sperm since it avoided scoring broken sperm and, therefore, provided more reliable data. Testes were dissected in Ringer solution and cysts were allowed to spread out. After adding a coverslip, the lengths of cysts were measured at the beginning of individualization, under a phase contrast microscope, using a video camera connected to a Macintosh computer. For other species, sperm were allowed to spread out from male seminal vesicles and slides were prepared in the same way for cysts. Measurements were realized using the public domain NIH Image program (written by Wayne Rasband at the U.S. National Institute of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). Fifty cysts or 100 sperm were measured for each species, isofemale line or population as indicated below in the different sections. In most of the data synthesized here, the sperm or cysts measurements have been made few generations after rearing the flies in the laboratory which limited various artifacts as laboratory drift or inbreeding depression.

For measuring the sperm nucleus, seminal vesicles of sexually mature males were opened directly into a drop of DAPI and sperm were allowed to spread out. The measures were performed under a fluorescent microscope connected to a computer as previously described for the cysts. Fifty nuclei were measured from five recently collected populations of *D. melanogaster* (Kenya 2001 lines K14, K41, K49, France 2001, Avignon and Gotheron) and *D. simulans* (Kenya 2001 line K7, France 2001, Avignon, Gotheron, Saint-Laurent and Pierrefeu).

Drosophila *stocks*

Sperm length means in the Drosophilidae family. A total of 108 species was analyzed and considered here (data from Yanders & Perras 1963, Sanger & Miller 1973; Hatsumi & Wakahama 1986; Hihara & Kurokawa 1987; Joly et al., 1991; Joly & Bressac 1994; Pitnick & Markow 1994a, b; Joly, Bressac & Lachaise, 1995; Pitnick, Markow & Spicer, 1995; Pitnick, Spicer & Markow, 1995; Pitnick 1996, plus seven additional species namely *D. adamsi*, *D. mercatorum*, *D. ercepeae*, *D. confusa*, *S. finitima*, *S. lebanonensis*, and *Z. inermis*).

Sperm length distributions in the six monomorphic species of the melanogaster *subgroup.* The species of the *yakuba* complex are excluded from this analysis because they are sperm heteromorphic (i.e., the sperm length distribution is characterized by one major and one minor peak). The species-specific distributions for all the six other sperm monomorphic species (i.e., the sperm length distribution has one peak) were determined using a various number of populations or isofemale lines. Considering the unequal number of measurements from one species to another, the frequency of each sperm length class was calculated to facilitate the comparisons. Species are listed below with the origin of the strains and the total number of cysts measured. *D. mauritiana*, $n = 200$, lines 67, 72, 74, 76 from Les Galets, Mauritius, 1985 Gif stock 275.1, *D. simulans*, $n = 200$, Seychelles, line 21, 1981 Gif Stock 229.3, Draveil, France, 1986, Gif stock 273.1, Brazzaville, Congo, 1981, Gif stock 246.1, Mt Ambre, Madagascar, 1980, Gif stock, 248.1, *D. erecta*, $n = 50$, Ivory Coast, 1980 Gif stock 220.5, *D. orena*, $n = 50$, West Cameroun, 1975, Gif stock 188.1, *D. sechellia*, $n = 200$, Cousin Is. Seychelles lines 2, 15, 25, Fri, 1985, Gif Stock 267.2, and *D. melanogaster*, $n = 200$, Ivory Coast, Taï, 1983, Gif stock 255.1, South Africa, Cape Town, 1984, Gif stock 263.2, and France, Yquem, 1986, Gif stock 272.1.

Intraspecific variation in D. simulans *and* D. melanogaster *species.* The following comparison had multiple objectives. Firstly, we compared four distinct populations of each species from geographically distant populations, in order to investigate the effect of gene pool divergence on sperm length. Secondly, the corresponding sperm length distributions were compared to the majority of wild and laboratory strains already analyzed. And finally, we discriminated against the between-individual effect from that of between-populations in these four populations. Individuals for which more than five cysts were measured have been taken into account in this analysis,

totalizing three to six individuals, at the minimum and the maximum, for each population. The mean number of mature cysts obtained in males is 8.4 ± 0.50 for *D. simulans* and 6.92 ± 0.47 for *D. melanogaster*. In order to homogenize the sample, 10 cysts have been considered at the maximum for each male.

The four strains of *D. simulans* were chosen on the basis of their different mitochondrial patterns as recognized in Baba-Aissa and Solignac (1984) and in Solignac and Monnerot (1986): *si*I 'Indo-Pacific race' from Mahé Is., Seychelles, 1981 (Gif Stock 229.3, four individuals), *si*II 'Cosmopolitan race' from Draveil, France, 1986 (Gif stock 273.1, three individuals) and from Brazzaville, Congo, 1981 (Gif stock 246.1, five individuals), *si*III from Mt Ambre, Madagascar, 1980 (Gif stock, 248.1, four individuals). Additional sperm length distributions of the following strains were compared to the previous four populations: wild populations from Tunisia, Nasrallah 1983, line ST, Israel 1993 line 2.2, Seychelles Line 9, 11, 13, 21 (Gif stock 229.3), Kenya 2001 line K7 and laboratory strains *Ubx*, *2119*, *PW8N8*, f^2 *nt pm st e*.

The strains in *D. melanogaster* are as follows: France, Yquem, 1986 (Gif stock 272.1, six individuals), Ivory Coast, Taï, 1983 (Gif stock 255.1, five individuals), Kenya, Nairobi 2001 (line K49, four individuals), and South Africa, Cape Town, 1984 (Gif stock 263.1, three individuals). Additional sperm length distributions of the following strains were compared to the previous four populations: Israel 1993 line 1.51, Kenya 2001 line K15, K41, France 2001, Avignon and Gotheron.

Sperm length variation under different environmental conditions. Flies were collected in 1993 from 'Evolution Canyon' in the Lower Nahal Oren, on Mount Carmel in Israel (E. Nevo's project) and were analyzed a few generations after being raised in the lab. This area involves opposing slopes with contrasting ecology due to the higher solar radiation (up to six-fold) during a certain time of the year on the south-facing slope (SFS) than on the north-facing slope (NFS). The 'tropical', savannoid SFS is warmer, drier, ecologically harsher, spatio-temporally more heterogeneous, microclimatically more fluctuating, and less predictable than the 'temperate' maquis brushwood NFS (Nevo 1995, 1997, 2001). From top to bottom, the SFS includes Stations 1–3 and the NFS includes from bottom to top Stations 5–7. The stations are at a distance of 30 m from each other and Station 4 at the valley bottom is only 40 m above sea level. The altitudes of the stations are 60 m (3 and 5), 90 m (2 and 6) and 120 m (1 and 7) on the SFS and NFS, respectively (Harry et al., 1999). For each station four isofemale lines were measured for *D. simulans* and two for *D. melanogaster* (except for Station 3).

Results

Sperm morphology

According to the species, the number of gonial divisions in *Drosophila* varies from 3 to 6, giving rise to 2^3 (=8), 2^4 (=16), 2^5 (=32) and 2^6 (=64) first spermatocytes per cyst. The two subsequent meiotic divisions generate 32, 64, 128, and 256 spermatids per cyst, respectively (Dobzhansky, 1934; Kurokawa & Hihara, 1976; Lindsley & Tokuyasu, 1980; Hanna, Liebrich & Hess, 1982; King & Büning, 1985). The majority of the species belonging to the *Sophophora* subgenus, including *D. melanogaster* and *D. simulans*, has 64 spermatocytes per cyst (Joly & Lachaise, 1994).

The *Drosophila* sperm is characterized by a filiform shape, an elongated nucleus, cylindrical posteriorly, with highly condensed DNA, followed by a disproportionately long tail (Perotti, 1969). Although some constituents of the sperm vary in shape or position, the overall sperm structure, in the Drosophilidae family, is relatively well conserved among species. In contrast, the sizes of nucleus and tail are highly variable morphological traits from one species to another and were extensively analyzed between the two sibling species *D. melanogaster* and *D. simulans* studied here.

The mean nucleus length is significantly different between the two species ($10.009 \pm 0.034\,\mu$m in *D. melanogaster* and $9.727 \pm 0.031\,\mu$m in *D. simulans*). However, there are also some statistically significant differences among populations within each species (ANOVA, $F_{(4,245)} = 17.749$, $P < 0.001$, and $F_{(4,245)} = 22.024$, $P < 0.001$, respectively). It can be suspected that the male body size could account for within as well as between species variability. Indeed, Pitnick and Miller (2000) have find consistent positive correlated responses between body size (thorax length) and sperm length in *D. hydei* species. Moreover, the body size, as well as the wing length, is greater in *D. melanogaster* than in *D. simulans* for almost all populations from higher latitude (Morin et al., 1999) which was the case here. Then, the discrepancy observed between our set of data and the previous one from the literature for *D. melanogaster*

($9.2 \pm 0.273\,\mu$m, Beatty & Sidhu, 1967) could be relevant to the male body size since the strains they considered were selected for smaller wing size. Unfortunately, the data about the sperm length from Beatty and Sidhu (1967) report were lacking and cannot be compared to our own results. Further investigations on the correlated response between body size, sperm length and sperm nucleus length are presently under consideration.

There is also a main difference in the length of the sperm tail between *D. melanogaster* and *D. simulans*. The overall sperm length varies from 1.404 to 2.198 mm in the former and 0.847 to 1.360 mm in the latter, with a global interspecific difference of 0.865 mm. The intraspecific variation in the tail and nucleus lengths clearly shows an allometric growth between these two traits. This characteristic is widespread in the drosophilid species that has only one type of sperm, in contrast to sperm heteromophic species where a correlation exists between the length of the nucleus and that of the tail (Bircher & Hauschteck-Jungen, 1997).

Among the 108 species analyzed so far in the Drosophilidae family (Figure 1), the two cosmopolitan species *D. melanogaster* and *D. simulans* have intermediate values of sperm length (smaller than the male body size), as compared with the seven other species of the *melanogaster* subgroup whose distributions are all smaller than that of *D. melanogaster*.

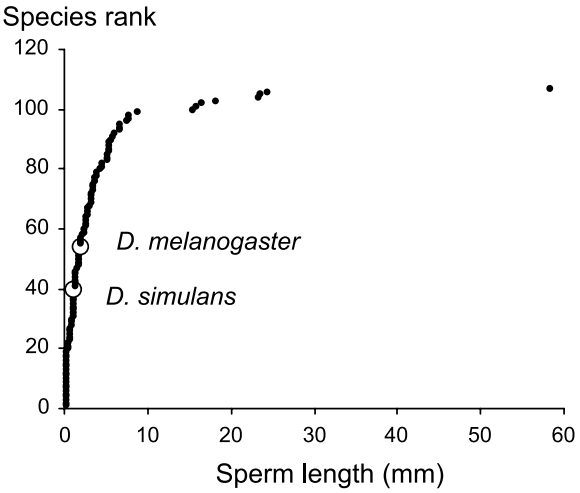

Figure 1. Sperm length distribution in the Drosophilidae family locating *D. melanogaster* and *D. simulans* among the 108 species analyzed so far. The *Y*-axis represents simply the rank of the species.

Interspecific variation in the melanogaster *species complex*

The range of individual sperm length variation in the six monomorphic species of the *D. melanogaster* subgroup is from 0.8 to 2.3 mm (Figure 2). The extent of the overlap between the distributions varies from one species to another, being large between *D. simulans* and *D. erecta* while limited between *D. sechellia* and *D. melanogaster*. Some species exhibit narrow distributions, for example, *D. mauritiana*, *D. simulans*, or *D. orena*, while others show extensive variation, for example, *D. sechellia*, or *D. melanogaster*.

Intraspecific sperm length variation in the sibling species D. simulans *and* D. melanogaster

Intraspecific sperm length variation has been previously investigated in *D. simulans* and *D. melanogaster* (Joly, 1987). Data are presently completed in order to investigate the sperm length variation at the between-population and between-individual levels. The results show strong interspecific discrimination between four wild distant geographic populations, but similar sperm length distribution for each species (Figure 3(a)). The range of the variation is slightly different between-populations, and higher in *D. melanogaster*. However, additional data from wild and laboratory strains show a divergent pattern concerning the mode of the distributions between the two species (Figure 3(b)). While all strains of *D. simulans* show the same mode, those of *D. melanogaster* exhibit significant variation from one population to another. The between-population variation in the same geographic region

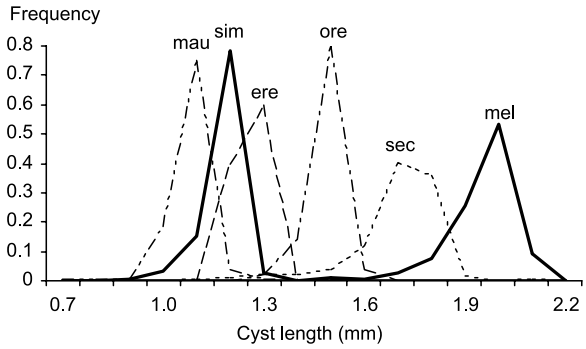

Figure 2. Sperm length distributions in the six sperm monomorphic species of the *D. melanogaster* subgroup. From the smallest to the highest values of the mean, *D. mauritiana* (mau, 1.036 ± 0.004), *D. simulans* (sim, 1.124 ± 0.002), *D. erecta* (ere, 1.210 ± 0.004), *D. orena* (ore, 1.436 ± 0.006), *D. sechellia* (sec, 1.649 ± 0.008), *D. melanogaster* (mel, 1.989 ± 0.008).

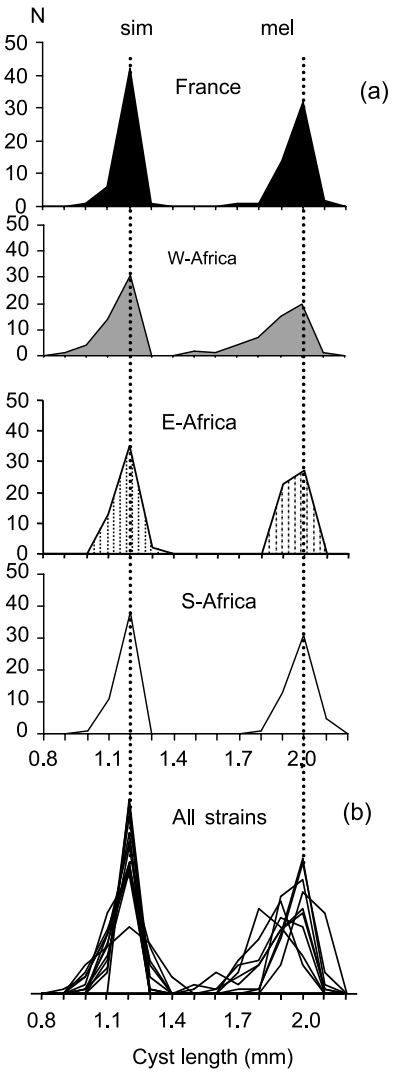

Figure 3. Sperm length distributions in the two cosmopolitan species *D. simulans* (from top to bottom: Draveil, France; Brazzaville, Congo; line K7, Kenya; line 21, Seychelles) and *D. melanogaster* (Yquem, France; Taï, Ivory Coast; line K49, Kenya; Cape Town, South Africa) showing the stability of the mode from one population to another. The graphs in the bottom summarized all the strains analyzed with additional populations for *D. simulans* (e.g., wild individuals originated from Israel line 2.2, lines 9, 11, and 13 from Seychelles Island and Tunis ST, and laboratory strains of *2119*, *PW8N8*, *Ubx* and f^2*ntmpste*) and *D. melanogaster* (e.g., wild individuals originated from Israel line 1.51, Kenya line K15, K41, and France Avignon and Gotheron).

(between Avignon and Gotheron from France, e.g.) in *D. melanogaster* could be as great as that observed between geographically distant populations. Accordingly, there is no correlation between sperm length and latitude for the populations analyzed here in this species ($r^2 = 0.0018, n = 7, P > 0.05$).

Table 1. Between-population ANOVA for sperm length in *D. simulans* and *D. melanogaster* from Figure 3(b)

Species	Source	df	Mean square	*F*
D. simulans	Populations	13	0.0268	7.8791***
	Error	686	0.0034	
D. melanogaster	Populations	8	0.1799	20.1169***
	Error	441	0.0089	

*** $P < 0.001$.

Table 2. Nested ANOVA for sperm length considering 3–6 individuals within populations and 5–10 cysts per male in *D. simulans* (4 strains and 16 individuals) and *D. melanogaster* (4 strains and 18 individuals) from Figure 3(a)

Species	Source	df	Mean square	*F*
D. simulans	Populations	3	0.0497	30.4023***
	Population: males	12	0.0088	5.3793***
	Error	119	0.0016	
D. melanogaster	Populations	3	0.0403	6.4587***
	Population: males	14	0.0217	3.4862***
	Error	111	0.0062	

*** $P < 0.001$.

In spite of the divergence in the variation of the mode of the distributions, the two species exhibit highly significant mean sperm length differences between-populations (Table 1) indicating geographic variation. In that respect, *D. melanogaster* appears more polymorphic than *D. simulans* (Test of variance equality, $F_{(1.398)} = 10{,}782.003$, $P < 0.001$).

To discriminate the between-individual variation from the between-population variation, a hierarchical analysis was performed between-individuals within each of the four populations from Figure 3(a). Results show that a significant polymorphism is superimposed on the between-population variation in both species (Table 2).

Effect of local biotic and abiotic conditions on sperm length

A first attempt to study the effect of the environmental conditions on the variation of sperm length in *Drosophila* was conducted with flies from 'Evolution Canyon'. Results of mean sperm length were displayed for each isofemale line, each station, and

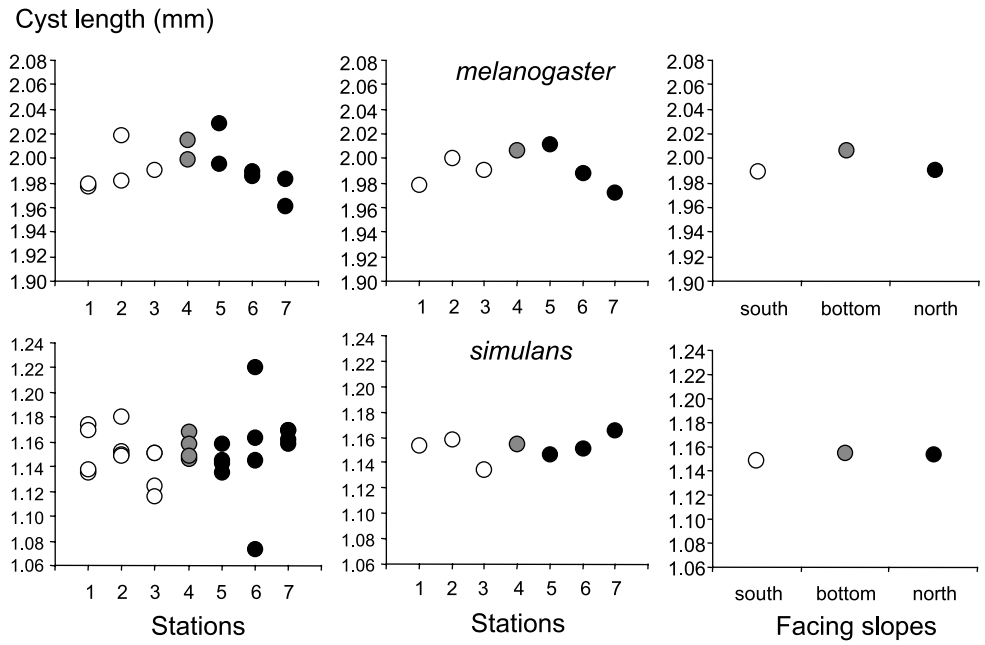

Figure 4. Cyst length means for *D. melanogaster* (above) and *D. simulans* (below) at 'Evolution Canyon'. The left graphs indicate values for each isofemale line, the middle ones give the means for each station and the rights represent the means for each slope. The standard errors cannot be represented because they are smaller than the diameter of the marks. Note that the *Y*-axes differ between the species, but the range is similar.

Table 3. Nested ANOVA on sperm length for testing the effect of north- and south-facing slopes from 'Evolution Canyon' at the Lower Nahal Oren microsite for *D. simulans* (24 isofemale lines) and *D. melanogaster* (11 isofemale lines)[a]

Species	Source	df	Mean square	*F*
D. simulans	Slope	1	0.0157	4.9063*
	Slope:station	4	0.0191	5.9739***
	Slope:station:line	18	0.0380	11.9082***
	Error	1176	0.0032	
D. melanogaster	Slope	1	0.0002	0.0246
	Slope:station	4	0.0266	4.0823**
	Slope:station:line	5	0.0150	2.2975*
	Error	539	0.0059	

[a] The isofemale lines from the 'bottom' station have not been considered here.
*** $P < 0.001$.

the north- and south-facing slopes for both species in Figure 4. The between-line difference in one station could be as great as the difference between stations. There is no correlated variation among stations for both species and the variation among slopes is much smaller than the variation between-lines, even within a station. The sperm length analyses show significant differences between-lines for *D. simulans* and *D. melanogaster* (ANOVA, $F_{(27,1372)} = 8.648$, $P < 0.001$ and $F_{(12,637)} = 2.778$, $P < 0.01$, respectively). The effect of environmental conditions was tested using a nested analysis opposing the north- and south-facing slopes (Table 3). Results for the two species show great intraspecific variation with significant line and station effects. However, the slope factor is not significant in *D. melanogaster* and *D. simulans.* These results suggest that the between-line and between-station differences have a possible greater effect on sperm length than the local climatic conditions opposing the north- and south-facing slopes.

However, remarkable contrasting patterns are exhibited in the two species according to the altitudes within each opposing slope. In *D. simulans*, the mean sperm length increases with altitudes whereas the reverse is observed in *D. melanogaster* (Figure 5(a) and (b)). Results show interestingly that the NFS always has a greater coefficient of correlation than the SFS, even if they are not statistically significant with this limited data. In spite of the opposing trends between the two species, the mean differences of sperm length decreases with altitudes on both slopes with a significant coefficient of correlation found in NFS (Figure 5(c)).

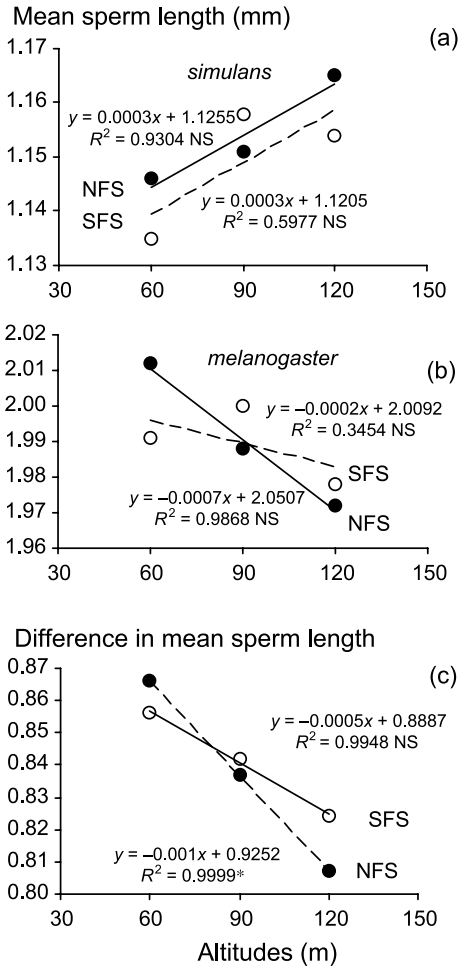

Figure 5. Sperm length means plotted against the altitudes are given for *D. simulans* (a) and *D. melanogaster* (b) within each slope. Note the contrasting pattern between the two species. Differences in the mean sperm length between the two species are given according to the altitudes (c) and show the same pattern for *D. melanogaster*. For each series, the equation of the tendency curve and the R^2 coefficients of correlation are given on the graph. NS: non-statistically significant; * significant at $P < 0.05$.

These findings show that intraslope altitude divergence is more effective on sperm size than interslope divergence. This suggests a very complex genetic/environmental determination of sperm length.

Sex contribution to the sperm length variation

The effects of both paternal and maternal lineages can be investigated by comparing the variation of sperm length from males derived from the same female (paternal effect) and from males derived from different females mated with the same male (maternal effect). However, data for the last case are not available, but the maternal effect can be nevertheless investigated by comparing male progeny sired by different females from lines of the same population. This was realized with the four Seychelles lines of *D. simulans* for which data on sperm length from different male offspring derived from the same female were available. The nested analysis shows more significant difference among lines than among individuals within line (ANOVA, $F_{(3,120)} = 29.295$, $P < 0.001$ and $F_{(13,120)} = 2.622$, $P < 0.01$, respectively). The present findings suggest that both maternal and paternal effects account for sperm length variation, as was already determined in cricket and *Scatophaga* (Ward, 2000; Morrow & Gage, 2001).

Discussion

Sperm size variation occurs between species, individuals, and within-individuals in the sibling species *D. melanogaster* and *D. simulans*. The major finding of the sperm size analysis at the intraspecific level is the contrasting pattern between *D. simulans* and *D. melanogaster*. The former species exhibits an extremely stable mode of sperm length distributions across populations while it is more variable in the latter species. This observation is not relevant to the two-fold difference in sperm size between *D. simulans* and *D. melanogaster* since their coefficients of variation are very similar (5.40 v.s. 5.85, calculated in the 14 and 9 populations from Figure 3(b), respectively). In that respect, sperm size appears more variable in *D. melanogaster* than in *D. simulans*, as in numerous other morphological traits. A number of various hypotheses has been invoked to explain the apparently lower level of within-population variation

and geographic differentiation observed for *D. simulans* versus *D. melanogaster* and were synthesized by Capy in the Introduction section of the present volume.

The selective pressures acting on sperm size in *D. simulans* restrain the variation, but do not prevent significant between-individual differences. Then geographic variation of mean sperm size is superimposed in both species to a within-population polymorphism, even at a microscale level, as was exemplified at 'Evolution Canyon'. These observations could account for selection acting at the level of the male, favoring diploid control of sperm size variation. There is clearly a large, complex, genetic element in the determination of sperm size in fruit flies, as was already shown in a variety of species (Beatty & Sidhu, 1970; Woolley, 1970; Joly et al., 1997; Roff, 1997), implying male and female heritability (Ward, 2000; Morrow & Gage, 2001). The adaptive significance of variation in sperm size has been extensively investigated at the interspecific level, but not at the intraspecific level. The present study points out that sperm size variation is probably under different selective pressures related to both physiological and biological (reproductive isolation, sperm competition, fertilization) constraints.

Within-individual sperm length range

The species-specific range of the cyst length distribution is a remarkable phenomenon. Each individual possesses a sample of the whole species-specific range of the distribution. But what is the significance of the within-individual variation? Does it represent epigenetic variability or differential fertilizing potentials? Very few studies on invertebrates were devoted to the analysis of within ejaculate sperm quality in terms of viability and fertility features (Peng et al., 1990; Collins, 2000; Hunter & Birkhead, 2002), while many studies have been done on birds or mammals. The ratio between the number of sperm used for fertilization to the number of sperm transferred to the female is much higher in insects than in mammals, suggesting a higher sperm efficiency. However, almost three-quarters of the sperm inseminated are lost in *D. melanogaster* for example (Gromko, Gilbert & Richmond, 1984). Two questions arise: What is the size of these lost sperm? Are they fertile?

In the present case, no available data show that the smallest or longest sperm from an individual distribution are fertile or not. However, studies in *sex-ratio* traits have shown that abnormal elongated spermatids within a full mature cyst are associated with progeny deficiency (Cazemajor, Joly & Montchamp-Moreau, 2000). Therefore, between-cyst variability could be superimposed to within-cyst micro-variability that could occur during spermatogenesis and more specifically during the elongation process. Recently, Hunter and Birkhead (2002) demonstrated a considerable intraspecific variation in the proportion of dead sperm in seven pairs of species including *Drosophila* and other insects, which influence their respective ability to fertilize ova. It seems then crucial to control for the fertility of sperm depending on their length at the within-individual level.

It is, nevertheless, also possible that the variation observed in *Drosophila* may simply be due to non-genetic variation, for example, phenotypic plasticity. A within-cyst variation could then be superimposed on the between-cyst variation without affecting the fertility of sperm regardless of their overall length. The major argument in favor of this hypothesis is that lines exhibiting statistically significant differences in sperm length do not appear to present significant differential fertility traits.

Sperm size and assortative fertilization

It has been tentatively suggested that the length and structure of the sperm tail are species-specific discriminators that are important in sperm compatibility with the female tract, and function more as a sexual isolating mechanism rather than a factor for the efficient propulsion of sperm from the uterus to the site of fertilization. Indeed, sperm in *Drosophila* exhibit no actual straightforward progression (Bressac et al., 1991b), in contrast to sperm in vertebrates and even in other Diptera as *Ceratitis capitata* (Baccetti, Gibbons & Gibbons, 1989). The sperm size variation presently observed in *Drosophila* could represent a response to environmental heterogeneity as defined by different females providing different environments for sperm of different sizes. The question could even be addressed to the possibility of positive assortative mating (Thoday & Gibson, 1962; Capy et al., 1999; Korol et al., 2000; Iliadi et al., 2001; Michalak et al., 2001) depending on sperm length. Postmating sexual selection has received very little attention in discussions of speciation. However, recent research has demonstrated that postmating sexual conflict can impel divergent postmating sexual selection among males by sperm competition, and/or cryptic female choice (Arnqvist et al., 2000 and references therein). It is noteworthy, that in the foregoing material from

'Evolution Canyon' (collections of 1997 and 1995), a strong indication of incipient premating sexual isolation between *D. melanogaster* flies from the opposite slopes was found, displayed as mate choice (Korol et al., 2000). Likewise, significant differentiation was revealed for the courtship song patterns (Iliadi et al., submitted). This finding calls for further in-depth analysis of the sperm length differentiation as a possible contributing factor to sexual isolation, the pre- and postmating mechanisms of isolation being not necessarily exclusive from each other. Below, we focus on the effect of within- and between-individual sperm size variation upon the chain of reactions that bring about the actual unions of gametes and discuss possible ways to investigate the reproductive isolation observed between *D. melanogaster* and *D. simulans*.

Sperm size in sperm competition. In *Drosophila*, the genetic compatibility between male and female strongly influences the retention and movement of sperm into the female storage organ (including resistance and displacement capabilities) and their release for fertilization (Civetta, 1999; Clark, Begun & Prout, 1999; Civetta & Clark, 2000). Clearly, both female (Clark & Begun, 1998) and male (Prout & Bundgaard, 1977; Clark et al., 1995) genotypes affect sperm displacement, but the effect of intraspecific sperm size variation on sperm competition has been poorly investigated in animals (Joly, Cariou & Lachaise, 1991; Ward & Hauschteck-Jungen, 1993; LaMunyon & Ward, 1999). From the viewpoint of ejaculation, the sperm length could have some influence on the outcome of reproduction by interacting with the female organ or by competing with rival sperm, in species where remating can occur before the sperm from the first mate are stored.

There is very little data on *Drosophila* that consider remating speed and sperm storage. In *D. melanogaster*, it has been reported that sperm storage requires 15 min–9 h, depending on the strains used (Gromko, Gilbert & Richmond, 1984). Since some species exhibit frequent polyandry (Markow, 1982; Bressac et al., 1991a; Pitnick & Markow, 1994b), we can suspect possible interaction in the uterus between different male ejaculates. In *D. melanogaster* and *D. simulans*, remating typically occurs 4–5 days after the first mating (Markow, 1996). In *D. melanogaster* the storage delay of the first sperm mate and the existence of a mating plug (Lung & Wolfner, 2001), which disappear in 24 h (Alonso-Pimentel, Tolbert & Heed, 1994), can favor sperm of a particular length to pass through in order to reach the sperm storage organs. In that respect, the kinetic parameters exhibited by long sperm in *Drosophila* (Bressac et al., 1991b) could explain why they are preferentially stored (Bressac & Hauschteck-Jungen, 1996). In our previous discussion of *D. melanogaster* from the mid-slope Stations 2 (SFS) and 6 (NFS) from 'Evolution Canyon' (collection 1997), the females derived from the south-facing slope displayed a much shorter average time of remating than NFS females (Iliadi et al., 2001), whereas in the present study (collection 1993) we found relatively small differences between these stations in mean sperm size (2.001 mm for Station 2 and 1.989 mm for Station 6). Comparing sperm length distributions between male and female storage organs would determine whether or not a selective process favors longer sperm in the storage process and, finally, influence the precedence mechanisms.

Sperm size in reproductive isolation. One of the major issues in evolutionary biology is to understand the selective pressures contributing to the differentiation of natural populations. Reproductive isolation is thought to be achieved, either in sympatry or in allopatry, as the product of postmating isolation which is widely accepted as a result from evolutionary divergence, or as the product of premating isolation that is associated with its initial stages (Coyne, 1992; Coyne & Orr, 1998; Nevo, 1999, and references therein). Evidences of natural hybridization between *D. melanogaster* and *D. simulans* are rare, but hybrids are repeatedly caught in various wild habitats (Sperlich, 1962; Tracey, Pavlovsky & Green, 1973; Capy et al., 1987; Inoue, Watanabe & Watada, 1990). However, whenever natural hybridization occurs the selective pressure is expected to act on reproductive traits to prevent cross-fertilization. The longer size (double in length) of the *D. melanogaster* sperm compared to that of the *D. simulans* (and then to the length of the *D. simulans* female seminal receptacle) could be large enough to dramatically reduce the sperm use.

Sperm length has a major role in the reproductive isolation between species, mainly because of its amazing variation among species in the Drosophilidae family (Figure 1). It is unlikely that this reproductive trait could simply be a by-product of gene pool divergence, according to Darwin, Muller, Mayr and Carson (see the discussion in Nevo, 1999), mostly because of the strong correlation to the length of the female ventral receptacle (Joly & Bressac, 1994; Pitnick & Markow, 1994b) which implies complex physiological

and biological co-adaptation between the sexes (e.g., the non-reproductive adult phase is much longer since the sperm is larger, Pitnick, Markow & Spicer, 1995). It is more probable that sperm length variation is under a direct selection process (Dobzhansky, 1937). An interesting point concerning the putative role of microclimatic conditions in reproductive isolation follows from the comparison of sperm size variation in *D. melanogaster* and *D. simulans* from 'Evolution Canyon'. Although we have not found any clear pattern in size variation between the slopes, the difference of the average scores displays a Λ-type pattern (Figure 5(c)), which we found in other studies of *Drosophila* in the Canyon (see Nevo et al., 1998). In interspecific crosses, sperm size should be particularly crucial during both storage and fertilization events, which is discussed below.

During the storage process, selection should favor sperm shorter than or equal to the length of the storage site. First, sperm that cannot fit entirely into sperm storage organs are likely to be swept out of the genital tract when eggs are laid. Second, sperm that completely fill a sperm storage organ could exclude other sperm from entering and thereby prevent the safe storage from potential competitors (Briskie & Montgomerie, 1992). This phenomenon could be particularly relevant in Drosophilidae, including the *D. melanogaster* subgroup species. In the present case, almost any of the *D. melanogaster* sperm can enter into the *D. simulans* receptacle, which is 1.541 ± 0.029 mm long, while the *D. simulans* sperm are roughly two times shorter than the *D. melanogaster* receptacle, which is 2.249 ± 0.038 mm long (Joly & Bressac, 1994). This could explain why the cross of *D. melanogaster* female to *D. simulans* males is far easier than its reciprocal (Lachaise et al., 1986; Ashburner, 1989). However, the inadequacy of lengths between sperm and storage organs could not account alone for low hybridization between these two species since the *Lethal hybrid rescue* gene in *D. simulans* (Watanabe, 1979) and the *Hybrid male rescue* in *D. melanogaster* (Barbash, Roote & Ashburner, 2000) have been shown to significantly enhance the production of progeny, recovering the two sexes in the F_1.

During the fertilization process, sperm length incompatibility can occur at the peculiar moment of the nuclear fusion after the oocyte penetration. Indeed, as previously shown by Karr (1991), the sperm enters the egg intact (in most species including *D. melanogaster* and *D. simulans*, Karr & Pitnick, 1996) and localizes within the anterior region of the egg where it forms a stereotypical folded structure. Numerous observations confirm the species-specificity of this structure (Karr 1991, 1996; Karr & Pitnick, 1996), suggesting that sperm–egg interactions are necessary for the observed folding and coiling (Karr, 1991). This structure positions the male pronucleus in the proper region of the egg in anticipation of pronuclear fusion. Therefore, the typical folded structure varies according to sperm length. In the case of interspecific crosses, the sperm length differences, allowing differential localization of the male pronucleus, can prevent karyogamy. Such phenomenon will merit further investigations on the hybridization process between *D. simulans* and *D. melanogaster* for which these data are lacking.

In conclusion, disentangling genetic and environmental effects together with the quality assessment of ejaculates deserve further studies devoted to the analysis of reproductive strategies and speciation.

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